Isolation of the Yeasts from the Spoilt Soft Drinks

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Japanese soft drinks, such as *Saidâ* and *Remon*, derived from cider and lemon respectively, are carbonated water, to which syrup and apple essence are added. It should contain 10 % of the sugars and 0.7 % of the acids (as citric acid), and a pressure of 40 lb/inch^2 of carbon dioxide at room temperature should be applied on it. Up to 1968, sodium cyclohexyl sulfamate (sodium cyclamate) had been employed as a sweetening agent in place of sugars, but use of this sweetening agent was forbidden in 1969. Since then, glucose or saccharose, or both, have been employed for the sweetening agent of Japanese soft drinks. Such preservatives, as benzoic acid and sorbic acid, have been employed in some countries of Europe[1], but recently in Japan, application of preservatives to foods and drinks has been, if possible, kept away. In 1968, SAND has isolated 150 strains of yeasts from soft drinks placed on the market in Europe. 49 % of the isolates belonged to the genus *Candida*, 35 % to the genus *Saccharomyces*, 5 % to the genus *Torulopsis*, 4 % to the genus *Rhodotorula*, 2.5 % to the genus *Hansenula*, 2.5 % to the genus *Cryptococcus*, and 2 % to the genus *Pichia*[1]. SAND also has isolated 13 strains of yeasts from *Kola*-drinks placed on the market in Europe, difficult to be attacked by microorganisms. Three strains of the isolates from *Kola*-drinks were identified as *Candida parapsilosis*, 2 strains as *Can. solani*, 2 strains as *Saccharomyces willianus*, and each strain of the rests respectively as *Sacch. cerevisiae*, *Sacch. carlsbergensis*, *Can. reukaufii*, *Can. robusta*, *Can. tropicalis*, and *Torulopsis famata*.

Table 1. Values of chemical analysis of Japanese soft drinks.

Soft drinks	pH	direct reducing sugar	total sugar*	Brix
Saidâ	3.02～3.04	2.30%	8.60～9.02%	10.5～10.7%
Remon	2.85	2.30%	8.25%	9.8%

* These values are shown as amounts of saccharose.

The values of analysis of Japanese soft drinks are shown in Table 1. Recently these soft drinks have become cloudy very often, and a deposit has been formed in the bottles of soft drinks. They seemed to be microbiological spoilage of soft drinks. Microbiological investigation of these spoilt soft drinks has been carried out and 3 species of yeasts were isolated.

This paper describes the isolation and determination of 3 species of yeasts from 2 kinds of Japanese soft drinks.

Isolation. The culture media employed for isolation are potato saccharose agar (pH4.2,

Psa), potato glucose agar (pH 7.2, *Pgl*), yeast extract potato glucose agar (yeast extract, 1 g/L of Pgl, pH 7.2, *y-Pgl*), Czapek agar (*Cz*), yeast extract malt extract agar (pH 7.2, *Ymx*), and malt extract agar (Difco malt extract, 25 g/L, pH 7.2, *Mx*)[2]. Incubation were carried out at 30° C in a decicator, in which air was replaced with carbon dioxide. Most of the isolates were obtained from all samples on the yeast extract malt extract agar in carbon dioxide. The rest were isolated on the potato saccharose agar and the potato glucose agar in carbon dioxide from the sample B, and on the potato saccharose agar in air from the sample C, as shown in Table 2. Twenty four isolates were arranged each in their respective cultural groups with their colony appearances, fermentation of sugars, and sugar assimilation. The first group consisted of 16 strains, respective 2 strains from each of the samples A, B, C, D, E, F, and H, on the yeast extract malt extract agar in carbon dioxide. The next group consisted of 4 strains on the potato saccharose agar (B-s-1, 2, 3, and 4), and 2 strains on the potato glucose agar (B-g-1, and 2) from the sample B in carbon dioxide. The last group consisted of 2 strains on the potato saccharose (C-s-1, and 2) from the sample C in air.

Table 2. Isolation of yeasts from Japanese soft drinks.

samples cultivation		*Saidâ* A	B	C	E	F	*Remon* D	G	H
in CO_2 gas	*Ymx*	+	+	+	+	+	+	+	+
	Pgl		+						
	Psa		+						
in air	*Ymx*								
	Pgl								
	Psa			+					

+ : Isolated.

Determination. Determination of the isolates was carried out according to LODDER and KREGER-VAN RIJ's methods and their modification[3]. Twenty strains served for determination.

Candida scottii Diddens et Lodder, 1942.

B-s-1, 2, 3, and 4. B-g-1, and 2.

These strains are almost similar to this species, shown in LODDER and KREGER-VAN RIJ's descriptions. They produce oval blastospores and cannot grow in litmus milk, while this species in LODDER and KREGER-VAN RIJ's descriptions has long oval blastospores, and peptonize litmus milk. These differences between two results seemed to be not important. (Fig. 1 a, b, c, d, and e. Fig. 4 a, and b.)

Candida brumptii Langeron et Guerra, 1935

A-1, and 3. B-1, and 3. C-1, and 3. D-1, and 3. E-1, and 3. F-1, and 3. G-1, and 3. H-1, and 3.

These strains were identified with *Candida brumptii.* (Fig. 2 a, b, c, d, e, and f. Fig. 4 c, and d.)

Rhodotorula glutinis (Fresenius) Harrison, 1928.

C-r-1, and 2.

These strains were identified with *Rhodotorula glutinis.* (Fig. 3 a, b, and c.)

Physiology of the isolates. Relations between growth of the isolates and pH values in the

Table 3. Growth in various values of initial pH of the yeast extract malt extract culture medium.

pH	Growth in optical density at 660 mμ		
	E-1		B-s-1
	in CO_2	in air	in air
2.5	0.316	0.520	0.183
3.0	.430	.540	.170
3.5	.420	.538	.190
4.0	.450	.520	.185
5.0	.405	.590	.183
6.0	.375	.590	.240
7.0	.325	.325	.250
8.0	.307	.166	.230

culture medium are shown in Table 3. Readings of optical density at 660 mμ in 5 days were given as an index for growth. A considerable growth has been obtained with every strain of *Can. brumptii* in low values of pH, such as 2.5 to 4.0, similar to the pH value of Japanese soft drinks. This result indicated that multiplication of *Can. scottii* B-s-1 would be inhibited in carbon dioxide, but that of *Can. brumptii* E-1 would not be inhibited in carbon dioxide and low pH value of soft drinks. Toleration of yeasts against high sugar concentrations was shown in Table 4. Most of the isolates were found to tolerate a high sugar concentration of 10 % and multiply on glucose, but not on saccharose. Analyses of metabolic products of some isolates were shown in Table 5. The used culture medium consisted of: yeast extract, 3 g; polypepton, 7.5 g; glucose, 20 g; distilled water, 1 l; pH 7.2. The strain B-s-3 produced a larger quantity of volatile acid (as acetic acid), when cultivated in air, than that of ethanol, while the strains C-1 and E-1 produced a large quantity of ethanol both in air and in carbon dioxide. There is a question whether the strain B-s-3 belongs to the genus *Brettanomyces* or to the genus *Candida*, because of its production of a large quantity of volatile acid in air, but then the strain B-s-3 would belong to the genus *Candida*, for it has no ogive-shaped cell.

Table 4. Growth in various concentrations of glucose in the culture medium.*

glucose in %	E-3	B-g-1
0	0.035	0.150
0.1	.205	.270
0.5	.430	.318
1	.570	.285
2	.582	.275
5	.675	.263
10	.680	.200
20	.570	.170

* 10 days growth.

Table 5. Metabolites of the yeast isolates.*

strains	cultivation	dried cell weight	consumed sugar	neutral distillates as ethanol	volatile acids as acetic acid
B-s-3	in air	47.6 mg	324.0 mg	59.6 mg	193.6 mg
	in CO_2	3.4	17.4	10.5	16.3
C-1	in air	159.7	1996.8	913.7	37.2
	in CO_2	54.0	630.8	138.1	24.2
E-1	in air	148.0	1737.2	718.8	42.3
	in CO_2	72.9	861.3	348.6	35.0

* cultivated in 100 ml of the culture medium at 30° for 10 days.

Multiplication of yeasts in Japanese soft drinks. Multiplication of yeast isolates and authentic strains were observed in Japanese soft drink Saidâ, filter-sterilized, in air and in carbon dioxide. As shown in Table 6, the strains of *Can. scottii* multiply better than the

Table 6. Growth of the yeast isolates and authentic strains in the filter-sterilized Japanese soft drink.

strains	days, required for determination of growth	
	in air	in CO_2
B-s-4.	4	—
B-s-2, 3. B-g-1.	6	—
B-s-1.	9	—
B-1, C-3, F-3,	4	4
A-3, B-3, C-1, E-1, 3. F-1, G-1, 3. H-3.	4	6
A-1, D-1, 3.	4	9
H-1.	4	13
C-r-1.	6	—
Candida guilliermondii IAM 4412, *Can. lipolytica* IAM 4947, *Can. utilis* IAM 4215, *Endomyces magnusii* IAM 4754, *Hansenula anomala* IAM 4213, *Pichia membranaefaciens* IAM 4025, *Saccharomyces cerevisiae* Wl-11	±	—
Can. parapsilosis IAM 4488	—	—

—: No growth in 18 days,
±: A slight growth in 18 days.

strains of *Can. brumptii* do in air, but cannot multiply in carbon dioxide. The strains of *Can. brumptii* can multiply on glucose both in air and in carbon dioxide, though they are not capable to assimilate saccharose at 30°C within 18 days. Of 8 authentic strains tested, *Can. parapsilosis* IAM 4488 could multiply slightly in air in 18 days. A turbidity and a deposit formed in Japanese soft drinks might be due to multiplication of such yeasts as *Can. brumptii.*

SUMMARY

Twenty four strains of the yeasts were isolated from spoilt soft drinks, becoming cloudy, or a deposit being formed in. They were identified each with *Candida scottii,* 6 strains, *Can. brumptii,* 16 strains, and *Rhodotorula glutinis,* 2 strains, respectively.

A considerable growth of *Can. brumptii* was observed in low values of pH, in a high sugar concentration, and in carbon dioxide. This strain was capable to multiply also in filter-sterilized soft drinks, while any of the authentic strains tested could not or hardly multiply.

REFERENCES

1) SAND, F. E. M. J., *Brauwelt Jg.*, **110** 225-236, (1970).
2) IIZUKA, H., TANABE, I., FUKUMURA, T., and KATO, K., *J. Gen. Appl. Microbiol.*, **13**, 125-137, (1967).
3) LODDER, J. and KREGER-VAN, RIJ N. J. W., "*The Yeasts, A Taxonomic Study*", (1952).

Fig. 1. Cells of *Candida scottii* in *Ymx* at 30°C for 4 days.
a. The strain B-s-1.
b. The strain B-s-2.
c, d, and e. The strain B-s-3.

Fig. 2. Cells of *Candida brumptii* in *Ymx* at 30°C for 3 days in air, and for 7 days in carbon dioxide.
a. Bottom cells of the strain F-3 in air.
b. Cells in a thin pellicle of the strain F-3 in air.
c. Bottom cells of the strain F-3 in carbon dioxide.
d. Cells in a thin pellicle of the strain F-3 in carbon dioxide.
e. Bottom cells of the strain A-1 in air.
f. Bottom cells of the strain A-1 in carbon dioxide.

Fig. 3. Cells of *Rhodotorula glutinis* C-r-1.
a. Bottom cells in air.
b. Cells in a yeast ring in air.
c. Bottom cells in carbon dioxide.

Fig. 4. Slide cultures of the yeast isolates.
a, and b. Pseudomycelium of the strain B-g-1.
c, and d. Pseudomycelium of the strain A-3.

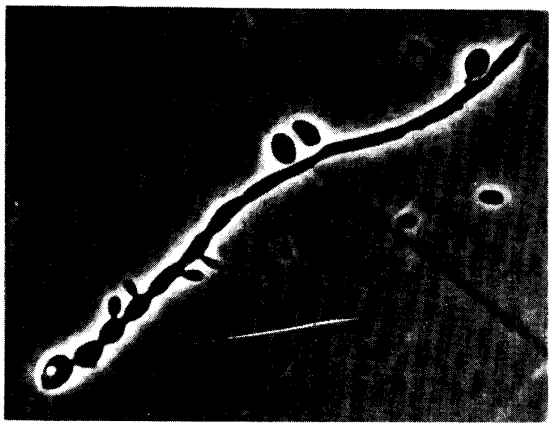


Fig. 1a

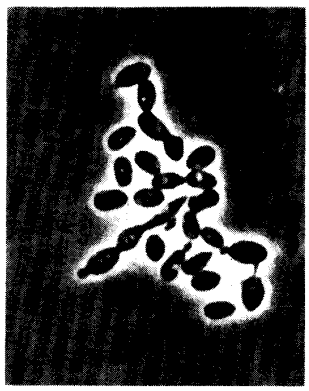


Fig. 1b

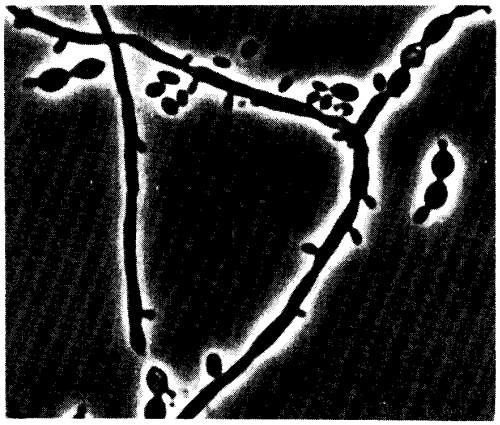


Fig. 1c

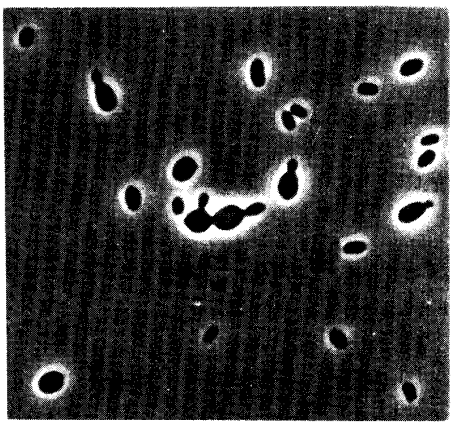
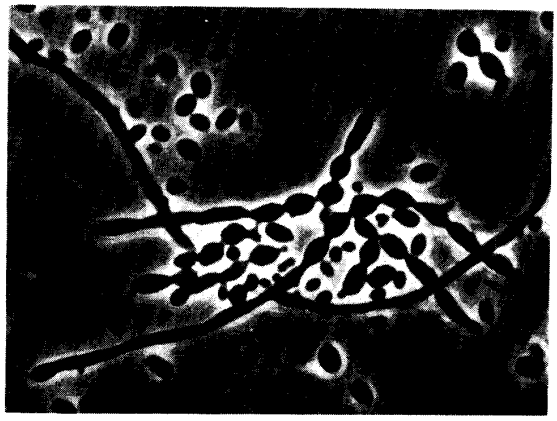


Fig. 1d



10 μ

Fig. 1e

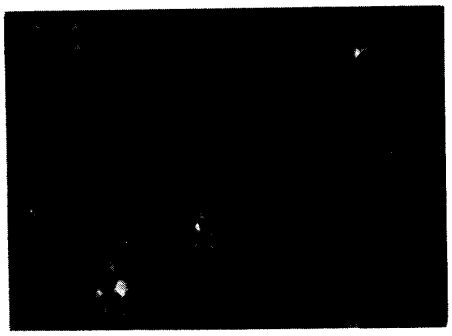


Fig. 2 a

Fig. 2 b

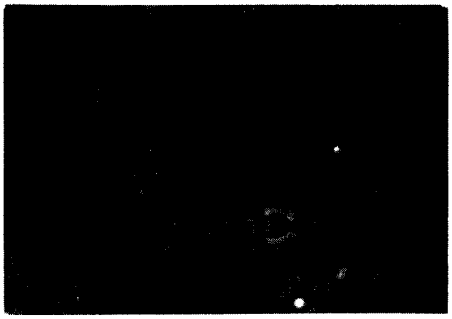


Fig. 2 c

Fig. 2 d

Fig. 2 e

Fig. 2 f 10 μ

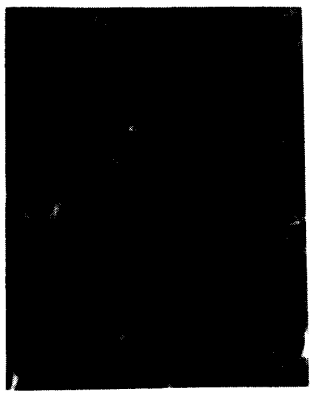


Fig. 3 a

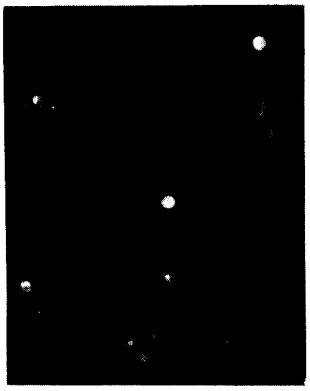


Fig. 3 b

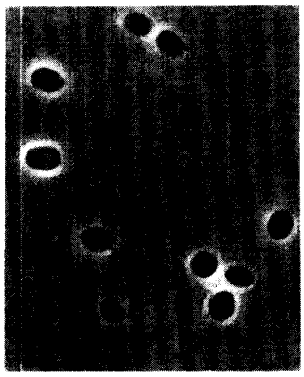


Fig. 3 c 10 μ

Fig. 4 a

Fig. 4 c

10 μ

Fig. 4 b

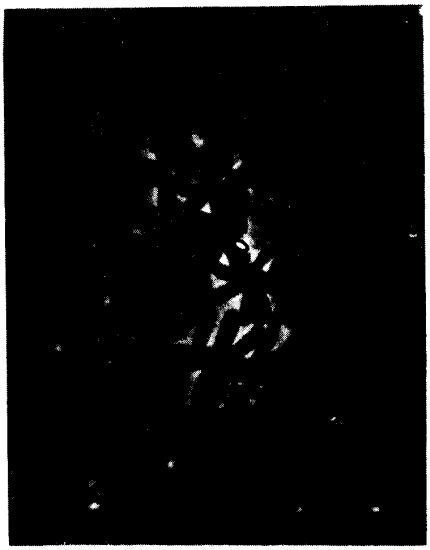


Fig. 4 d

10 μ