STUDIES ON THE UNFERMENTABLE SUGARS (I-III)

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By

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[I] On the Sugars in Sake and its Moromi

Introduction

"*Sake*" is the widely used alcoholic beverrage of the Japanese, and is a yellow rice wine brewed from mold rice known as "Kôji" ; *Aspergillus oryzae* is generally used as the mold. The starch of the rice is converted to fermentable sugars by mold enzymes. The thick liquid resultant from this enzyme hydrolysis and its subsequent fermentation is known as "*Moromi*".

The authors analysed the changes in composition of Moromi during fermentation (pH, acidity, total nitrogen, amino nitrogen, total and reducing sugar), and also examined the changes of organic acids, amino acids and sugars by the method of paper partition chromatography.

From the first stage of Moromi, a strongly colored spot which was not that of maltose appeared in addition to the glucose-spot in the paper chromatograms and the sugar remained without fermentarion in the Sake which is a filtrate of fermented Moromi.

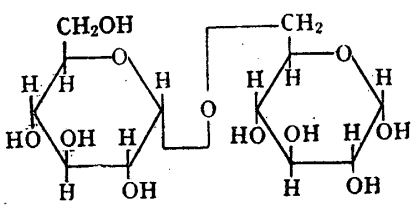
A survery of literatures shows that it corresponds with "isomaltose" (0.11% in Sake as glucose) which was discovered in Sake and Kôji-extract by HENMI and TSUKIASHI in 1932.[1] As shown in Table 1, the physical constants of isomaltose were remarkably different from the constants which were fully proved by WOLFROM[2] and MONTGOMERY[3] in 1949.

Therefore, we compared the sugars in Sake with the crystal isomaltose and panose (we are indebted to Drs. L. M. WOLFROM, EDNA M. MONTGOMERY and

Table 1. Physical constant of isomaltose.

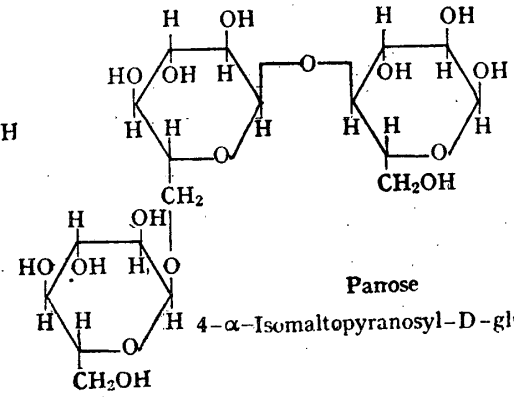
	HENMI[1] TSUKIASHI	MONTGOMERY[3] WOLFROM[2]
[α] D	+86.17°C in H_2O C=6.29%	+120°C
Octaacetate [α] D	+135.97°C in chloroform C=4 74%	+98.2°C in chloroform C=1.50%
" M.P.	72~78°C	143~144°C

ALLENE JEANES for the sugar samples) by using the method of paper chromatography and then the sugars in Sake and its Moromi were analysed separately using the same method.



Isomaltose

6-α-Glucopyranosyl-D-glucose



Panose

4-α-Isomaltopyranosyl-D-glucose

Though many analyses of the varieties and quantities of sugars in Sake and Moromi have been reported, our results proved different. The noteworthy difference was that maltose which was hitherto belived be next to glucose in quantity, was very low while isomaltose, panose and etc. were present.

Isomaltose is very hygroscopic and is a little sweet (our sample of isomaltose from Sake gave a little bitter taste owing to its impurities), m.p.120°C. WOLFROM[2] and MONTGOMERY[3] proved in 1949, that isomaltose was 6-(α-D-glucopyranosyl)-D-glucose linked by α-D-1, 6-linkage.

Panose is the crystalline trisaccharide of d-glucose obtained in 1950 by PAN, KOLACHOV and his associates[4] through the action of maltose in the cultures of the mold *Asp. niger* NRRL 337. WOLFROM, THOMPSON and their associates[5] proved in 1951 that it was composed of three glucose units linked by a α-D-1,4 and a α-D-1,6 linkage and was 4-α-isomaltopyranosyl-D-glucose and they observed that those sugars were not fermented by bakers' and distillers' yeast, but that panose was fermented by *Schizosaccharomyces Pombe*.

Experimental

(1) Sugar Analyses of Moromi

The filtrates of Moromi on brewing were divided into 10 ml portions, placed in test-tubes, neutralized, vaporized alcohol, sterilized, and inoculated with *Fleishman yeast* and *Sacch. praecisus* (which fermented only glucose but not maltose, isomaltose and panose etc.). The inoculated filtrates were incubated for 4 days at 30°C. The mixture of glucose, maltose and unfermentable carbohydrate were analysed using the method of PAVIS-DARCH.[6] The results are shown Table 2. (A) and (B).

Table 2. Sugar analyses of "Moromi."

[A] "Moromi"

ferment. age in day	total sugar	reducing sugar	dextrin	as glucose	as maltose	as unfermentable sugar
7	30.68%	25.91%	4.01%	24.02%	1.96%	1.15%
14	32.89	29.36	2.90	26.78	3.25	0.88
21	16.89	13.38	2.93	11.77	1.94	0.69
25	13.32	9.61	2.38	8,23	1.72	0.58
33	10.59	7.84	2.16	7.01	1.01	0.52
41(after addition)	15.63	9.94	3.88	8.74	2.50	1.09
51	4 64	3.55	0.96	3.28	0.56	0.56
59	2.24	1.36	0.87	1.00	0.23	0.51

[B] "Ginjō Moromi."

ferment. age in day	total sugar	reducing sugar	dextrin	as glucose	as maltose	as unfermentable sugar
14	30.68%	26.76%	3.21%	25.03%	1.76%	1.08%
21	28.93	24.64	3.63	22.45	2.79	0.74
27	14.03	11.06	3.57	10.07	1.36	0.30
33(after addition)	17.11	9.00	7.19	7.33	1.34	1.49
40	9.33	3.92	5.76	2.19	1.31	1.59
49	6.32	2.42	3.59	1.09	1.30	1.02
58	4.76	2.00	2.68	1.09	0.11	0.62

(2) Sugar Analyses of Sake

The five upper classes, five middle classes, and the four lower classes of Sake and special Sake "Ginjo" were taken as samples. The sugars were analysed using the same method as (I). The results are shown in Table 3.

(3) Sugar Analyses of Sake and its Moromi on Paper Chromatography

(A) General Procedure

The method used in our laboratory for the identification of sugars by paper chromatography was reported previously.[7] For the elution solvent, a mixture of pyridine, buthanol and water (2:3:1.5, respectively) was used. Other solvents

Table 3. Sugar analyses of "Sake"

No.	pH	total sugar	reducing sugar	dextrin	as glucose	as maltose	as unfermentable sugar
upper class 1	4.0	4.21	3.63	0.52	3.51	0.11	0.09
〃 2	4.2	4.21	3.77	0.39	3.70	0.03	0.09
〃 3	4.2	5.18	4.46	0.62	4.32	0.13	0.10
〃 4	4.0	4.62	4.12	0.43	4.00	0.10	0.10
〃 5	4.0	4.09	4.35	0.64	4.23	0.10	0.09
middle class 1	3.8	3.69	3.12	0.51	3.11	0.01	trace
〃 2	4.2	4.87	4.48	0.33	4.39	0.05	0.11
〃 3	4.2	5.35	5.02	0.28	4.88	0.11	0.12
〃 4	4.0	4.54	3.96	0.50	3.88	—	0.05
〃 5	4.0	4.26	3.91	0.39	3.85	0.04	0.06
lower class 1	4.0	3.69	3.22	0.42	3.22	trace	trace
〃 2	4.0	5.40	5.36	0.04	5.20	0.17	0.11
〃 3	3.8	4.48	4.07	0.36	4.00	0.03	0.10
〃 4	3.6	4.84	4.23	0.43	4.12	0.07	0.11
Ginjō 1	—	4.88	3.69	1.19	3.42	0.24	0.26
〃 2	—	5.38	4.66	0.72	4.39	0.01	0.67

were also tried, but they caused greater overlapping of the spots in chromatograms of the mixtures of sugars having similar R_f values. For the developing agent, aniline hydrogenphtalate was used. After preliminary drying, the papers were heated for 20～25 minutes at 125～130°C in a special oven to develop the weak reducing oligosaccharides.

Commonly, we obtained satisfactory results by the onedimension method and the ascending method. Because Moromi posesses a large quantity of dextrin and has a high viscosity, the R_f values of its sugars were low and in addition, somewhat elongatated spots were often formed.

Table 4. Rf values of sugars

Sugars	JEANES et al[8]		Authors
	1	4 ※	
Rhamnose	—	—	0.60
Xylose	47	84	0.50
Mannose	41	79	0.42
Arabinose	41	79	0.40
Fructose	40	79	0.42
Glucose	35	75	0.35
Galactose	31	70	0.31
Sucrose	—	—	0.29
Maltose	25	60	0.26
Cellobiose	23	56	0.24
Isomaltose	19	48	0.19
Gentiobiose	19	49	—
Lactose	18	47	0.19
Melibiose	16	43	0.17
Panose	—	—	0.14
Raffinose	—	—	0.12
Maltotriose	17	45	—
Maltotetraose	10	31	—
Maltopentaose	—	20	—

Therefore, for their identification, spots were formed after a preliminary treatment by removed of dextrin and etc. Spotted samples were also seen mixing with the authentic sugars parallel with the same samples. The data in Table 4 are the R_f values of sugars obtained by us and JEANES et al.[8] (1 is R_f values obtained by one dimension method; 4 is that obtained by 4 times multiple chromatography).

(B) Paper Chromatography of Sugars in Sake and Moromi

0.015~0.050 ml of Sake and the filtrate of Moromi were spotted on a strip of "Tôyô" No. 2 filter paper this was eluted for 15 hours at 25°C.

The obtained chromatograms are shown in Fig. 1 (①~⑥).

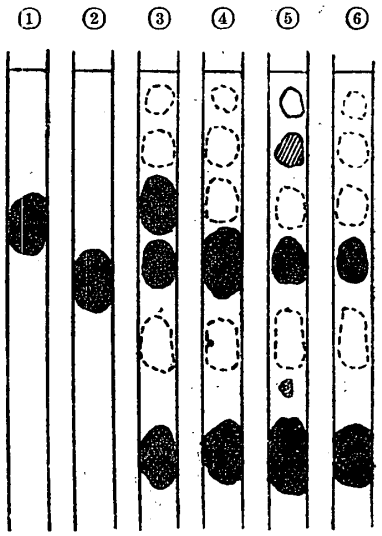


Fig. 1. ① Chromatogram of Sake ; Two spots appeared, but when concentrated Sake (1/5-1/10) or a large quantity of it was spotted, five or six spots appeared. They were designated as A, B, C, D, E and F according to thier R_f values. The spot A had the largest and strongest color, and C was next in runk.

② Chromatogram of Moromi before press : Six or seven (A~F) spots appeared, the spots E and F were stronger than Sake

③ The mixed chromatogiam of Sake with 300 γ isomaltose

④ 〃 with 300 γ panose

⑤ Chromatogram of isomaltose

⑥ 〃 of panose

Fig. 1. Paper-chromatograms of sugars in Saké and its Moromi.

● intense. ▨ middle.
○ weak. ○ very weak.

From the R_f values, colors and the mixed chromatography in the authentic samples, it was found that the spots A and B were identical with glucose and maltose.

From the following experiments, it was discovered that the B spot was not maltose, but was overlapped with two sugars having somewhat lower and higher Rf values than maltose.

There is almost no report about oligosaccharides in Sake and Moromi, except for "*isomaltose*" of HENMI and TSUKIASHI.[1] We investigated on the C~G spots as follows. 10 ml of Sake was hydrolysed by heating it together with 0.6 ml of 35 per cent hydrochloric acid for 2.5 hours on a boiling water bath. When the paper chromatogram of its hydrolysate was produced, the spot A (glucose) became larger than before and other spots (B~G) were not observed. The hydrolysate was adjusted to pH 5.0 with caustic soda and then inoculated with *Fleishman yeast*. When the inoculated hydrolysate was incubated at 30°C, carbon dioxide was produced, and the chromatogram of the fermented hydrolysate cleary showed that the spot A also disappeared. Those facts mean that the sugars of the spots C~G were polymers of glucose. Otherwise, the Sake was

evaporated up alcohol and was adjusted to pH 5.0 and was inoculated with *Fleishman yeast.* On its chromatogram of the fermented solution, A spot (glucose) disappeared and the other spots B~G remained. Therefore, it is clear that the sugar of the spots B~G are unfermentable. The Rf values of C and D resemnbled isomaltose and panose respectively, and the mixed chromatograms (Fig. 1 ③~⑥) showed that the sugars of C and D spots were isomaltose and panose respectively.

(C) Multiple Chromatography of Sugars in Sake and Moromi

As above mentioned, the sugar of the spot B was recognized as maltose by onedimension chromatography, but it was unfermentable by yeast.

We investigated this problems using a modification of the multiple development technique described by ALLENE JEANES et al.[8]

The obtained 3~4 times multiple chromatograms and mixed multiple chromatograms of the sugars in Moromi are shown in Fig. 2 ; ⑦~⑫.

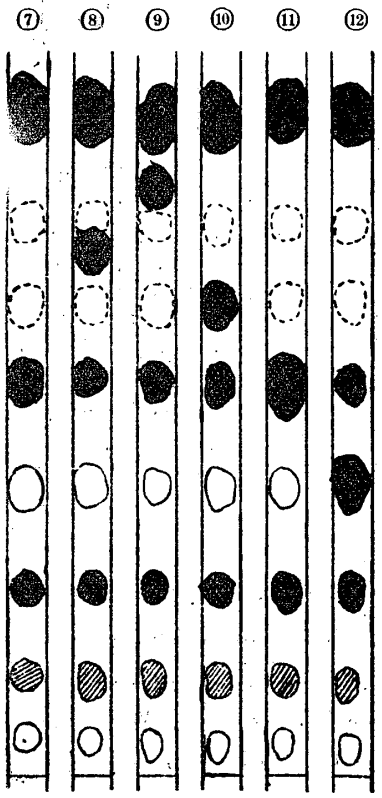


Fig. 2. Multiple-paper-chromatograms of sugars in Sakè and its Moromi.

⑦ Multiple chromatogram of sugars in Moromi before press
⑧ The mixed multiple chromatogram with 300 γ maltose
⑨ 〃 with 200 γ galactose
⑩ 〃 with 150 γ cellobiose
⑪ 〃 with 200 γ isomaltose
⑫ 〃 with 300 γ panose

From the results, the sugars of the spots A, C and D were identified to be glucose, isomaltose and panose respectively. The spot B was divided into two spots which were designated as B_1 and B_2 according to their R_f values, neither one agreed with the spot of maltose.

The Rf value of B_1 was higher than that of maltose (Fig. 2 ; ⑧) and it did not agree with the spot of galactose. (Fig. 2 ; ⑨).

The Rf value of B_2 was lower than that of maltose (Fig. 2 ; ⑧) but identica with the spot of cellobiose (Fig. 2 ; ⑩).

The identification of the sugars of the spots Bi, E, F and G will be reported elsewhere. That those unfermentable sugars might be formed directly from rice-starch by enzymic decompostion, or from glucose and maltose by synthesizing action of other enzymes will be discussed in the IInd report.

We shall also investigate the mold-bran in relation between its enzymes and the chemical constitution of its digestion products.

(D) Analyses of Sugars in Sake by Paper Chromatography

The eight upper classes, four middle classes and two lower classes of Sake were taken as samples. The sugars were analysed using a modification of the paper chromatography technique described by Flood et al[9] as already described above.

The respective parts of sugars appearirry on the paper chromatogram were compared with the colored spots and then extracted with water.

The extracted sugars were analysed using the method of Stark and Somogyi.[10] The obtained data are shown in Table 5.

Table 5. Sugar analyses of Sake gr./100 ml.

Sugars \ No.	Upper Class							
	1	2	3	4	5	6	7	8
Glucose	2.86	2.54	3.30	3.22	3.84	2.48	3.76	3.42
Unknown disaccharide	0.04	0.03	0.06	0.12	0.05	0.04	0.04	0.02
Cellobiose	0.03	0.02	0.10	0.07	0.07	0.01	0.06	0.04
Isomaltose	0.12	0.08	0.36	0.25	0.06	0.07	0.13	0.07
Panose	0.04	0.05	0.07	0.03	0.03	0.02	0.01	0.03
Tetra-saccharide ?	0.02	0.02	0.05	0.04	0.02	—	0.03	0.04
Reducing dextrin	0.11	0.14	—	—	0.13	0.14	0.05	0.08
Total	3.22	2.88	3.94	3.73	4.20	2.76	4.08	3.70

Sugars \ No.	Middle Class				Lower Class	
	1	2	3	4	1	2
Glucose	2.78	3.28	3.68	3.08	3.28	3.44
Unknoun disaccharide	0.00	0.01	0.05	0.02	0.04	0.04
Cellobiose	0.00	0.01	0.02	0.03	0.03	0.04
Isomaltose	0.03	0.09	0.11	0.08	0.10	0.11
Panose	0.02	0.01	0.01	0.03	0.03	0.03
Tetra-saccharide ?	0.03	0.02	0.02	0.02	0.02	0.04
Reducing dextrin	0.11	0.07	0.08	0.09	0.05	0.12
Total	2.97	3.49	3.97	3.35	3.55	3.82

Summary

(1) We analysed the sugars of "Sake" and its "Moromi", using the general method and paper chromatography.

(2) Though it was generally believed that maltose was abundantly contained in quantity secondly to glucose in Sake and Moromi, the multiple paper chromatograms showed that glucose and unfermentable sugars (namely, isomaltose, cellobiose, panose and few unknown oligosaccharides which are consist of glucose) were present, but almost no maltose could be detected.

[II] Production of Unfermentable Sugars by Fungal Enzyme

Introduction

We studied on the sugars contained in *Sake*, *Moromi* and *Kôji-extract* and observed that glucose and several unfermentable sugares, namely isomaltose, cellobiose, panose and a few unknown sugars, were present.

We investigated the mechanism of the production of those unfermentable sugars and found that they were produced by the action of fungal enzymes on maltose and glucose. WOLFROM et al[2)11)] and[12)] MONTGOMERY et al[3)13)] have reported on isomaltose since 1947.

Isomaltose was first isolated from the enzymic hydrolysate of amylopectin (waxy corn starch) as a crystalline substance by MONTGOMERY, WEAKLEY and HILBERT[14)] (1949). WOLFROM and his associates[15)] (1951) also isolated it by acid degradation of amylopectin (waxy maize starch) and recently[16)] (1952) from commercial hydrol.

PAN et al[4)] (1950) reported that the submerged culture of *Asp. niger NRRL* 337 contained an enzyme which synthesized an unfermentable trisacchsaride from maltose. WOLFROM et al[5)] demonstrated that this trisaccharide was 4–α–isomaltopyranosyl-D-glucose, and named it *"panose"*

PAZUR[17)18)] (1952) reported that the enzyme concentrate prepared from *Asp. oryzae* by alcohol precipitation procedure contained transglucosidase which synthesized isomaltose, panose, dextrantriose (6–α–isomaltosyl-D-glucose) and 4–α–dextrantriosyl-D-glucose from maltose.

TSUCHIYA et al[19)] demonstrated by paper chromatohraphic techniques that *Asp. niger* enzymes synthesized isomaltose and cellobiose from maltose.

PFANNMULLER[20)] (1952) reported that the enzyme preparation derived from *Asp. oryzae* synthesized isomaltose and a small quantity of an oligosaccharide. WOLFROM (1951) et al[21)] isolated panose for the first time by mild acetolysis of amylopectin. ONO (1950)[6)] reported that unfermentable reducing dextrin increased when mold-bran (*Asp. oryzae*) was added as a saccharify agent on alcohol fermentation of sweet potatoes.

Experimental

(1) Enzymic Synthesis of Oligosaccharides

We used a modification of the method described by PAN et al.[4] A submerged culture of *Asp. niger NRRL* 337 was prepared in a medium containing the following ingredients per liter; corn 20 g, ammonium sulfate 2g, ferrous sulfate 0.01g, calcium carbonate 5g, potassium dihydrogen phosphate 1g and magnesium sulfate 0.5g. The medium was divided into 40 ml portions, placed in 300 ml shaking flasks, sterilized, and inoculated with spores of the mold (*Asp. niger NRRL* 337). The inoculated medium was incubated for 66 to 96 hours at 27–28°C in a shaker operating at 150 cycles per minute with a stroke length of 6 cm.

One volume of filtrate of the fungal culture was added to two volumes of a buffered maltose solution. The resulting mixture contained approximately 100 mg. of maltose per ml. and 10 ml. of MCILVAINES standard buffer (pH 4.5) per 100 ml. A small amount of toluene was added and the reaction mixture was incubated for 48–72 hours at 55°C. We inoculated similarly with *Asp. niger sp.*, *Asp. oryzae sp.*, *Asp. oryzae* S4–15, *Asp. Usamii*, *Asp. Awamori*, *Rhizopus javanicus* TAKEDA and *Rhizopus Peka* II.

The products, which were synthesized by culture filtrates of those strains from maltose, were examined on a paper chromatography.

The obtained four times multiple chromatograms are shown in Fig. 3 (① ~⑬).

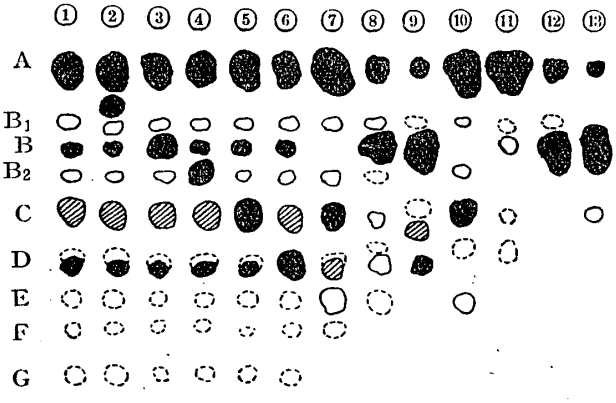


Fig. 3. Paper chromatogrames of digestion products from maltose.

①	Buffered maltose solution	+	*Asp. niger* NRRL 337 filtrate			
②	〃	+	〃	+	galactose	200 γ
③	〃	+	〃	+	maltose	300 γ
④	〃	+	〃	+	cellobiose	200 γ
⑤	〃	+	〃	+	isomaltose	200 γ
⑥	〃	+	〃	+	panose	300 γ

⑦ Buffered maltose solution + *Asp. niger sp.* filtrate
⑧ " + *Asp. oryzae sp.* filtrate
⑨ " + *Asp. oryzae* S4–15 filtrate
⑩ " + *Asp. Usamii* filtrate
⑪ " + *Asp. Awamori* filtrate
⑫ " + *Rhizopus javanicus* TAKEDA filtrate
⑬ " + *Rhizopus Peka* II filtrate

No spot appeared on the chromatograms of respective filtrates and only the maltose spot appeared on that of the buffered maltose solution.

In Fig. 3 ①, *Asp. niger* NRRL 337; eight spots appeared. They are designated as *A*, B_1, B_2, *C*, *D*, *E*, *F* and *G* according to their Rf values.

Comparative tests (Rf value, color and etc.) with the pure glucose, galactose, maltose, cellobiose, isomaltose and panose by the technique of the mixed chromatography showed that *A* was glucose, B_2 cellobiose; *C*, isomaltose; and *D*, panose respectively (Fig. 3 ② ③ ④ ⑤ ⑥).

B_1, *E*, *F* and *F* and *G* are unknown sugars. PAZUR[17)18)] reported on the production of 6–α–isomaltosyl-D-glucose (designated by him as dextrantriose) and 4–α–dextrantriosyl-D-glucose from maltose. We could not obtain those pure sugars, and it is now impossible to say with the data available at present, whether the sugars *E*, *F* and *G* are identical with them. Work is in progress concerning those sugars. PAN[4)] reported on the production of panose from maltose, and we observed that many oligosaccharides were synthesized from maltose by the culture-filtrate of *Asp. niger* NRRL 337.

In Fig. 3 ⑦, *Asp niger sp.*; seven spots appeared, namely *A* (glucose), B_1 (unidentified sugar), B_2 (cellobiose), *C* (isomaltose), *D* (panose) *E* and *F* (unidentified sugars).

In Fig. 3 ⑧, *Asp, oryzae sp.*; seven spots appeared, namely *A* (glucose), B_1 (unidentified sugar), *B* (maltose), B_2 (cellobiose), *C*(isomaltose), *D* (panose) and *E* (unidentified sugar).

In Fig. 3 ⑨, *Asp. oryzae* S4–15; eight spots appeared, namely *A* (glucose), B_1 (unidentified sugar), *B* (maltose), B_2 (cellobiose), *C* (isomaltose), *D* (panose) *E* (unidentified sugar) and a large spot between isomaltose and panose which would be maltotriose from its Rf values, but we could not identify it because of the luck of authentic maltotriose.

In Fig. 3 ⑩, *Asp. Usamii*; six spots appeared, namely *A* (glucose), B_1 (unidentified sugar), *B* (maltose), B_2 (cellobiose), *C* (isomaltose) *E* (unidentified sugar) and the spot which accured between isomaltose and panose had a somewhat higher R_f value than panose.

In Fig. 3 ⑪, *Asp. Awamori*; five spots appeared, namely *A* (glucose), B_1 (unidentified sugar), *B* (maltose), *C* (isomaltose) and *D* (panose).

In Fig. 3 ⑫, *Rhizopus javanicus* TAKEDA; three spots appeared, namely A (glucose), B_1 (unidentified sugar) and B (maltose); the isomaltose spot did not appear.

In Fig. 3 ⑬, *Rhizopus Peka* II; three spots appeared, namely A (glucose), B (maltose) and C (isomaltose).

The Rf values of those synthesized sugars resembled each other, thus satisfactory results could be obtained by using a larger sheet of paper by which greater movement is given to the solvent front. Investigatios were advanecd using a modification of the multiple development technigue described by ALLEE JEANES.[8] Always, four times multiple chromatography were used, The sheet of paper used for Fig. 3 and Table 6 obtained by the ascending technique was 40 cm long. The solvent mixture was pyridine, buthanol and water (2 : 3 : 1.5).

On the paper chromatograms, it is necessary that the spots B, B_1 and B_2 overlap on the single one dimension chromatogram. The data were obtained by using the submerged culture-filtrates. The same results were obtained similarly by using the surface culture-filtrates or CZAPECK solution instead of the semi-synthetic medium described by *Pan*.[4]

(2) Quantitative Analyses of Synthesized Oligosaccharides

The quantitative analyses of the oligosaccharides which were synthesized through the action on maltose of cultures of the molds have been made by the method described by FLOOD et al.[9] The method of paper multiple development chromatography was mentioned already. The respective spots of sugars on paper chromatogram were cut off and then extracted with water.

The extracted sugars were analysed using the method of STARK and SOMOGYI.[10] The obtained data are given in Table 6

Table 6. Digestion product from maltose g/100 ml.

Sugars \ Enzyme		1	2	3	4	5	6	7	8
A	Glucose	4.095	5.090	0.922	0.432	5.258	4.831	0.771	0.095
B_1	Unidentified sugar	0.036	0.155	0.178	0.060	0.112	0.076	0.127	5.587
B	Maltose	0.832	—	5.952	5.405	—	2.113	5.601	
B_2	Cellobiose		0.175	0.069		0.158	?		
C	Isomaltose	0.549	0 910	0.282	0.174	0.950	0.086	—	0.241
—	Maltotriose ?	—	—	—	0.312	—	—	—	—
—	Unidentified sugar	0.822	0.138	0.087	—	0.105	0.125	—	—
D	Ponose			0.207	0.337	—	—	—	—
E	Dextrantriose ?	trace	0.267	0.047	—	0.126	—	—	—
F	Unidentified sugar	〃	trace	—	—	—	—	—	—
G	Unidentified sugar	〃	—	—	—	—	—	—	—

1. *Asp. niger* NRRL 337.
2. *Asp. niger sp.*
3. *Asp. oryzae sp.*
4. *Asp. oryzae* S4-15.
5. *Asp. Usamii.*
6. *Asp. Awamori.*
7. *Rhiz. javanicus.*
8. *Rhiz. Peka* II

(3) Production of Isomaltose and Panose by Taka-distase

We investigated the action of Taka-diastase (*Sankyo* Co. Ltd.) on maltose. 10 ml of Taka-diastase solution was added to 20 ml of a buffered maltose solution (pH 4.5). The resulting mixture contained approximatly 50 mg. of maltose per ml and 10 ml of McILVAINES standard buffer per 100 ml. The reaction mixture was incubated for 72 hours at 55°C. Taka-diastase was used in the amounts of 0.05, 0.25, 0.5 and 1.0 per cent for maltose. The obtained paper chromatograms are shown in Fig. 4 (①~③).

The procedure was as above menitoned.

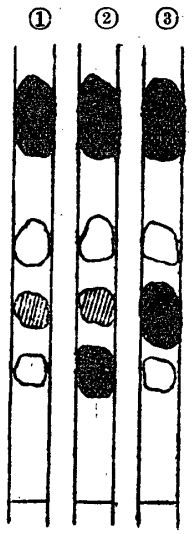


Fig. 4. Paperchromatograms of sugars produced by the action on maltose of Taka-diastase.

① Taka-diastase solution + buffered maltose solution
② 〃 + 〃 + panose 300 γ
③ 〃 + 〃 + isomaltose 200 γ

Four spots appeared and they were designated as A, B, C and D according to their Rf values. It was found from the mixed chromatograms (②, ③) that A, B, C and D were glucose, maltose, isomaltose and panose respectively. Therefore, it indicated that the Taka-diastase synthesized panose and isomaltose from maltose.

(4) Production of Isomaltose and Panose from Glucose, Starch and many Sugars

(A) *Glucose*: The procedure was the same as for maltose. The filtrate from *Asp. niger* NRRL 337 culture and Taka-diastase solution were added to a buffered glucose solution.

The reaction mixtures were incubated for 72 hours at 55°C. It was found from the mixed paper chromatograms that isomaltose was synthesized from glucose, but in smaller amount than in the case of maltose, and panose was not clearly proved.

(B) *Starch and Rice Powder*:

The procedure was the same as for maltose. The filtrates from *Asp. niger* NRRL 337 culture, *Asp. oryzae sp.* culture and Taka-diastase were used.

Isomaltose, panose and few oligosaccharides were found in the digested solution of starch and rice powder by the mixed paper chromatography.

(C) *Sugars*:

The procedure was the same as for maltose. The filtrates from *Asp. niger* NRRL 337 culture, *Asp. oryzae sp.* culture and Taka-diastase were used. No spot appeared on the chromatograms of the digested solution of arabinose, xylose, galactose, fructose, mannit, sucrose and inulin.

Discussion

We investigated on the sugars contained in Sake and Moromi and found glucose and many unfermentable sugars, namely isomaltose, cellobiose, panose besides a few unidentified sugars. We also studied on the mechanism of the production of those unfermentable sugars and found that they were produced by the action of fungal enzymes on maltose and glucose.

Those unfermentable sugars contained in Sake and its Moromi might be formed directly by enzymic decomposition from starch of rice as parts of it or by the synthesizing action of enzymes from glucose and maltose. From our results and literatures described in the introduction, it seems that the unfermentable sugars contained in Sake and its Moromi are mainly the sugars which were synthesized by enzymic action from maltose and glucose.

Summary

We studied the production of unfermentable sugars by the action of fungal enzymes on maltose, glucose, starch, etc., and obtained the following results:

(1) Filtrates from the fungal culture (*Asp. niger* NRRL 337, *Asp. niger sp.*, *Asp. oryzae sp.*, *Asp oryzae* S4–15, *Asp. Usamii*, *Asp. Awamori*, *Rhizopus javanicus* T. and *Rhizopus Peka* II were added as fungal enzymes to the buffered maltose solution and the reaction mixtures were incubated for 48–96 hrs. at 55°C. We have demonstrated by paper partition chromatography that those filtrates synthesized unfermentable sugars (isomaltose, cellobiose, panose and 2 or 3 unknown sugars) from maltose and starch. The constituents of sugars which were synthesized differed according to the kind of molds. A quantitative analyses of those sugars have been made.

(2) Maltose spot which was recognized as maltose by the one dimension paper chromatography, was divided into two spots by multiple chromatography. The sugars of these two spots were unfermentable by yeast and we observed that the one was cellobiose and the other was an undetermined disaccharide.

(3) Taka-diastase synthesized isomaltose and panose from maltose.

(4) The filtrates from the fungal culture (*Asp. niger* NRRL, 337 *Asp. niger*

sp. and Asp. oryzae sp.) and Taka-diastase synthesized isomaltose from glucose but not from arabinose, xylose, galactose, fructose, mannit, sucrose and inulin.

[III] Assimilation and Fermentation of Isomaltose and Cellobiose by Yeasts

We have observed previously that unfermentable cellobiose, isomaltose panose and a few unidentified sugars were produced by the action of fungal enzymes on maltose, glucose and starch. Further, it was found that these sugars were present in Sake *Moromi*.

We now observed that the assimilability and fermentability of these sugars were considerably different according to the strain of the yeasts.

It has been reported in literature that cellobiose was unfermentable by ordinary yeasts. WOLFROM et al report[2] that isomaltose was unfermentable by bakers' yeast. MONTGOMERY et al[14] described that crude isomaltose was purified by removing fermentable sugars using baker's yeast and distiller's yeast (*Sacch. cerevisiae* NRRL 567).

PAN et al[4] reported that panose was not fermented by baker's or distiller's yeasts but was fermented by *Schizosaccharomyces Pombe*. Maltose is commonly fermented by ordinary yeasts, but HARRIS et al[22] observed that maltose and maltotriose were fermented by *pale ale yeast* (normal top-fermentation yeast) and *larger yeast* (bottom-fermentation yeast), but by poor attenuator (the poorly attenuating mutant), maltose was fermentable with difficult and maltotriose was unfermentable.

Some kinds of fungi produce limit dextrinase, amylo-glucosidase and etc. which hydrolyse 1, 6-linkage. It is unknown that yeast hydrolyzes 1, 6-linkage and ferments the hydrolysate. Recently, MARUO et al[23] found that the "amylosynthease" of yeast did not synthesize starch but attacked the branching points of glucosidic chains. They proposed for this enzyme a new name *"isoamylase"*. This enzyme dose not hydrolyze dextran and differs from dextranase. In addition, it is unknown whether this enzyme might hydrolyze isomaltose. NEUBREG[24] observed that an enzyme which hydrolyzed α–1, 6-linkage of limit-dextrin was present in the extract of yeast and proposed for this enzyme the name of α–1, 6-glucosidase (amylo-glucosidase).

BERNFELD[25][26] reported that isophospholylase which synthesized and attacked α–1, 6-glucoside linkage was present in yeast, muscle and potato juice. SUZUKI[27] reported that β-glucosidase in yeast synthesized or hydrolysed isomaltose.

Experimental

(1) Preparation of Samples

The assimilation and fermentation of cellobiose and isomaltose by many kinds of yeasts were investigated. For cellobiose pure chemicals (*Takeda* Co.) were used. Isomaltose was prepared from dextran and Kôji-extract using the following procedures.

(A) Preparation of dextran

We prepared dextran from sucrose by the action of *Leuconostoc mesenteroides* B. (We are indebted and express our thanks to EDWARD J. HEHRE for this strain).

The procedure adopted for the preparation of dextran was a modification of the method described by KOBAYASHI[28] and JEANES.[29]

Namely, a culture of *Leuconostoc mesenteroides* was prepared in a medium containing the following ingredients per liter; sucrose 100 g., ammonium sulfate 0.6 g, magnesium sulfate 0.2 g, sodium chloride 1 g, dipotassium hydrogen-phosphate 5 g and autolysate of 8 g dry yeast.

The medium was divided into 800 ml portions, placed in 1 liter flasks, sterilized, and inoculated with 20 ml seed of *Leuconostoc mesentercides* which had been grown in the same medium for three days. The inoculateed medium was incubated for one week at 28–30°C. The formation of dextran was paralleled by an increase in viscosity, until after about five days incubation, the viscosity reached a maximum and dextran formation appeared to be completed. 95 per cent Ethanol to make 20 per cent by volume was stirred into the culture to remove the bacterial cells and then it was centrifuged. When the ethanol per cent of the centrifugate was made up to 50 per cent by volume, dextran separated as a gummy mass from which the supernatant was decanted. The dextran was kneaded with 50 per cent ethanol. It was then dissolved in water and reprecipitated by addition of an equal volume of ethanol. This cycle of reprecipitation and washing were repeated twice. The obtained dextran was dried in the vacum decicator.

(B) Preparation of isomaltose from dextran

The dextran was hydrolyzed partially on the boillng water bath using many kinds of concentration of hydrochloric acid, sulphic acid and oxalic acid. But yields of isomaltose were low. Thus we used a modification of the method described by WOLFROM et al.[11] Six grams of dextran was added to 60 ml of 35 per cent hydrochloric acid and it was kept at 28–30°C for 48 hours. It was then diluted with 150 ml of water and the acid was removed by passing over AMBERITE resins IR-4B and then it was treated with active carbon. The

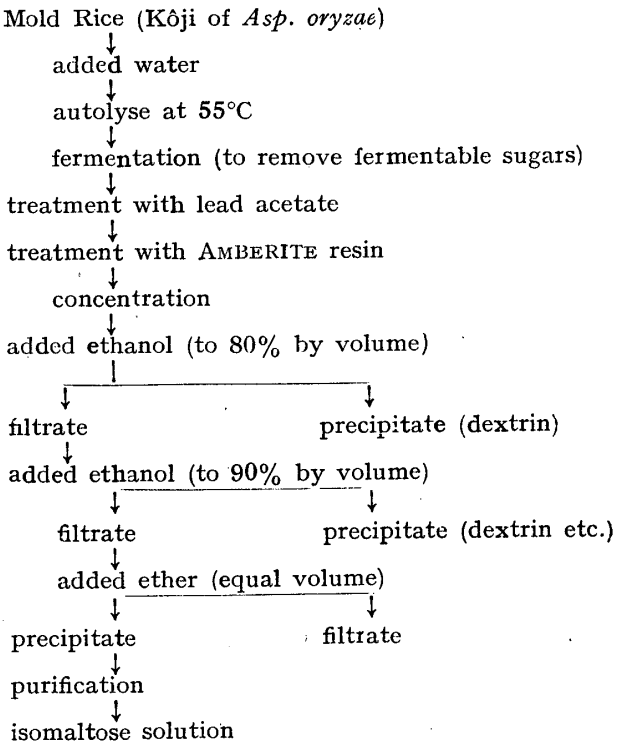
effluent was conecntrated to 120 ml under reduced pressure. 0.12·g Of dipotassium hydrogen phosphate was added to the solution and glucose was removed from it by baker's yeast (*Wakamoto*) fermentation. It was stirred into the centrifugate to remove the yeast cells and was concentrated to a sirup under reduced pressure.

Isomaltose was extracted with 95 per cent ethanol from the syrup.

(C) Preparation of isomaltose from Moromi

We have previously found that isomaltose was present in Sake and its Moromi.

The outline of preparation of isomaltose from Moromi was as follows:



(2) Assimilation and Fermentation by Yeasts

The assimilation and fermentation of cellobiose and isomaltose by many kinds of yeasts were investigated. The distillers' yeasts (*Rasse* II, *Rasse* XII, *Sacch. formosensis*), the *Sake yeasts* (*Kyokai* No. 6, *Kyokai* No. 7), the bakers' yeasts (*Fleishman, Sankyo, Oriental*), the newly isolated bakers' yeasts from commercial compressed yeasts (*N-Wakamoto, N-Red Star, N-Sankyo, N-Oriental*), *Torula utilis, Mycotorula japonica, Schizosaccharomyces Pombe* No. 1, *Schizosacchromyces Pombe* No. 2 and *Saccharomyces praecisus* which is fermentable only by glucose, were used.

The procedure was as follows; for fermentation, the spits of the ordinary thick-glass injection tubes were used and a small gum stopper was adjusted up to the fixed depth of this spit. 1.5 ml Of isomaltose solution (0.1 per cent, dipotassium hydrogen phosphate, 0.25 per cent asparagin, 0.3 per cent magnesium sulfate and pH 5.0, 1 per cent isomaltose.)were taken in the tube, sterilized and inoculated with abundant yeast.

The inoculated isomaltose solution was incubated for 48–72 hrs. at 27–28°C. The volume of produced CO_2 was measured. The residual sugars were analysed using the method of STACK and SOMOGYI[10] and that of paper chromatography. The obtained results are shown in Table 7.

Results and Discussion

From the above experiments it has been found that the Sake yeast fermented a considerable amount of isomaltose, and so it seemed that isomaltose which remained in Sake and its Moromi was not unfermentable, but remained with glucose in Sake without complete fermentation. The fermentation of isomaltose was slower than glucose.

Some of the distillers' yeast fermented a considerable amount of isomaltose and others fermented incompletely. The bakers' yeast which had been grown continuously on agar slants of Kôji-extract for long time, fermented isomaltose remarkably, but the bakers' yeast which had been isolated newly from commercial compressed yeasts, were very unsuitable to ferment isomaltose.

Schizosaccharomyces Pombe No. 1, No. 2 and *Mycotorula Japonica* fermented remarkably but *Torula utilis* and *Sacch. praecisus* did not ferment.

Schizosacch. Pombe No. 1 and No. 2 fermented cellobiose about 50 per cent for 72 hours, but the other yeasts did not ferment cellobiose.

We examined the residual sugars from time to time by paper chromatography. It was found that the spot of isomaltose disappeared gradually by many yeasts, but by *Schizosacch. Pombe* No. 2, the spot of isomaltose disappeared and yet the spot of glucose appeared and then gradually disappeared. It was considered that α-1,6-glucosidase of *Schizosacch. Pombe* No. 2 was strong or its zymase was weak.

Because the volume of CO_2 in Table 7 was measured by the scale of the injection tube, it was not so accurate. The consumption of sugars did not always parallel the production of CO_2 in our condition of experiment. For example, there were example in which CO_2 was not produced in spite of the consumption by the sugars.

It seems that the sugars were assimilated in the cells and that they charged in glycogen and trehalose or were sorped in cells. EKELUND[30] reported about the sorption of sugars in sulfite wasteliquir by yeast cells.

Table 7. Assimilation and fermentation

Substrate / Yeast	Isomaltose (prepared from dextran)				
	CO_2 ml	pH	Initial sugar %	Residual sugar %	Consumption % of sugar
Kyôkai yeast No. 6	>2.0	4.0	0.840	0.269	68.04
〃 No. 7	>2.0	4.0	〃	0.387	53.93
Sacch. formosensis	1.7	4.1	〃	0.193	76.52
Rasse II	0.1	4.3	〃	0.794	5.43
Rasse XII	0.2	4.1	〃	0.770	8.33
Fleishman yeast	0.8	4.1	0.940	0.389	58.67
Oriental 〃	>2.0	4.0	〃	0.381	59.42
N*-Oriental 〃	0.2	4.1	〃	0.932	0.85
Sankyo 〃	>2.0	4.1	〃	0.340	63.89
N-Sankyo 〃	0	4.1	〃	0.920	2.18
N-Wokamoto 〃	0	4.3	〃	0.889	5.48
N-Red Star 〃	0.9	4.1	0.840	0.506	39.82
N-Nitten 〃	0.4	4.1	〃	0.708	15.71
Sacch. Praecisus	0.1	4.1	〃	0.832	0.95
Torula utilis	0	4.3	0.940	0.940	0
Mycotorula japonica	>2.0	4.1	〃	0.313	66.75
Schizosacch. Pombe No. 1	>2.0	4.2	〃	0.241	74.42
Schizosacch. Pombe No. 2	>2.0	3.9	〃	0.281	69.79

* N; newly isolated yeast from commercial compressed yeast.

(3) Change of Fermentability by Taming

Although it is said that the fermentability of glucose by yeast does not change during storage, there is no report about that of isomaltose. Judging from the results of Table 7, the baker's yeasts which were newly isolated from compressed yeasts (e.g. *N-Sankyo, N-Oriental, N-Wakamoto* etc.) were very different in their fermentability of isomaltose from the bakers' yeasts which had been grown continuously on agar slant of Kôji-extract (which containes isomaltose).

We examined the isomaltose-fermentability of Sake yeast "*Kyôkai* No. 7" using the same procedure as in (2).

The three kinds of Sake yeasts "*Kyôkai* No. 7" of the same origin, which had been in our laboratory (TOHOKU UNIVERSITY), in The GOVERMENTAL INSTITUTE of BREWING TOKYO and in the APPRAISERS ROOM SENDAI, were used.

The obtained data are shown in Table 8.

Table 8. Isomaltose-fermentability of Sake yeasts.

Sake yeast "Kyôkai" No. 7	Initial sugar %	Residual sugar %	Consumption % of sugar	CO_2 ml	Chromatogram of isomaltose
our labolatory	0.857	0.266	69.10	>2.0	++
Sendai	〃	0.576	39.79	1.0	+++
Tokyo	〃	0.200	79.13	>2.0	+

of cellobiose and isomaltose.

Isomaltose (prepared from Kôji-extract)					Cellobiose				
CO_2 ml	pH	Initial sugar %	Residnal sugar %	Consumption % of sugar	CO_2 ml	pH	Initial sugar %	Residual sugar %	Consumption % of sugar
1.6	4.0	0.906	0.276	70.09	0.2	4.6	1.265	1.074	15.09
0.9	4.1	〃	0.465	48.67	0.6	4.0	〃	0.884	30.35
1.9	4.2	〃	0.252	72.24	0.5	4.0	〃	1.062	16.10
0.4	4.2	〃	0.669	26.32	0.2	4.4	〃	1.195	5.35
0.8	4.2	〃	0.519	42.76	0.3	4.4	〃	1.142	9.96
>2.0	4.0	0.836	0.175	79.07	0.5	4.2	〃	1.026	20.32
>2.0	4.0	〃	0.091	89.09	0.4	3.7	〃	1.108	13.59
0.5	4.2	〃	0.614	26.55	0.5	4.2	〃	1.100	14.35
2.0	4.0	〃	0.137	83.61	0.4	4.0	〃	1.035	19.58
0.5	4.0	〃	0.666	20.34	0.4	4.1	〃	1.062	17.48
0.5	4.2	〃	0.718	14.18	0.5	4.1	〃	1.069	16.94
1.2	4.2	0.906	0.514	43.33	0.5	4.6	〃	1.170	7.63
1.0	3.9	〃	0.496	45.31	0.6	4.5	〃	0.949	24.98
0	4.2	〃	0.713	21.36	0.4	4.1	〃	1.170	7.63
0.4	4.1	0.836	0.752	10.11	0.4	4.0	〃	1.053	18.18
>2.0	4.0	〃	0.052	93.12	0.8	3.8	〃	1.062	17.48
1.4	4.0	〃	0.026	96.77	0.8	3.9	〃	0.668	48.10
1.5	3.9	〃	0.043	94.86	1.0	4.0	〃	0.659	48.80

Remarkable differences were recognized between the fermentability of isomaltose by those yeasts. Although the reason for this difference is not clear, the medium in storage may be related. We examined the change of the fermentability of isomaltose when the newly isolated yeasts, *N-Wakamoto, N-Sankyo, N-Oriental, Sake yeast* (*Kyôkai* yeast No. 7 Sendai) and *Saccharomyces praecisus* had been tamed continuously in Kôji-extract. The constituents of Kôji-extract were as follows ; total sugar 11.62 per cent, reducing sugar 10.66 per cent, glucose 8.82 per cent, isomaltose 0.87 per cent, cellobiose and the others 0.53 per cent.

The medium was divided into 8 ml portions, placed in 250 ml test tubes, sterilized and inoculated with yeasts which had been grown on agar slants of wort for one week. Inoculating with the culture of yeasts for every twenty four hours, they were incubated at 27-28°C for 20 days continuously. Using the procedure described in (2), the fermentability of the tamed yeasts was examined. The obtained data are shown in Table 9.

It was found that the tamed yeasts (*N-Oriental, N-Sankyo* and Sake yeast *Kyôkai* No. 7) increased their fermentability of isomaltose remarkably, but *N-Wakamoto* and *Sacch. praecisus* did not change their fermentability in spite of taming with isomaltose.

In our country, yeasts for storage have been generally grown on agar slants of Kôji-extract containing isomaltose, while cane mollasses and wort are used commonly for the production of commercial compressed bakers' yeast.

Table 9. The changes of fermentability by Tameness.

Yeast	No.	Initial sugar %	CO_2 ml	pH	Residual sugar %		Consumption % of sugar	
N-Oriental Control	1	0.881	0.2	4.3	0.696	average 0.681	21.68	average 22.66
	2	〃	0.3	4.3	0.672		23.64	
〃 incubated for 10 days	1	〃	0.4	4.2	0.416	0.419	52.78	52.50
	2	〃	0.8	4.3	0.421		52.21	
〃 incubated for 20 days	1	〃	>2.0	4.2	0.171	0.180	80.59	79.63
	2	〃	>2.0	4.2	0.188		78.66	
N-Sankyo Control	1	〃	0.5	4.3	0.612	0.612	30.53	30.53
	2	〃	0.5	4.2	0.612		30.53	
〃 incubated for 10 kays	1	〃	>2.0	4.1	0.181	0.176	79.33	79.96
	2	〃	>2.0	4.2	0.171		80.59	
〃 incubated for 20 days	1	〃	>2.0	4.2	0.183	0.189	78.09	77.98
	2	〃	>2.0	4.2	0.195		77.86	
N-Wakamoto Control	1	〃	0	4.2	0.854	0.847	3.06	3.86
	2	〃	0	4.3	0.840		4.65	
〃 incubated for 10 days	1	〃	0	4.3	0.820	0.812	6.91	7.88
	2	〃	0	4.2	0.803		8.85	
〃 incubated for 20 days	1	〃	0.2	4.2	0.857	0.852	2.72	3.29
	2	〃	0.1	4.2	0.847		3.86	
Kyôkai yeast No. 7 (Sendai) Control	1	〃	0.5	4.0	0.644	0.640	26.89	27.35
	2	〃	0.6	4.1	0.636		27.81	
〃 incubated far 10 days	1	〃	1.8	4.1	0.404	0.410	54.14	53.46
	2	〃	1.0	4.2	0.416		52.78	
〃 incubated for 20 days	1	〃	1.7	4.1	0.178	0.175	79.68	80.13
	2	〃	>2.0	4.1	0.171		80.58	
Sacch. Praesisus Control	1	〃	0	4.3	0.747	0.747	15.21	15.21
	2	〃	0.3	4.3	0.747		15.21	
〃 incubated for 10 days	1	〃	0.1	4.4	0.684	0.693	22.36	21.34
	2	〃	0.1	4.5	0.702		20.31	
〃 incubated for 20 days	1	〃	0.2	4.5	0.722	0.728	18.04	17.36
	2	〃	0	4.4	0.734		16.68	

From those facts, the differences of fermentability by the different kinds of yeasts may largely depend on the medium in storage.

Summary

(1) Isomaltose was prepared from Kôji-extract and bacterial dextran which was obtained from sucrose by a strain of *Leuconostoc mesenteroides*.

(2) We studied the assimilation and fermentation of isomaltose and cellobiose by yeasts.

(3) Effects of tameness were experimented on assimilation and fermentation of isomaltose by yeasts.

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