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Molecular Phylogeny and Phenotypic Characterization of Yeasts with a Broad Range of pH Tolerance Isolated from Natural Aquatic Environments

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Abstract

In this study, yeasts with broad range of pH tolerance were isolated and characterized from natural aquatic environments in Japan. Only a few basic and application studies of alkali-tolerant yeasts have been reported, despite the unmet industrial needs. First, we surveyed alkali-tolerant yeasts from natural aquatic environments at pH 7.6 - 9.4. We isolated 35 yeast strains that grew in pH 9.0 medium, from seven genera and nine species: 25 strains (N1, N2, through N6, N9, K1, K3 through K19) were *Rhodotorula mucilaginosa*; one (N7) was *Rhodosporidium fluvial*; one (N8) was *Scheffersomyces spartinae*; two (N10 and N13) were *Wicherhamonyces anomalus*; one (N11) was *Cyberlindnera saturnus*; one (S1) was *Candida* sp.; two (S2 and S4) were *Candida intermedia*; one (S3) was *Candida quercuum*; and one (K2) was *Cryptococcus liquefacience*. We examined the effects of pH on the growth of representative yeast strains. Strains K12 and S4 showed high growth at pH 3 - 10. Strains N7, N8, N10, N11, and S3 showed high growth at pH 3 - 9. Strains K2 and S1 showed high growth at pH 4 - 8. All nine of these strains had neutralizing activities from acidic media at pH 3 - 5 to pH 6 - 8. We previously isolated acid-tolerant yeasts (*Cryptococcus* sp. T1 [1] and *Candida intermedia* CeA16 [2]) from extremely acidified environments; they showed high growth at pH 3 - 9 and neutralizing activities of acidic media by releasing ammonium ions. Thus, alkali-tolerant yeasts and acid-tolerant yeasts were found to be similar species and have both high growth at a broad range of pH and neutralizing activities of acid media. Previously, we also isolated acid-tolerant, acid-neutralizing yeasts

from neutral natural environments (26 strains, 12 species) [3]. Next, we constructed the phylogenetic trees of both acid-tolerant yeasts and alkali-tolerant yeasts. All were situated in the same classification position. Similar yeast species with a broad range of pH tolerance were living in natural aquatic environments at pH values from alkali to acid.

Keywords

pH Tolerance, Yeast Species, Aquatic Environment, Phylogenetic Tree, Fermentation

1. Introduction

Various types of yeasts and yeast-like microorganisms are widely spread in nature, and some have been used since 2000BC for fermented products (e.g. alcoholic beverages and the leavening of bread). Over the past several decades, suitable yeasts for different food processes have been repeatedly isolated and bred separately according to the type of fermentation desired. These efforts revealed that all industrial-use yeasts should have similar characteristics of high fermentative activity and high tolerance activities under various types of stress. Identification methods for yeasts were developed after the 18th century, and most of the yeasts used in the fermentation industries were found to be a species of *Saccharomyces cerevisiae*. From ancient to modern times, *S. cerevisiae* has been the most important microorganism species in the history of humans.

However, with the progress of the bioethanol industry in recent years, the breeding of novel yeast strains other than *S. cerevisiae* with higher fermentative activity under several stress pressures is needed, because various types of wasted biomass materials are used as fermentation substrates, and from an economical point of view the concentration of the biomass should be higher. Above all, in bioethanol production, acids or alkalis are used for the hydrolysis of a cellulosic biomass, and the direct fermentation of hydrolysates using yeasts without neutralizing themselves is desirable. Fermentative yeasts with pH tolerance are thus considered potential beneficial candidates for efficient bioethanol production. Several studies of acid-tolerant yeasts have been conducted, as *S. cerevisiae* and other high-fermentative yeasts show high activity in neutral or acid environments (pH 4 - 7) [4] [5] [6]. Only a few basic and application studies of alkali-tolerant yeasts have been reported, despite the unmet industrial needs [7].

In previous investigations, we isolated and characterized two strains of acid-tolerant yeasts from extremely acidic environments. One strain was *Cryptococcus* sp. T1 from Lake Tazawa in Japan's Akita prefecture, is a caldera lake polluted with hydrochloric acid from an upstream hot spring [1]. The other yeast strain is *Candida fluviatilis* CeA16 from Agatsuma River in Japan's Gunma prefecture, which is polluted with sulfuric acid from Mount Kusatsu-Shirane [2]. Both strains T1 and CeA16 showed high growth at pH 3 - 9,

and both showed neutralizing activity from acidic media to pH 6 - 8 by releasing ammonium ions.

We also previously isolated and characterized 26 strains and 12 species of acid-tolerant yeasts that had neutralizing activities of acidic media from neutral environments in the city of Yokohama, Japan [3]. The acid-tolerant and acid-neutralizing yeasts were found to exist in both acidic and neutral environments.

In the present study, we attempted to isolate alkali-tolerant yeasts from alkali environments (pH 7.6 - 9.4) in Japan's Kanto region. We characterized the yeast strains' growth and fermentation activities, and we constructed the strains' phylogenetic trees and compared them with those of acid-tolerant yeasts.

2. Materials and Methods

2.1. Collection of Environmental Samples

We surveyed alkali aquatic environments near metropolitan areas and selected four stations in Japan in order to isolate alkali-tolerant yeasts. In May 2016, we collected water and sediment samples from four aquatic environments in Japan's Kanto region: Station #1 (a pond on the campus of Tokyo University of Marine Science and Technology, Tokyo), Station #2 (at the coast of Kasairinkai Park, Tokyo), Station #3 (at the coast of Senbongi Park in Shizuoka Prefecture, Japan), and Station #4 (at the coast of the Shioiri River in Tateyama of Chiba Prefecture, Japan). All samples were collected in sterile plastic tubes. We measured the samples' temperature, pH values, and NaCl concentration. The samples were immediately transferred to and stored at Tokyo University of Marine Science and Technology at <4˚C.

2.2. Medium Culture

For the cultivation of yeasts in the water and sediment samples, we used a YPD medium consisting of 1.0% w/v yeast extract, 2.0% w/v proteose peptone (Becton Dickinson, Lincoln Park, NJ, USA), and 2.0% w/v D-(+)-glucose (Kokusan Chemicals, Tokyo). For the isolation of yeasts from the environments, 0.01% w/v chloramphenicol (Wako Pure Chemical Industries, Tokyo) was added to the YPD medium to prevent the growth of bacteria. The pH of the media was adjusted with sulfuric acid or sodium hydroxide (Wako). Solid media were constructed by adding 2.0% w/v agar (Kanto Chemicals, Tokyo) to the YPD liquid medium.

2.3. Isolation of Alkali-Tolerant Yeast Strains

The water samples were filtered through a 0.45-μm FTFE membrane-filter (Advantec, Tokyo), and microorganisms were trapped on the filter. The microorganisms were dispersed into the portion of the filtrate by a mixer; thus, we obtained an approx. 100-fold-concentrated population of the microorganisms in the water samples. To obtain a moderate population of the microorganisms, we

diluted sediment samples to 10-fold with physiological saline (0.8% w/v NaCl).

For the first screening of alkali-tolerant yeasts, a 200-μl volume of each preparation was spread on the YPD solid medium containing chloramphenicol at pH 8.0 and incubated at 25˚C. After several days' cultivation, growing yeast-like colonies were picked up, and we observed their cells under a light microscope. Cells that were yeast-like morphologically were isolated and stored at −80˚C.

For the second screening of alkali-tolerant yeasts, the isolates obtained by the first screening were inoculated on the YPD solid medium at pH 9.0 and incubated at 25˚C. The growing colonies were obtained as candidate alkali-tolerant yeasts, stored at −80˚C, and numbered as members in the yeast collection.

2.4. Yeast Identification

The 28S rRNA genes of the isolates in the yeast library 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') and Premix Ex Taq (Takara Bio, Shiga, Japan). The 28S rRNA phylogenetic tree was constructed in the molecular evolutionary genetics analysis (MEGA) tool 6.06 using the maximum likelihood method with a 1000 replicate bootstrap resampling. The D1/D2 domain sequences of the 28S rRNA genes in the yeasts were deposited in DDBJ, EMBL, and GenBank.

2.5. Fermentation Activities of the Yeasts

Each isolate in the yeast library was inoculated into 10 ml of YPD liquid medium with a Durham pipe in a test tube and then anaerobically incubated at 25˚C. After 7 days' cultivation, the yeast fermentative activity in the medium was examined by the naked eye based on the storage of gas (CO_2) evolving from the cells into the Durham pipe.

2.6. Yeast Growth Tests

Each strain in the yeast library was precultured in YPD medium at 25˚C for 24 hr. The growing cells were precipitated by centrifugation at 3000 rpm for 5 min and then washed with physiological saline. The centrifugation/washing procedure was conducted three times, and the cell precipitates were obtained. A 100-μg portion of the wet cells was inoculated into 10 ml of YPD liquid medium with the pH value 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, or 10.0. We measured the growth curves of the yeasts at 25˚C for 72 hr using a bio-photorecorder (temperature-gradient incubator, Advantec, Japan).

2.7. Measurement of pH in the Yeast Cultures

In the growth tests of the yeasts in the YPD liquid media with the initial pH values 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0, we measured the change of pH values in the media after 3 days' cultivation at 25˚C by using a LAQUA pH meter (F-71, Horiba Scientific, Fukuoka, Japan).

3. Results

3.1. Isolation and Identification of Alkali-Tolerant Yeasts

Table 1 shows the pH, temperature, and NaCl concentration of the sampling areas and the isolation of alkali-tolerant yeast-like microorganisms. The first screening by the isolation of growing colonies on the YPD solid medium containing chloramphenicol at pH 8.0 identified 22, 229, 37, and 21 yeast-like isolates from both water and sediment samples taken from Stations #1, #2, #3, and #4, respectively. In the second screening at pH 9.0 of the isolates identified by the first screening of growing yeast-like cells, 19, 12, and four strains were obtained from Stations #2, #3, and #4, respectively. Our microscopy observations of the cell morphology suggested that all 35 of the second-screening strains were yeast species with alkali tolerance. We numbered the strains as K1-K19 from Station #2, as N1-N11 and N13 from Station #3, and as S1-S4 from Station #4.

No alkali-tolerant yeasts were isolated from Station #1, even though the pH value of the water at that station was 9.4. At that station, we observed highly concentrated microalgae growing in the pond, the pH of which was 9.4 due to ammonium ions being released from the microalgae, and few alkali-tolerant yeasts seemed to be living at this station.

Table 2 shows the identification results for the 35 strains of alkali-tolerant yeast. These strains were identified as members of seven genera and nine species: 25 strains (N1, N2, N3, N4, N5, N6, N9, K1, K3, K4, K5, K6, K7, K8, K9, K10, K11, K12, K13, K14, K15, K16, K17, K18, and K19) were *Rhodotorula mucilaginosa.* One strain (N7) was *Rhodosporidium fluviale*. One strain (N8) was *Schefersomyces spartinae.* Two strains (N10 and N13) were *Wicherhamonyces anomalus*. One strain N11 was *Cyberlindnera saturnus*. One strain (S1) was *Candida* sp. Two strains (S2 and S4) were *Candida intermedia*. One strain (S3) was *Candida quercuum*. One strain (K2) was *Cryptococcus liquefacience*. Therefore, *Rhodotorula mucilaginosa* seemed to be a common species among alkali-tolerant yeasts.

3.2. The Fermentation Activities of the Alkali-Tolerant Yeasts

Table 3 summarizes the fermentation activities of the 35 strains of alkali-tolerant

Table 1. pH, temperature, and NaCl concentration of sampling area and isolation of alkali-tolerant yeasts.

Sampling area	pH	NaCl concentration (%)	Temp. ˚C	No. of isolates at pH 8.0	No. of isolates at pH 9.0	Strain
Station #1	9.40	0.01	26.0	22	0	-
Station #2	7.65	1.40	22.4	229	19	K1-K19
Station #3	7.55	2.70	20.3	37	12	N1-N11, N13
Station #4	8.08	0.25	ND	21	4	S1-S4

Station #1: A pond in Tokyo university of marine science and Technplogy in Tokyo, Japan; Station #2: A coast of the Kasairinkai Park in Tokyo, Japan; Station #3: The coast at Senbongi Park in Shizuoka Prefecture, Japan; Station #4: The coast of the Shioiri River in Tateyama of Chiba Prefecture, Japan.

Table 2. Identification results for 35 strains of alkali-tolerant yeasts.

Strain	Identification result	Identity (%)	Accession No.
N1			LC438608
N2			LC438609
N3			LC438610
N4			LC438611
N5			LC438612
N6			LC438613
N9			LC438614
K1			LC438615
K3			LC438617
K4			LC438618
K5			LC438619
K6			LC438620
K7	*Rhodotorula mucilaginosa*	99 - 100	LC438621
K8			LC438622
K9			LC438623
K10			LC438624
K11			LC438625
K12			LC438626
K13			LC438627
K14			LC438628
K15			LC438629
K16			LC438630
K17			LC438631
K18			LC438632
K19			LC438633
N7	*Rodosporidium fluviale*	100	LC438634
N8	*Scheffersomyces spartinae*	100	LC438635
N10	*Wickerhamomyces anomalus*	100	LC438636
N13		100	LC438637
N11	*Cyberlindnera saturnus*	100	LC438638
S1	*Candida* sp.	100	LC438639
S2	*Candida intermedia*	100	LC438640
S4		100	LC438641
S3	*Candida quercuum*	99	LC438642
K2	*Cryptococcus liquefaciens*	100	LC438616

Table 3. Fermentation activities of 35 strains of alkali-tolerant yeasts.

Sampling area	Species	Strain	Fermentation
	Rhodotorula mucilaginosa	K1	–
	Cryptococcus liquefaciens	K2	–
		K3	–
		K4	–
		K5	–
		K6	–
		K7	–
		K8	–
		K9	–
Station #2		K10	–
	Rhodotorula mucilaginosa	K11	–
		K12	–
		K13	–
		K14	–
		K15	–
		K16	–
		K17	–
		K18	–
		K19	–
		N1	–
		N2	–
	Rhodotorula mucilaginosa	N3	–
		N4	–
		N5	–
		N6	–
Station #3	*Rodosporidium fluviale*	N7	–
	Scheffersomyces spartinae	N8	+
	Rhodotorula mucilaginosa	N9	–
	Wickerhamomyces anomalus	N10	+
	Cyberlindnera saturnus	N11	+
	Wickerhamomyces anomalus	N13	+
	Candida sp.	S1	+
	Candida intermedia	S2	+
Station #4	*Candida quercuum*	S3	+
	Candida intermedia	S4	+

(+): with fermentation activity; (−): without fermentation activity.

yeast. Among them, 27 strains and three species had no fermentative activity, but eight strains and six species had fermentative activities in the media: *Schef-*

fersomyces spartinae N8, *Wicherhamonyces anomalus* N10 and N13, *Cyberlindnera saturnus* N11, *Candida* sp. S1, *Candida intermedia* S2 and S4, and *Candida quercuum* S3. We considered these eight yeast strains as candidates for bioethanol production.

3.3. Effect of pH in the Media on the Growth of Alkali-Tolerant Yeasts

Using the representative strains of nine species described above in Section 3.1, we examined the effect of pH on the growth of the strains. The growth curve of each of the nine strains is provided in **Figures 1-9**. Two strains (*Rhodotorula mucilaginosa* K12 and *Candida intermedia* S4) showed high growth at pH 3 - 10 (**Figure 1** and **Figure 2**). Five strains (*Rhodosporidium fluviale* N7, *Schefferso-myces spartinae* N8, *Wicherhamonyces anomalus* N10, *Cyberlindnera saturnus* N11, and *Candida quercuum* S3) showed high growth at pH 3 - 9 (**Figures 3-7**). Two strains (*Cryptococcus liquefacience* K2 and *Candida* sp. S1) showed high growth at pH 4 - 8 (**Figure 8** and **Figure 9**). Thus, all nine species isolated as alkali-tolerant yeasts in this study were found to have both alkali tolerance and acid tolerance.

Most importantly, *Rhodotorula mucilaginosa* K12 and *Candida intermedia* S4 had high growth activities at the broad range of pH (3.0 to 10.0), and strain S4 also had fermentation activities. Saito *et al*. reported that *C. intermedia* 4-6-4T2, an acid-tolerant mutant from parent strain *C. intermedia* 10601, was obtained from strain 10,601 by repeated fermentation and cultivation under the addition of acetic acid. They observed effective ethanol production by *C. intermedia* 4-6-4T2 from hemicellulose hydrolysate containing both glucose and xylose.

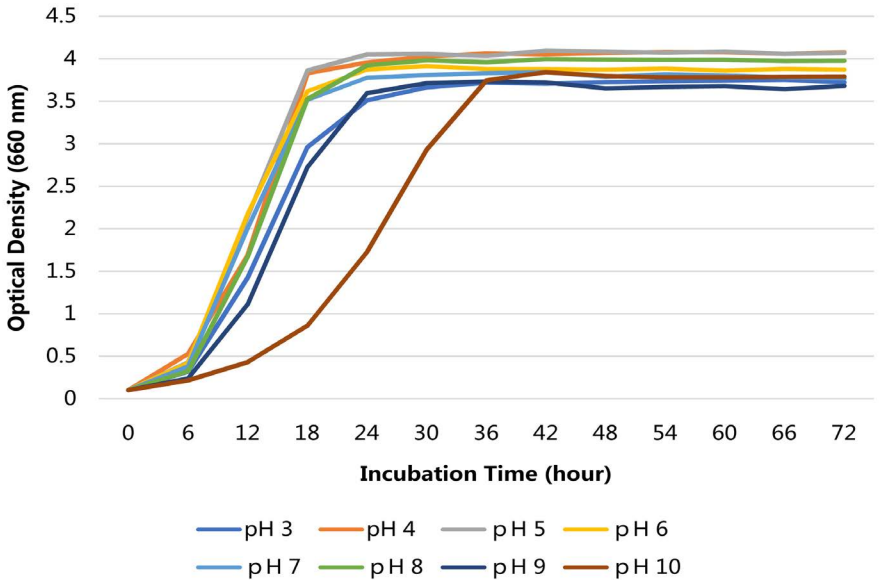

Figure 1. The growth of *Rhodotorula mucilaginosa* K12 in YPD medium at initial pH values ranging from 3.0 to 10.0. The values are the mean of triplicate cultures. The standard deviation (SD) values were omitted in order to clarify the growth curves.

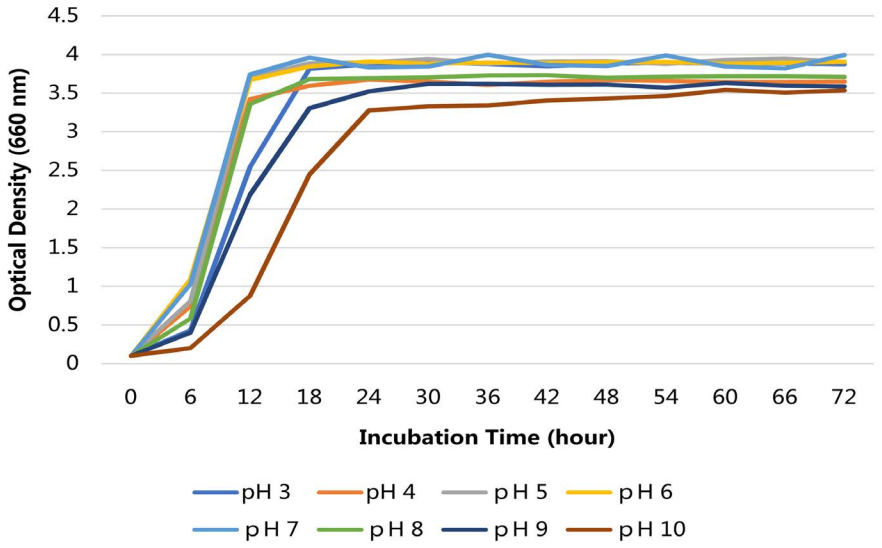

Figure 2. The growth of *Candida intermedia* S4 in YPD medium at initial pH values ranging from 3.0 to 10.0. The values are the mean of triplicate cultures. The SD values were omitted in order to clarify the growth curves.

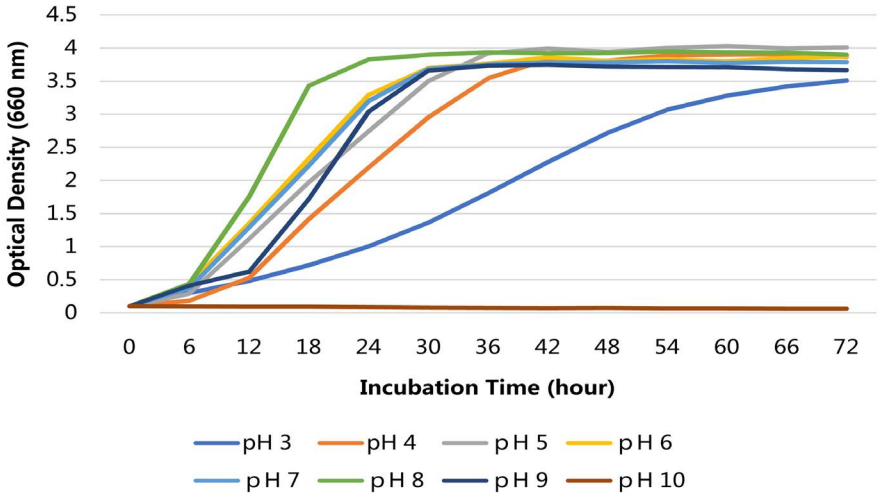

Figure 3. The growth of *Rhodosporidium fluviale* N7 in YPD medium at initial pH values ranging from 3.0 to 10.0. The values are the mean of triplicate cultures. The SD values were omitted in order to clarify the growth curves

Strain 4-6-4T2 simultaneously assimilated 25 g/L glucose and 25 g/L xylose and produced ethanol at 1.0 g/L/hr, with a total of 50 g/L [8]. We thus considered *Candida intermedia* S4 the most suitable candidate for effective bioethanol from waste cellulosic biomass.

Lisichkina *et al*. reported alkali-tolerant yeasts from natural biotopes, *i.e*. soda-rich soils of Armenia and the Transbaikal region of Russia [9]. They cultured the yeast in pH 10.0 medium, and the yeasts were identified as *Cryptococcus laurentii*, *Candida albicans*, *Rhodotolula glutinis*, *Rhodotolula mucilaginosa*, and *Sporobolemyces roseus*. We observed that these yeasts' species are in good agreement with those of the alkali-tolerant yeasts in the present study.

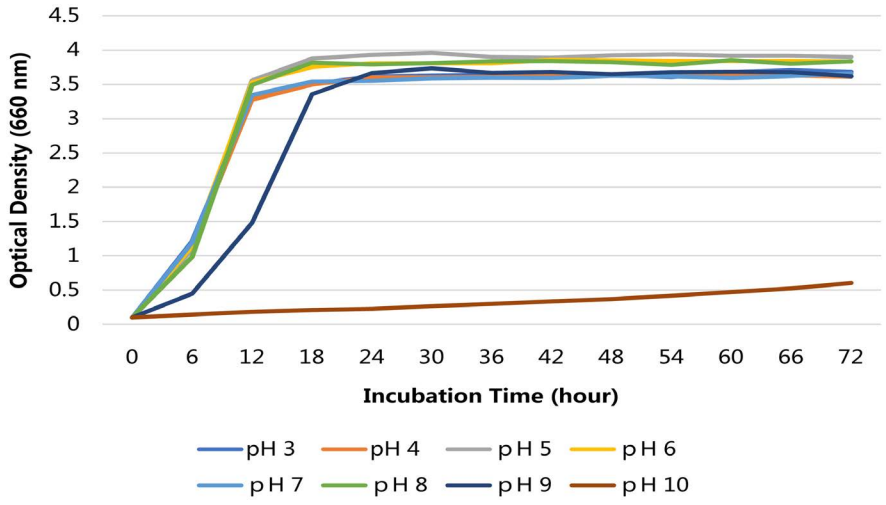

Figure 4. The growth of *Scheffersomyces spartinae* N8 in YPD medium at initial pH values ranging from 3.0 to 10.0. The values are the mean of triplicate cultures. The SD values were omitted in order to clarify the growth curves.

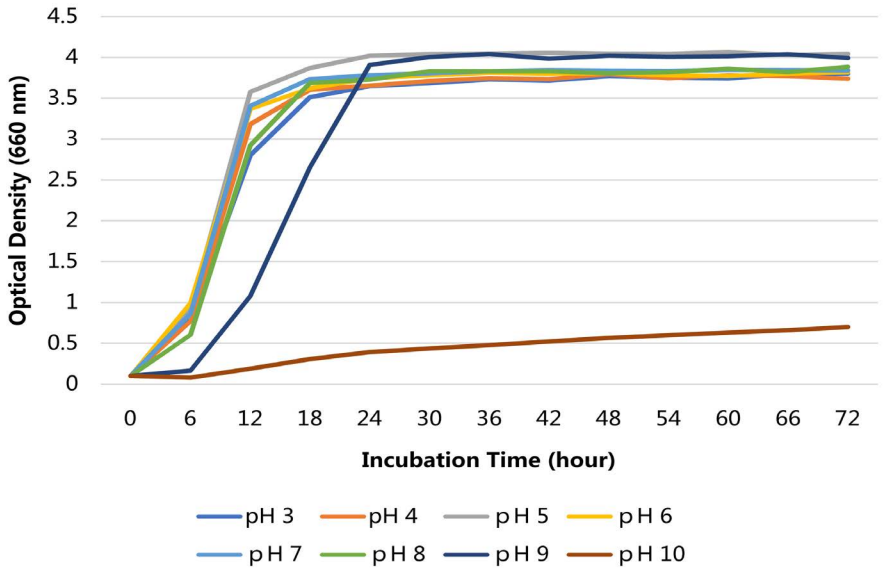

Figure 5. The growth of *Wicherhamonyces anomalus* N10 in YPD medium at initial pH values ranging from 3.0 to 10.0. The Values are the mean of triplicates (Standard deviation was omitted to clarify the growth curves).

3.4. Changes in the pH Values in the Yeast Cultures

We cultured representative strains of nine species at the initial pH values of 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 and then measured the changes of the pH values after 3 days' cultivation (Table 4). At initial pH 3.0, the pH of three strains (K2, K12, and N11) increased to >4.0 in the media. At the initial pH 4.0, the pH of two other strains (N7 and N11) increased to >7.0 in the media. At the initial pH 5.0, the pH of eight strains (S1, S4, K2, K12, N7, N8, N10, and N11) increased to >7.0 in the media. At the initial pH 6.0, the pH of all nine strains increased to >7.0 in the media. Therefore, all nine strains were observed to have

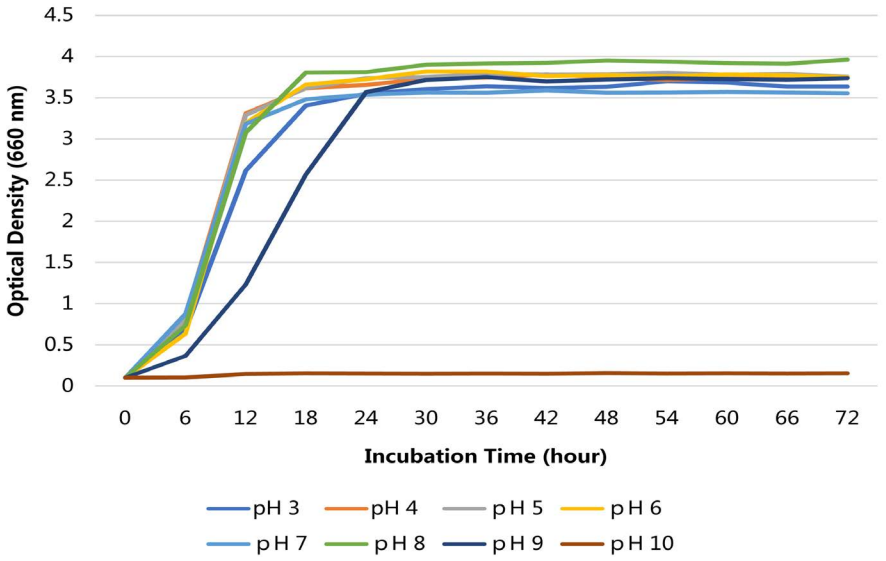

Figure 6. The growth of *Cyberlindnera saturnus* N11 in YPD medium at initial pH values ranging from 3.0 to 10.0. The values are the mean of triplicate cultures. The SD values were omitted in order to clarify the growth curves.

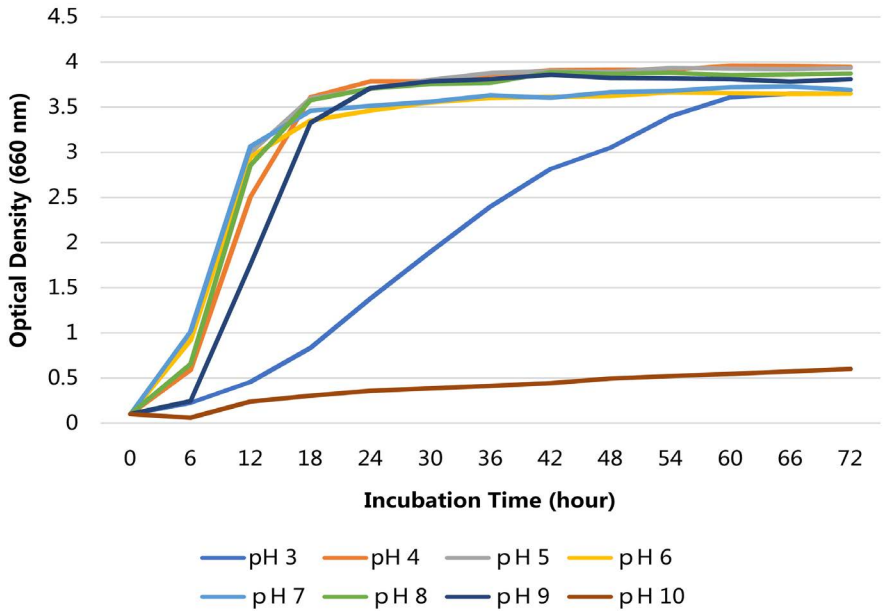

Figure 7. The growth of *Candida quercuum* S3 in YPD medium at initial pH values ranging from 3.0 to 10.0. The values are the mean of triplicate cultures. The SD values were omitted in order to clarify the growth curves.

acid-neutralizing activities by releasing ammonium ions (data not shown).

3.5. Polygenetic Tree of pH-Tolerant Yeasts

The nine species of alkali-tolerant yeasts had pH tolerance over a broad range of pH, and their genera and species were very similar to those of the acid-tolerant yeasts that were isolated previously [1] [2] [3]. The yeasts that we used for construction of the polygenetic tree are summarized in **Table 5**. The polygenetic

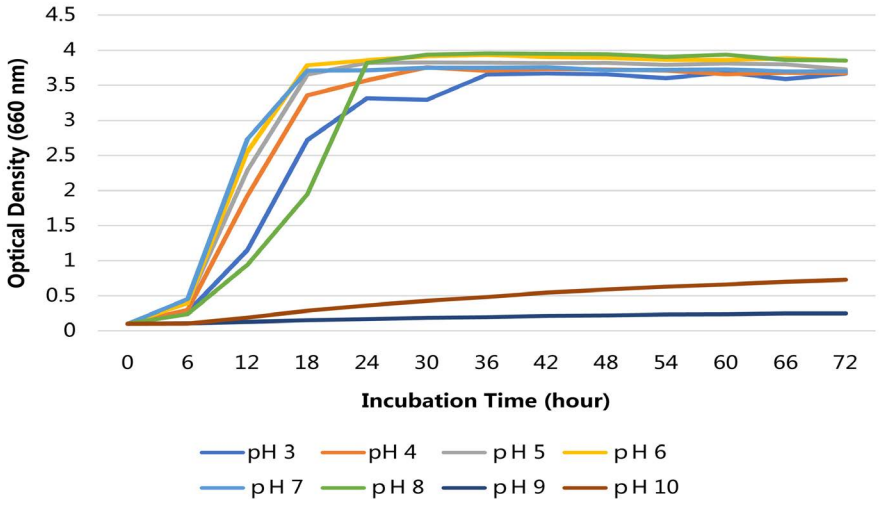

Figure 8. The growth of *Cryptococcus liquefacience* K2 in YPD medium at initial pH values ranging from 3.0 to 10.0. The values are the mean of triplicate cultures. The SD values were omitted in order to clarify the growth curves.

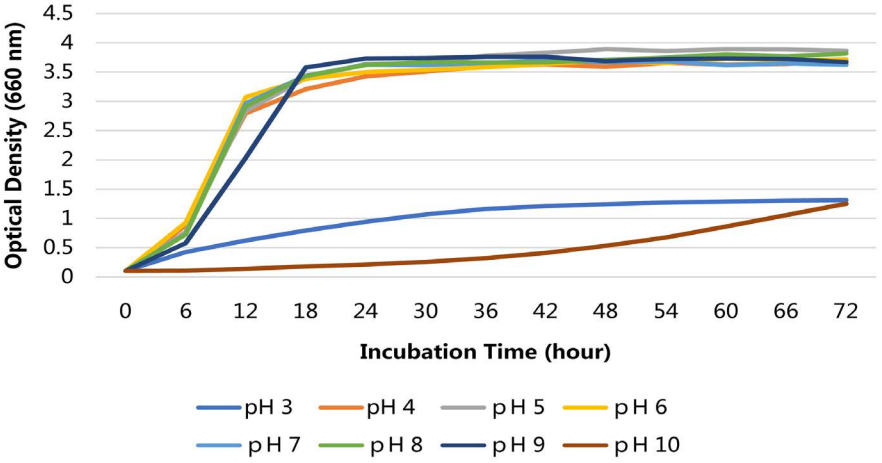

Figure 9. The growth of *Candida* sp. S1 in YPD medium at initial pH values ranging from 3.0 to 10.0. The values are the mean of triplicate cultures. The SD values were omitted in order to clarify the growth curves.

trees of the acid-tolerant yeasts (26 strains) and alkali-tolerant yeasts (nine strains) are shown in **Figure 10**. These trees were constructed on the 28s rRNA gene sequences by the maximum likelihood algorithm in MEGA version 6.06.

Based on these results, both acid-tolerant yeasts and alkali-tolerant yeasts were situated in the same classification position. Of the nine strains, K2 was the sister group of the clade that includes *Filobassidium magnum* mi-w16. K12 and N7 were the sister group that includes *Rhodotolura* sp. n-w29, si-w12, *Leucosporidium golubevii* si-w13, and *Microbotryozyma collariae* sm-w40. The strains S1, S3, S4, N8, N10, and N11 were the sister group that includes *Candida* sp. om-w46, h-m7, *Meyerozyma guilliemondii* mr-w1, *Candida parapsiosis* h-m7, and *Candida oleophila* n-w33. Thus, similar yeast species with a broad range of pH tolerance were found to be living in natural aquatic environments at a broad

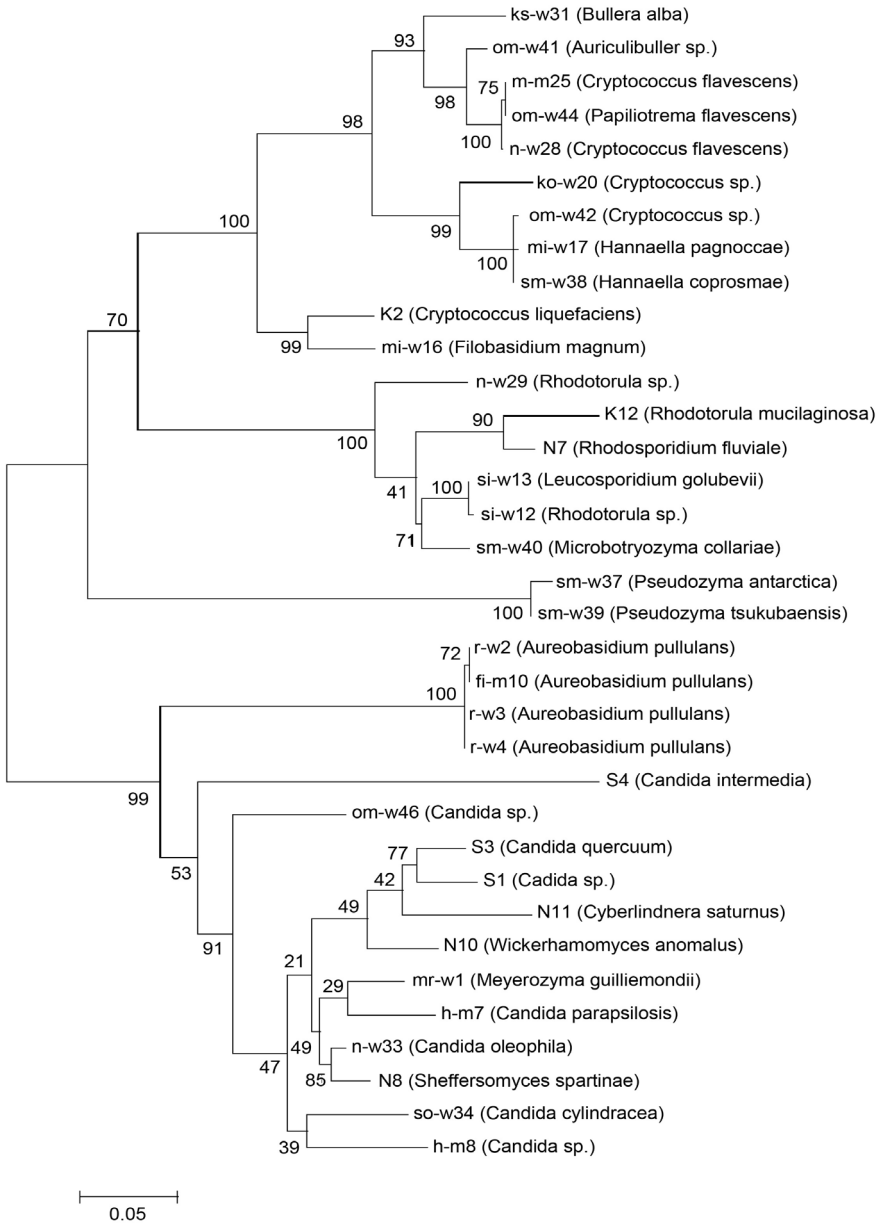

Figure 10. The polygenetic tree of both acid-tolerant yeasts (26 strains) and alkali-tolerant yeasts (nine strains). It was constructed on the 28s rRNA gene sequences by the maximum likelihood algorithm in MEGA ver. 6.06.

range of pH from alkali to acid.

4. Discussion

We have been studying the characterization and application of aquatic yeasts that could be suitable for bioethanol production, and we developed several types of bioethanol production systems that use cellulosic biomass such as seaweeds, *i.e. Undaria pinnafida* [10] [11] [12], *Ulva* spp., and *Costaria costata* [10] [11] [12], an alien aquatic plant in Japan, *Eichhornia crassipes* [13] [14]; and paper or wood scrap [11] [15] along with aquatic yeasts. In bioethanol production from a

Table 4. Change of pH after 3 days' cultivation of alkali-tolerant yeasts.

Strain		Starting pH of the medium							
		pH 3	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9	pH 10
Rhodotorula mucilaginosa	K12	4.875	6.873	7.989	8.171	8.129	8.440	9.189	9.597
Rodosporidium fluviale	N7	3.330	7.167	7.504	7.286	7.294	7.798	8.535	9.781
Cryptococcus liquefaciens	K2	4.842	6.858	7.650	8.084	8.543	8.825	9.513	10.242
Candida sp.	S1	3.178	5.046	7.484	7.920	7.879	8.410	9.096	9.540
Candida quercuum	S3	2.980	3.756	4.826	7.655	8.813	8.813	9.368	10.092
Candida intermedia	S4	2.865	4.188	7.112	7.658	8.047	8.649	9.434	10.019
Scheffersomyces spartinae	N8	3.703	6.648	7.377	7.532	7.600	8.209	9.079	9.628
Wickerhamomyces anomalus	N10	2.929	4.049	7.059	7.536	7.925	8.500	9.321	9.907
Cyberlindnera saturnus	N11	4.272	7.433	7.950	8.199	8.248	8.580	9.328	9.830

Each value is a final pH of the medium.

cellulosic biomass, acids or alkalis are used for the hydrolysis of the materials, and thus the direct fermentation of hydrolysates containing oligosaccharides by yeasts without neutralizing themselves is desirable, and the concentration of the biomass should be higher from an economical point of view. However, as highly concentrated hydrolysates contain high levels of oligosaccharides and rich salts and since their pH values are acid or alkali, the yeasts should have high fermentative activities under osmotic pressure stress, salt stress, and pH stress.

We previously surveyed yeasts from the coast of Tokyo Bay to isolate highly fermentative yeasts under concentrated substrates, and most of the superior yeast strains were *Saccharomyces cerevisiae* [16] [17] [18] [19]. We later isolated *Citeromyces matritensis* M37 from Tokyo Bay as a high salt-tolerant yeast that produces ethanol, and we observed its effective fermentation from salted algae [20].

The remaining problem is the application of pH-tolerant yeasts. We have surveyed pH-tolerant yeast strains in several types of environment. We isolated acid-tolerant yeasts from acidic or neutral streams in Japan; their classification is shown in **Figure 10** [1] [2] [3]. Gadanho *et al*. described the yeast diversity in the extreme acidic environments of the Iberian Pyrite Belt, and they tested the yeast community in terms of high, intermediate, and low environmental stress. They identified *Cryptococcus* sp. under high stress (pH 1.8 and a high concentration of heavy metals), *Rhodosporidium toruloides*, *Cryptococcus* sp., *Rhodotorula* sp., and *Candia fuviatilis* under intermediate stress (pH 2.5 - 3.0 and intermediate concentrations of heavy metals), and *Williopsis californica*, *Cryptococcus* sp., *Rhodotorula* sp., *Rhodotorula bogoriensis*, *Cryptococcus albidosimilis*, and *Bullera unica* under low stress (pH 2.5 - 3.0 and low concentrations of heavy metals) [21]. Thus, there seemed to be strong similarity regarding the types of yeast species in the acidic environments between Japan and Portugal. In the present study we obtained alkali-tolerant yeasts with pH tolerance that was very similar to that of acid-tolerant yeasts. Acid-tolerant yeasts and alkali-tolerant

Table 5. The yeasts used for construction of the polygenetic tree in **Figure 10**.

Strain	
Meyerozyma guilliermondii	mr-w1
Aureobasidium pullulans	r-w2, 3, 4, fi-m10
Rhodotorula sp./*Leucospridium golubevii*	si-w12, 13
Filiobasidium magunum	mi-w16
Hannaella pagnoccae	mi-w17
Cryprococcus sp.	ko-w20
Cryptococcus flavescens	m-m25, n-w28
Rhodotrula sp.	n-w29
Bullera alba	ks-w31
Pseudozyma antarctica	sm-w37
Hannaella coprosmae	sm-w38
Pseudozyma antarctica	sm-w39
Microbotryozyma collariae	sm-w40
Auriculibuller sp.	om-w41
Cryptococcus sp.	om-w42
Papiliotrema flavescens	om-w44
Candida sp.	m-w46
Candida sp.	S1
Candida quercuum	S3
Candida intermedia	S4
Cryptococcus liquefaciens	K2
Rhodotorula mucilaginosa	K12
Rhodosporidium fluviale	N7
Scheffersomyces spartinae	N8
Wickerhamomyces anomalus	N10
Cyberlindnera saturnus	N11

yeasts were also found to be situated in the same classification position.

5. Conclusions

We surveyed alkali-tolerant yeasts from natural aquatic environments at pH 7.6 - 9.4 in the Kanto region of Japan and isolated 35 strains of yeast that grew in medium at pH 9.0. These strains were identified as members of seven genera and nine species: 25 strains (N1, N2, N3, N4, N5, N6, N9, K1, K3, K4, K5, K6, K7, K8, K9, K10, K11, K12, K13, K14, K15, K16, K17, K18, and K19) were *Rhodotorula mucilaginosa*; one strain (N7) was *Rhodosporidium fluviale*; one strain (N8) was *Scheffersomyces spartinae*; two strains (N10 and N13) were *Wicherhamonyces anomalus*; one strain (N11) was *Cyberlindnera saturnus*; one strain (S1) was *Candida* sp.; two strains (S2 and S4) were *Candida intermedia*; one strain (S3) was *Candida quercuum*; and one strain (K2) was *Cryptococcus liquefa-*

cience.

We examined the effect of pH on the growth of representative strains of the nine species. Strains K12 and S4 showed high growth at pH 3 - 10. Five strains, *i.e*. N7, N8, N10, N11, and S3 showed high growth at pH 3 - 9. Strains K2 and S1 showed high growth at pH 4 - 8. All nine strains had neutralizing activities from acidic media at pH 3 - 5 to pH 6 - 8. The alkali-tolerant yeasts and acid-tolerant yeasts [1] [2] [3] were found to be similar species, to grow well in a broad pH range, and to have neutralizing activities from acid media to pH 6 - 8. We constructed the phylogenetic trees of the acid-tolerant strains and the alkali-tolerant strains, and all of the strains were situated in the same classification position. Similar yeast species with a broad range of pH tolerance were found to be living in natural aquatic environments at pH values from alkali to acid. Most importantly, *Candida intermedia* S4 showed high growth at pH values from 3.0 to 10.0, had fermentative activity, and seems to be a candidate for bioethanol production from cellulosic biomass.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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