Distribution of Halotolerant and/or Fermentative Yeasts in Aquatic Environments

Journal of Tokyo University of Fisheries, Vol. 87, pp. 23–29, 2001

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(Received July 31, 2000)

Abstract: This study deals with halotolerant and/or fermentative yeasts living in various aquatic environments: upper stream of Arakawa river, middle and lower streams of Tamagawa rivers and sea coasts of Kemigawa in Chiba prefecture and of Chemigahama in Choshi city. Colony forming units of the yeasts decreased with the increase of osmotic pressure or salt concentration and increased with the increase of total organic carbon in aquatic areas. The average limit concentration of NaCl for yeast growth and fermentation in the sea coasts were 2.3–2.5 M and those in the rivers were 1.2–1.9 M. The existence ratio in number of fermentative yeasts to total yeasts under 2.5 M NaCl was 17–31% in the sea coasts and 0–4% in the rivers, respectively. Therefore, high salt tolerant and fermentative yeasts were found to inhabit largely the sea coasts.

***Key words*:** *Yeast, Halotolerant, Aquatic environment, Fermentation*

Introduction

Many kinds of microorganisms have been utilized in the long history of mankinds. Above all, yeasts hold very important places in our lives. In the 19th century, Pasteur demonstrated the essential participation of live yeasts in the fermentation process. Since that time a small number of species of the genus *Saccharomyces* have been produced and used in the manufactures of food, drink, chemical products, fuel, and single cell protein. *Saccharomyces* spp. were originally isolated from the sap of trees in terrestrial area and purely cultivated in fermentation tanks in a long period. There were also known to live various kinds of genus with fermentative phenotype except for *Saccharomyces* in both terrestrial and aquatic environments. A large number of studies have been reported about terrestrial fermentative yeasts and on the other hand, there have been few reports about aquatic fermentative yeasts. In our study, several kinds of marine yeasts were isolated from Japanese coastal water and characterized as a new fuel resource which produces ethanol from biomasses[1]. However, there remains much unknown phenomena about aquatic yeasts including their origin and characters. This paper deals with isolation and characterization of salt tolerant and fermentative yeasts in various aquatic environments. These results are thought to elucidate the distribution of the yeasts in aquatic environments, followed by the establishment of isolation procedure for the superior yeasts in industries from aquatic areas.

Materials and Methods

Chemical Analyses of the Water

In order to investigate the environments living aquatic yeasts, three kinds of analyses of natural waters were performed; osmotic pressure, salt concentration , and total organic carbon. Osmotic pressure of each water was measured using an osmometer OSM-1 (Shimadzu Seisakusho Co. Ltd.), salt concentration was analysed by Fajans' method[2], and total organic carbon was detected using a TOC 5000 analyzer (Shimadzu Seisakusho Co. Ltd.).

Isolation of Aquatic Yeasts

Each 500 ml of water was collected into a sterilized polyethylene bottle from the surface of aquatic environment in Japan, ① Arakawa river at Nagatoro (ANA), Saitama prefecture on October, 24th, 1997, ② Tamagawa river at Den-enchofu (TDE), in Tokyo metalopolitan on June, 17th, 1997, ③ Tamagawa river around Haneda (THA), in Tokyo metalopolitan on June, 17th, 1997, ④ Kemigawa coast in Chiba city (KM) on January, 14th, 1998, ⑤ Chemigahama coast in Choshi city (CM) on December, 3rd, 1997. A portion of the water (400 m*l*) was passed through a membrane filter (pore size: 0.45 μm, Nihon Millipore Ltd.) and the filter was soaked in 1.5 m*l* of the fresh water, and shaked vigously. Each 150 μ*l* of the water was spread onto a YPD agar medium (2% glucose, 2% Bacto-peptone (Difco), 1% Bacto-yeast extract (Difco), and 0.01% chloramphenicol) and incubated aerobically at 20°C for 3 days. After incubation, the developed colonies on the plate were picked up and observed

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Table 1. Chemical analysis of sample water from aquatic environments

	ANA	TDE	THA	KM	CM
Osmotic pressure (Osm/kg)	>0.10	>0.10	0.57	0.90	0.90
Salt concentration (M)	0.30×10^{-2}	0.10×10^{-1}	0.32	0.50	0.50
Total organic carbon (ppm)	27	49	34	34	21

ANA: Arakawa river at Nagatoro TDE: Tamagawa river at Den-en-chofu
THA: Tamagawa river at Haneda KM: Kemigawa coast at Chiba
CM: Chimigahama coast at Choshi

under a microscope IX70 (Olympus Optical. Co.) with a CCD camera HCC 1,600A (Flovel Co. Ltd.), a still video recorder VR300 (Olympus Optical Co.), and a color video printer UP-5000 (Sony Co. Ltd.) and yeast strains were isolated by observing morphologically. In each aquatic environment, yeasts were isolated and the strains were randomly numbered as No. 1, 2,3···. From the numbered strains, the yeasts with morphologically different shapes in each environment were selected and they were tested for fermentation and halotolerance.

Fermentation Test of Yeasts

The isolated strains were inoculated into 10 m*l* of a YPD liquid medium in a 30 m*l* volume test tube with a Durham tube and incubated at 20°C for 3 weeks. The fermentation activity of yeast was tested by observing fullness of CO_2 gas in a Durham tube.

Halotolerance Test of Yeasts

Halotolerance of yeasts was measured by growth or fermentation under various concentration of NaCl. Each yeast strain was inoculated into a YPD liquid medium containing 0.5–3.5 M NaCl in a test tube with a Durham tube and incubated at 20°C for 3 weeks. Both growth and fermentation activities of the yeast were observed by naked eyes.

Results and Discussion

Chemical Analyses of the Water

Table 1 shows chemical analyses of the waters from ①–⑤. Both osmotic pressure (Osm/kg) and salt concentration (M) were very low at ANA and TDE and high at THA, KM, and CM. From these results, tolerant microorganisms against high osmotic pressure or high concentration of salt were thought to be in the lower reaches of the rivers or sea coasts. Total organic carbon (ppm) was the highest at TDE in the middle reach of the river. In sea coasts, total organic carbon at KM was higher than that at CM. Therefore, in this study, the effect of osmotic pressure, salt concentration or pollution in aquatic areas on the living yeasts were investigated.

Table 2. Colony forming units of yeasts collected from various areas

	ANA	TDE	THA	KM	CM
Colony forming units	177	650	170	63	15

Colony forming units: Number of colonies from 1L of sample water.
ANA: Arakawa river at Nagatoro
THA: Tamagawa river at Haneda
TDE : Tamagawa river at Den-en-chofu
KM : Kemigawa coast at Chiba
CM : Chimigahama coast at Choshi

Isolation of Yeasts from Various Aquatic Areas

Yeast strains were isolated from ANA, TDE, THA, KM, and CM. Colony forming units of the yeasts on the YPD agar medium are shown in Table 2. The unit of TDE strains was 650 which was the highest among those of the strains from the rivers. The unit of ANA and THA strains decreased with the decrease of total organic carbon. In the sea coasts, the unit of KM strains was also higher than that of CM. As the unit of yeasts was thought to be related to total organic carbon at the aquatic areas, it is expected to become new indexes of water pollution. Further detailed analyses about the relationship between the unit of yeast and total organic carbon should be carried out. As the units of yeasts from the sea coasts were 1/40–1/10 of those from the rivers, many yeasts were found to be existed in the aquatic areas with low osmotic pressure and low salt concentration such as the upper or middle reaches of river. On the other hand, the environment with high osmotic pressure, high salt concentration, and low total organic carbon was relatively severe for living yeasts as shown in the units at CM.

Morphological Characterization of Yeasts

The strains were characterized morphologically under the microscope. Figure 1 shows photographs of the por-

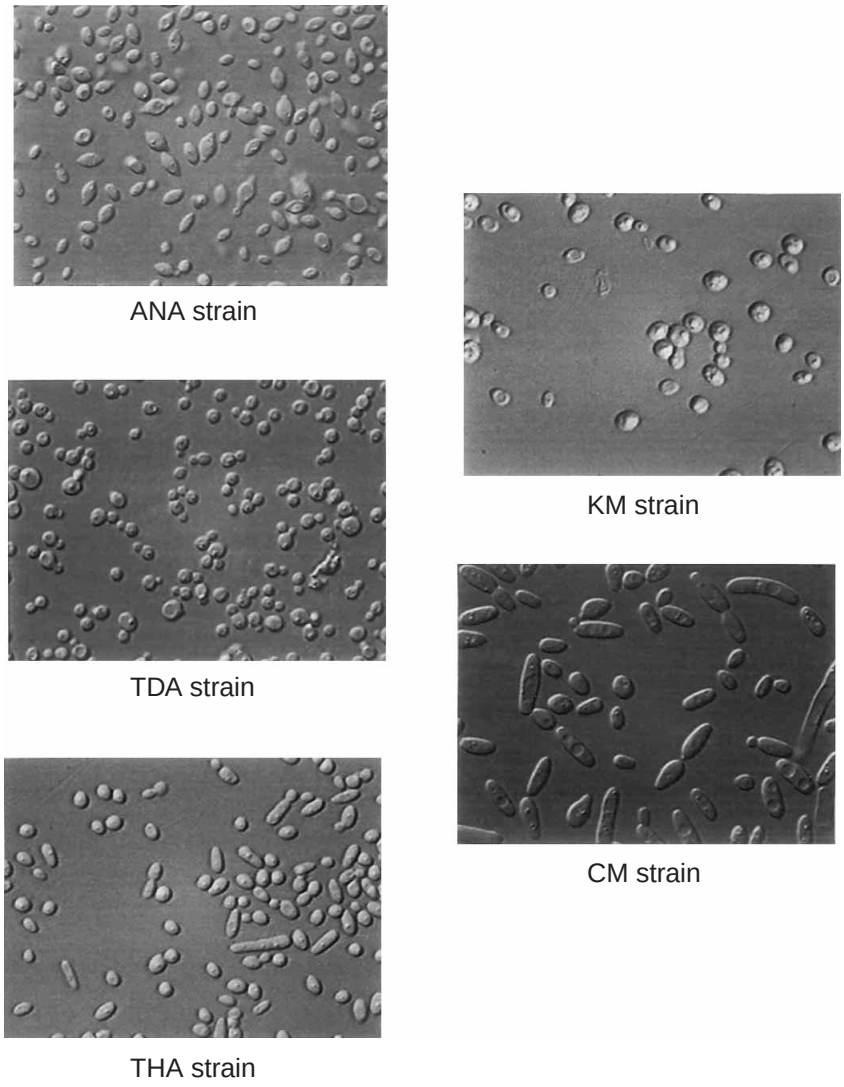

Figure 1. Photograph of the portion of isolated yeasts.

tion of isolated strains. Various kinds of yeasts with different shapes were observed under the microscope. Table 3 shows the number of morphologically different yeasts at various districts. Colony forming units of the yeasts from the rivers were over 10 folds higher than those from the sea coasts. On the other hand, the number of yeasts with different shapes was almost the same among various areas and there seemed to live many yeasts morphologically similar to each other in the rivers. However, there seemed to live different yeasts in the taxonomy in each aquatic area and further studies are directing towards detailed identification of the yeasts.

Table 3. Numbers of isolated yeasts with different shapes

	ANA	TDE	THA	KM	CM
Isolated yeasts	11	25	20	13	6

These values were obtained by counting yeasts under a microscope.

ANA: Arakawa river at Nagatoro
THA: Tamagawa river at Haneda
TDE : Tamagawa river at Den-en-chofu
KM : Kemigawa coast at Chiba
CM : Chimigahama coast at Choshi

Halotolerance of the Yeasts

Figures 2–6 show limit concentration of NaCl for growth (LNG) and fermentation (LNF) of the yeast strains. The axses of abscissa show the strain Nos. In every aquatic areas, LNG widely ranged 0–3.0 M except for strains Nos. 5 and 19 in KM which had 3.5 M of LNG. Otherwise, LNFs of ANA, TDE, and THA strains ranged 0–2.0 M except for strain No. 1 and those of KM and CM strains generally ranged 0–2.5 M. It is noteworthy that there were some yeasts which could only grow without NaCl out of the isolates from sea coasts though seawater contains about 0.6 M of salts. These yeasts seemed to be carried from other places such as rivers or soils to sea coasts and might not be fit for high salt condition. Table 4 shows the averages of LNG and LNF. The averages of ANA, TDE, and THA strains were 1.2–1.9 M and those of KM and CM strains were 2.3–2.5 M. The averages also increased gradually from upper to down streams of the river and reached the highest at the seawater. The yeasts were found to have acquired salt tolerance by adapting their environments. Recently, various studies about marine yeasts have been reported, i.e. isolation of oil-degrading and hydrocarbon assimilating yeasts[3–8], feed for *Brachionus plicatilis*[9], osmotic regulation[10,11], ecology of aquatic yeasts[12], isolation of marine killer yeasts[13], characterization of rRNA[14,15] and construction of host-vector system in marine *Debaryomyces hansenii*[16–21]. However, the detailed ecology of marine yeasts remains unknown and the correct difinition to the name of marine yeast has not been carried out, yet. We consider that the yeasts from seawater are divided into

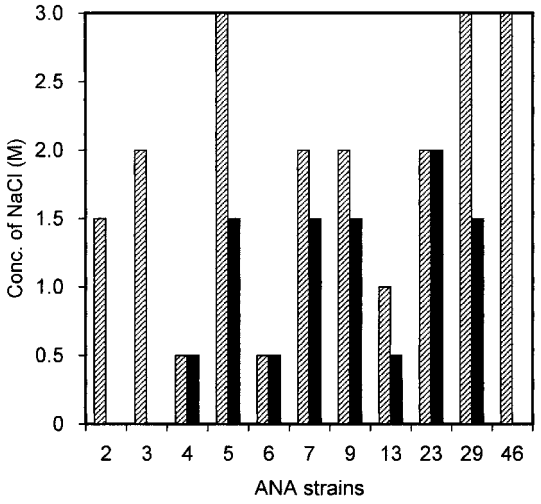

Figure 2. Limit concentration of NaCl for yeast growth and fermentation in ANA strains.
□ Growth
■ Fermentation

Table 4. Averages of limit concentration of NaCl for growth and fermentation of yeasts

	Limit conc. of NaCl (M)				
	ANA	TDE	THA	KM	CM
Growth	1.9	1.7	1.9	2.5	2.3
Fermentation	1.2	1.8	1.9	2.3	2.3

These values were calculated from Figs. 2–6.
ANA: Arakawa river at Nagatoro
THA: Tamagawa river at Haneda
TDE : Tamagawa river at Den-en-chofu
KM : Kemigawa coast at Chiba
CM : Chimigahama coast at Choshi

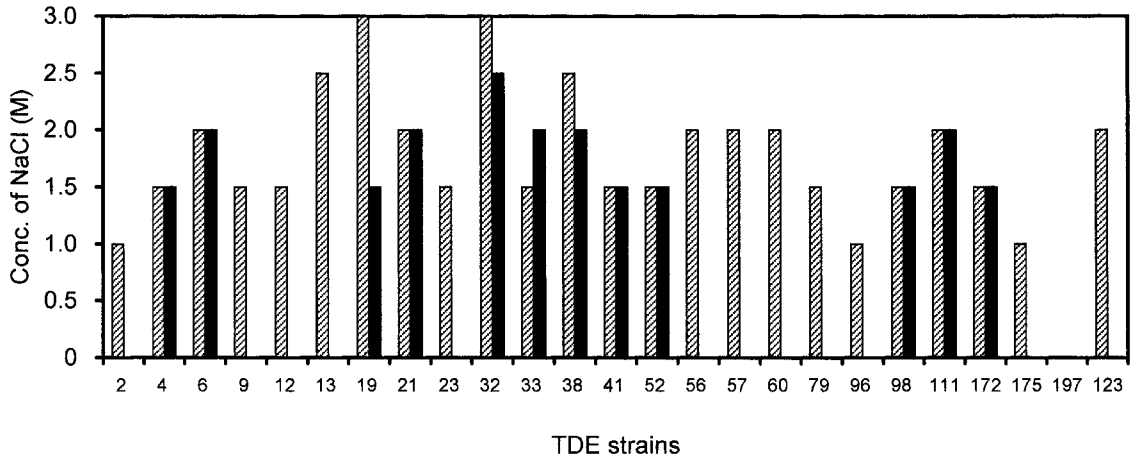

Figure 3. Limit concentration of NaCl for yeast growth and fermentation in TDE strains.
□ Growth
■ Fermentation

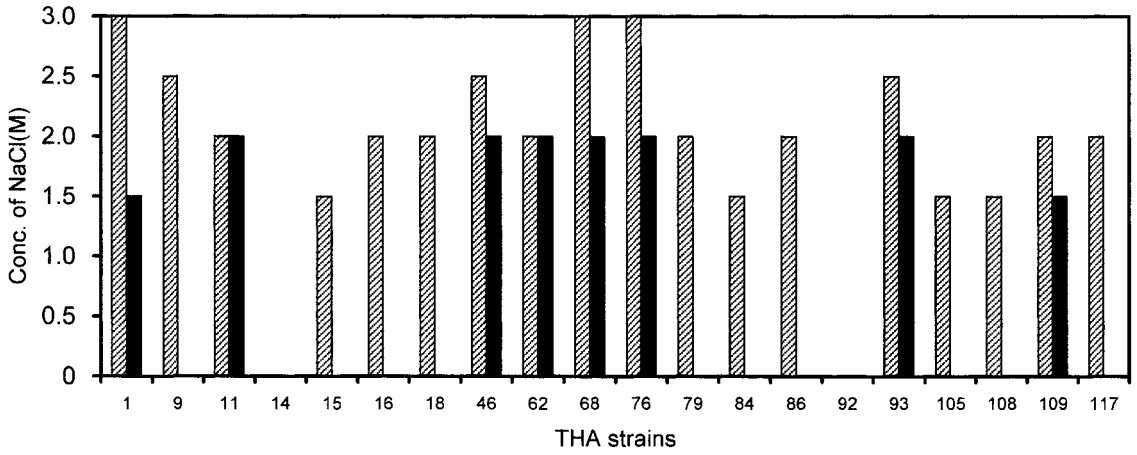

Figure 4. Limit concentration of NaCl for yeast growth and fermentation in THA strains.
□ Growth
■ Fermentation

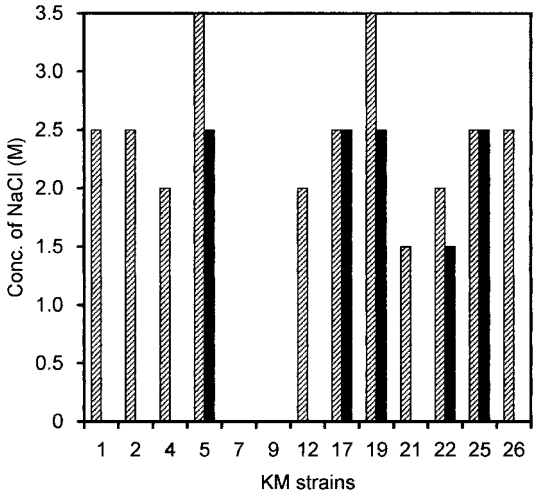

Figure 5. Limit concentration of NaCl for yeast growth and fermentation in KM strains.
□ Growth
■ Fermentation

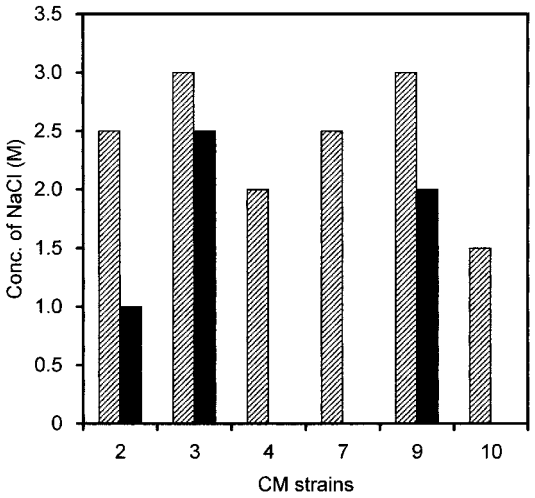

Figure 6. Limit concentration of NaCl for yeast growth and fermentation in CM strains.
□ Growth
■ Fermentation

two categories. One is strict marine yeasts; they originate from the marine and inhabit the seawater through their lives. The other is facultative marine yeasts; they originate from other environments such as rivers, soils, woods, or the surface of animals and are transported to marine environment. One of the main characters of strict marine yeasts was high NaCl tolerance[1)] and they seem to have the phenotypes, inherently. Otherwise, facultative marine yeasts seem to aquire high NaCl tolerance, gradually in long periods. In this study, the yeasts with no or weak salt tolerance from sea coasts might be facultative marine yeasts. In further studies, in order to demonstrate the hypothesis, repeated cultivations of no and weak salt tolerant yeasts in NaCl-rich media will be carried out and the transformation of them to high salt tolerant ones will be observed.

Fermentation Activities of the Yeasts

Table 5 shows ratio in number of fermentative yeasts to total yeasts under various concentration of NaCl. The ratios of fermentative yeasts to all ones gradually decreased with increase of NaCl. The ratios under 2.0 M

Table 5. Ratio in number of fermentative yeasts to total yeasts (%) under various concentration of NaCl

Conc. of NaCl (M)	ANA	TDE	THA	KM	CM
0.5	73	48	40	ND	50
1.0	45	48	40	ND	50
1.5	45	48	40	38	33
2.0	9	24	30	31	33
2.5	0	4	0	31	27
3.0	0	0	0	0	0

ANA: Arakawa river at Nagatoro
THA: Tamagawa river at Haneda
TDE : Tamagawa river at Den-en-chofu
KM : Kemigawa coast at Chiba
CM : Chimigahama coast at Choshi

NaCl increased in upper, middle, and lower streams of the river and sea coasts in turn. The ratios under 2.5 M NaCl were 0–4% in the rivers and 17–31% in the sea coasts. Therefore, fermentation activities under high salt conditions also seemed to be one of the main characters in marine yeasts

Further studies are directing towards more detailed charcterization of the ecological features of aquatic yeasts, followed by isolation of various yeasts. As we have been planning the construction of a new fuel resource which produces ethanol from marine biomass containing rich salt, we are aiming at the isolation of candidate strains with high slat tolerance and ethanol productivity.

Acknowledgements

This work was supported partly by The Fund for Research Encouragements in Celebration of the Cential Anniversary for the Founding of Tokyo University of Fisheries. We are grateful to Mr. Hiroyuki Hirai (Laboratory of Marine Biochemistry, Tokyo University of Fisheries) for experimental supports.

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水圏環境における耐塩性・発酵性酵母の分布

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水圏（荒川の上流，多摩川の中・下流，検見川浜海岸／千葉，君ケ浜／銚子）に生息する耐塩性および発酵性酵母の分布について調べ，栄養寒天培地上での酵母コロニー形成数は水圏の浸透圧や塩濃度の増加に伴い減少し、逆に有機炭素量の増加に伴い増大した。酵母の増殖および発酵における限界 NaCl **濃度の平均値は沿岸の酵母の場合** 2.3–2.5 M **であり、また河川の酵母の場合** 1.2–1.9 M **であった。分離酵母に占める発酵性酵母の割合は、**2.5 M NaCl **存在下で河川の酵母では** 0–4%**、沿岸の酵母では** 17–31% **であり，高塩耐性・発酵性の酵母は主として沿岸に生息することがわかった。**

キーワード：酵母，耐塩性，水圏環境，発酵