SUCCINIC ACID PRODUCTION BY THE SAKE YEAST AND ECOLOGICAL CONSIDERATIONS ON THE YEAST ACTION

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By

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I. Introduction.

Sake wine is characteristic in its high content of succinic acid. This acid accumulates during the Moromi-process (the chief fermentation process) of Sake-brewing, and attains a level of 0.1 per cent. This accumulation in the Sake-Moromi has been often researched, and it is now known that the succinic acid proceeds in its accumulation during the vigorously fermenting stage of the Moromi-process, while the lactic acid, one of the main acids contained in Sake wine, is accumulated rather later, i.e. during the decay stage of the fermentation(1).

On the other hand, it has been once ascertained by Kleinzeller (2) that the added glucose or glutamic acid is chiefly responsible for the succinic acid produced by the yeast.   He also recognized that quantities of this acid produced either aerobically or anaerobically are considerably higher only when the yeast is incubated in the bicarbonate buffer added with the glucose, whereas in the phosphate buffer the yeast is able to produce only small quantities of this acid from the added glucose when incubated aerobically and also its negligible quantities when anaerobically.

In contrast to Kleinzeller's result the Sake yeast strain so far tested could produce aerobically considerable quantities of the succinic acid from the added glucose even in the phosphate buffer, when the cells of this strain cultured with shaking were employed.   Furthermore, the yeast fraction obtained directly from the Sake-Moromi by differential centrifugation, which is then designated by us "Sake-Moromi-yeast"(3), could show this production ability even when incubated anaerobically in the phosphate buffer.   This was also the case in the isolated laboratory strain of the Sake yeast when it had grown in the deep layers of the cultural solution.   Then, anaerobic pathway to form the succinic acid from glucose by the Sake yeast was also researched.   From these results thus obtained was induced a discussion on the relationship between ecological actions of the microbes, especially the yeast and the concerned environments.

II. Methods

Organism.   For laboratory yeast we have adopted Sake yeast strain, *Saccharomyces Sake* (Kyôkai No. 6) throughout the experiments.   The so-called "Moromi-yeast", a yeast fraction directly separated from Sake-Moromi by differential centrifugation, was also employed.

Cultures and inocula.   Stock cultures of Sake yeast were carried out on Kôji-agar and inocula were prepared from 1 to 3 weeks old cultures. For vitamin deficiency experiment, cells of one tube were washed twice with sterile water, then diluted to 10 ml, and one drop of this suspension was inoculatated to each 100 ml of medium.

Culture media.   Kôji-juice was usually used, which was prepared by saccharification of rice-Kôji within 4–5 times volume of water.   Its Bllg. was 10° and its N-content 0.05 per cent.   The peptone-glucose-salts-medium was composed of 0.6 per cent peptone, 6.0 per cent glucose, 0.1 per cent KH_2PO_4, and 0.05 per cent $MgSO_4$.   For pantothenate deficiency experiments, the following compositions of both sufficient and deficient media were employed : glucose (for injection) 5 per cent, KH_2PO_4 0.1 per cent, $MgSO_4$ 0.05 per cent, vitamin-free casein hydrolysate (as total nitrogen) 0.05 per cent, thiamine 1 mg/L, inositol 2 mg/L, nicotinic acid 0.4 mg/L, pyridoxine 1 mg/L, biotin $10\mu g$/L., and Ca-pantothenate $500\mu g$/L in the case of sufficient medium, or $15\mu g$/L in the

case of deficient medium. Vitamin-free casein digest was prepared from milk casein, as follows. 500 g of the thoroughly washed milk casein (four times with each 2L of hot ethanol) were hydrolyzed for 20 hours at 120° with 2.5 L of 10 per cent sulfuric acid. Concentrated digest was treated twice at pH 3.0 with active carbon until an almost colorless solution was obtained.

Acid formation from the added substrate and assay procedure. Experiments of acid formation were carried out usually in a Warburg vessel, in which 1 ml of yeast suspension, 1 ml of buffer solution, and 0.5 ml of substrate solution (M glucose alone, M glucose plus M/5 glutamate, or 40 per cent [v/v] ethanol) were mixed. The reaction mixture was incubated either aerobically (in air) or anaerobically (in N_2) at 30° for 3 hours and then centrifuged. The supernatant was either deproteinized or extracted with ether after acidifying with sulfuric acid. The latter ether extraction was done at 55° for 50 hours in a microextracter. These deproteinized or extracted solutions were permitted to assay the formed acid. The formed succinic acid was determined mano-metrically with pig heart succinic dehydrogenase preparation (4), which was lyophilized and stored in vaccum for three months with no loss of activity. The formed lactic acid was estimated colorimetrically by the modified method of Barker *et al.* (5). Glucose was determined by the method of Willstätter and Schudel (6). Respiration and fermentation were examined at 30° by the ordinary manometric method in the Warburg vessel, which was usually composed of 1 ml of yeast suspension, 1 ml of phosphate buffer, and 0.5 ml of substrate with or without 0.3 ml of KOH. For the inhibition test, 0.2 ml of substrate instead of 0.5 ml was employed with the addition of 0.2 ml of inhibitor (or water as blank test). Final concentrations of each substrate except ethanol were employed in M/20 unless otherwise noted, whereas M/150 ethanol was used as the res-piration substrate. Final concentrations of inhibitors were cited in each case. Other details of experimental conditions were noted if neccessary.

III. Results

1. Aerobic production of the succinic acid from the added glucose or glutamate by the Sake yeast.

A. Cells from the stationary culture.

A stock strain of the Sake yeast (Kyôkai No. 6) was grown on Kôji-juice at 30° as a stationary culture. Cells were harvested from 24, 70, and 130 hours' culture, respectively, washed twice with distilled water, and then aged in an ice box for a night before the test. Aerobic incubation of these cells was done at 30° for 3 hours in either the phosphate buffer (pH 7.4) or 0.5 per cent bicarbonate solution with glucose or glucose plus glutamate.

As shown in Table 1, all the cells tested except those from 70 hours' culture were able to produce appreciable quantities of the succinic acid from the added

Table 1.   Succinic acid production from the added substrate by the Sake
yeast of different ages (stationary culture)

Buffer	Culture period (hrs)	Succinic acid formed from (μg/mg dry cell)		
		Endogenous	Glucose	Glucose + L-glutamate
Phosphate (pH 7.4)	21	20	38(18)	105(85)
	70	0	0	101(101)
	130	7	14(7)	105(87)
Bicarbonate (0.5%)	24	86	108(22)	118(32)
	70	41	51(10)	61(20)
	130	55	85(30)	—

Figuress in parentheses show the volume subtracetd by autoformation.

substrates when incubated in either of both buffers, whilst on the contrary the
latter cells when incubated in the phosphate buffer could produce only a scanty
volume of this acid and its smaller but discernible quantities when incubated in
the bicarbonate buffer.   Then, the aerobic production ability of this acid of
the yeast varied along with its growth periods, regardless of which the cells so
far tested produced the acid in much larger quantities from either their
endogenous substrate or the added glucose when incubated in the bicarbonate
buffer than when in the phosphate buffer.   The results so obtained accorded
well with those of Kleinzeller.   When the glutamate was the added substrate,
however, it occurred conversely, and the acid quantities then produced in the
bicarbonate buffer were smaller than those in the phosphate buffer.   It might
be then supposed that the cells would require more phosphorus for the glutamate
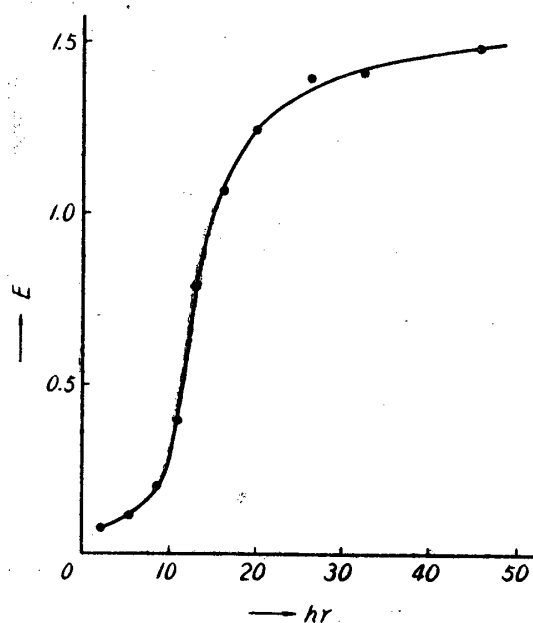breakdown because of its loss by the aging treatment.

Fig. 1.   Growth-curve of the
Sake yeast in the shake culture
(in Kôji-juice at 30° incubated).

B. Cells from the shake culture.

Acording to their growth periods shown in Fig. 1, the yeast cells were harvested from the shake culture after 12, 18, 24, 60, and 130 hours' growth, respectively, and used to the test after aging in similar manner as above noted.

Table 2 indicates that aerobic production of the succinic acid from each added

Table 2. Succinic acid production from the added substrate by the Sake yeast of different ages (Shake culture)

Buffer	Culture period (hrs)	Succinic acid formed from (μg/mg dry cell)		
		Endogenous	Glucose	Glucose+ L-glutamate
Phosphate (pH 7.4)	12	55	78(23)	161(106)
	18	23	65(32)	183(160)
	24	29	60(31)	233(194)
	60	45	74(29)	194(74)
	130	41	89(48)	120(78)
Bicarbonate (0.5%)	12	130	157(26)	
	18	80	117(37)	
	24	170	270(100)	
	60	72	79(7)	
	130	143	147(4)	

Figures in parentheses show the volume subtracted by autoformation.

substrate by these cells was considerably intensified throughout their growth periods, and accordingly the quantities of this acid produced by them were much higher than those by the cells from the stationary culture. Especially, when the glutamate was supplied as the added substrate and the incubation was carried out in the phosphate buffer, the cells of 24 hours' growth showed the most active ability among all so far tested. These cells also could produce more abundantly the succinic acid from either the added glucose or their endogenous substrate than could other cells when incubated in the bicarbonate buffer. From these results it may be well mentioned that at the end stage of its logarithmic phase the yeast tested possesses an intense ability of this acid production both from the added glutamate in the phosphate buffer and from the added glucose in the bicarbonate buffer. Furthermore, these yeast cells also showed extremely high endogenous acid production, and the acid quantities then produced were about two or three times higher than those by the cells from the stationary culture. Since such yeast cells as those from the shake culture was able to produce aerobically the succinic acid in considerable quantities from the added glucose even in the phosphate buffer, which were almost identical with those by the cells from the stationary culture in the bicarbonate buffer, Kleinzeller's concept on a special rôle of the bicarbonate buffer to promote the carbon dioxide fixation for this acid formation by the yeast is rather questionable.

2. Isolation of the succinic acid produced aerobically by the Sake yeast from the added glucose as incubated in the phosphate buffer.

The fact that in contrast to Kleinzeller the yeast cells from the shake culture could produce aerobically the succinic acid in quantities sufficient to be isolated even when incubated in the phosphate buffer with glucose, was verified by isolation of this acid, as attempted to carry out this reaction in large scale.

A large volume of the yeast cells was collected from 45 hours' culture in Kôji-juice with shaking; at first it was washed three times with distilled water, then once with 0.5 per cent bicarbonate solution for the purpose of removing the endogenous succinic acid, furthermore once with the phosphate buffer (pH 5.4), and finally once again with distilled water.

The yeast cell suspension so prepared was incubated at 30° for 3 hours in the phosphate buffer (pH 7.2) with or without the substrates. After centrifugation of the reacted mixture solution the supernatant was concentrated, acidified with sulfuric acid, and then extracted with ether. This ether solution was treated with active carbon, added with ligroin, then evaporated on the warm bath, and the plate-shaped crystalls then formed were collected. MP of the crystalls was 183°, which showed no depression when mixed with the authentic succinic acid.

Experiment A. Glucose as the added substrate. 100 ml of the yeast suspension (dry weight of the cells, 6.2g), 30 ml of M glucose (5.4g), and 200 ml of the phosphate buffer were used for the reaction. Yield of the succinic acid crystalls then isolated was 34 mg. When the same reaction of 1/100 scale was carried out in Warburg vessel under the same conditions, the total quantities of the produced succinic acid were estimated to be 0.51 mg.

Experiment B. Glucose plus glutamate as the added substrate. 100 ml of the yeast suspension (dry weight of the cells, 4.3 g), 34 ml of M glucose (5.4 g), 50 ml of M/5 glutamate (1.7 g), and 200 ml of the phosphate buffer were used for the reaction. Yield of the succinic acid crystals then isolated was 134 mg. Total quantities of the produced acid in 1/100 scale as above noted, were estimated to be 1.92 mg.

In the experiment without any substrate, only a scanty volume of the crystalls was then formed, but too small to be isolated. The experimental results with the added substrates agreed well with Kleinzeller's ones, in which quantities of the succinic acid produced aerobically from glucose plus glutamate were three- or fourfold larger than those from the glucose alone when the yeast reacted with these substates in the bicarbonate buffer.

3. The endogenous succinic acid

When the cultured cells of the Sake yeast were washed with 0.5 per cent bicarbonate solution, the succinic acid was found in this washing. This might be probably supposed to result in an abundant endogenous production of this acid when the yeast is incubated for 3 hours in the bicarbonate buffer, as above

observed. Then, attempts to research effects of wash procedure with the bicarbonate solution upon such a succinic acid accumulation were made with the yeast cells cultured at 30° for 45 hours in Kôji-juice with shaking.

Each about 0.9 g (dry weight) of the yeast cells preliminarily washed twice with distilled water, was again washed with distilled water, 0.5 per cent bicarbonate solution, and M/10 Na$_2$HPO$_4$ solution (pH 8.5), respectively. In each wash-treatment 25 ml of these agents was employed. The succinic acid then transferred into these agents was determined by the ordinary dehydrogenase method.

As seen from Table 3, the bicarbonate solution was the most active stripping agent of this acid among them, and indeed had eluted it exhaustively from the

Table 3. Succinic acid found in various washings of the cultured yeast cells

Washing solution	Succinic acid found in washing (μg/ml)				
	Washing round				
	1 st	2 nd	3 rd	4 th	5 th
Distilled wster	0	0	50		90
M/10 Na$_2$HPO$_4$ (pH 8.5)		50	30	30	20
0.5% bicarbonate	160	145	130	25	0

Cells in each case (ca. 0.9 g dry wt.) are harvested from 45 hours culture in Kôji-juice with shaking.

cells at the fifth wash-round, whereas the Na$_2$HPO$_4$ solution was an incomplete wash-agent as to the acid quantities then eluted as well as continuity of elution. With distilled water the succinic acid was scarcely found till at the second wash-round, and then it appeared slightly in the wash-water, tending rather to increase by further wash-treatment. Thus it might be apparently pointed out that the succinic acid contained in the cells is easily eluted out into weakly alkaline solution, and also that the stronger the alkalinity of the agent employed, the more abundantly the succinic acid is eluted out from the cell body. The adhesion ability of the succinic acid to the yeast cell body was also investigated in the following manner. Each about 0.8 g (dry weight) of the yeast cells was washed five times with 25 ml of either 0.5 per cent bicarbonate solution or M/10 Na$_2$- HPO$_4$ solution, respectively (primary wash). The cells so preliminarily washed were then soaked into M/20 succinic acid solution, and left alone for a moment, where thereafter the cells were re-washed twice with distilled water, and then treated three times with each of the above agents, respectively (secondary wash). The results are given in Table 4. The bicarbonate solution had washed out exhaustively the succinic acid from the unsoaked cells already at the third wash-round in the primary wash-procedure, whilst with this wash-agent the soaked

Table 4.   Soakage of the yeast cells after washing with buffers into M/20
succinic acid solution and the re-eluted succinic acid

Washing buffer	Succinic acid eluted from the cells (μg/ml)					
	In washing of the cultured cells			In washing of the cells after soaking in M/10 succinic acid solution.		
	1st round	3rd round	5th round	1st round	2nd round	3rd round
Bicarbonate (0.5%)	105	0	0	405	380	315
Na$_2$HPO$_4$ (M/10)	20	25	10	185	65	40

cells were continued to be stripped off still at the third round in the next secondary wash-procedure, in which the acid quantities then eluted were strikingly high though gradually decreased. This means that the cultured yeast cells are in unsaturated state with the succinic acid and the more they are stripped off, the more the succinic acid they can absorb when soaked into the concentrated succinic acid solution. Thus it was difficult to elute out this acid thoroughly from the saturated cells even with such an active agent as the bicarbonate solution. This might be also seen from the experimental results with an incomplete wash-agent such as an alkaline phosphate solution. It may be then reasonably stated that with the yeast cell body unites the succinic acid in its large part loosely enough to be eluted out with only simple procedure like wash-treatment with the slightly alkaline solution.

4. Aerobic production of the succinic acid from the endogenous substrate by the Bicarbonate-washed yeast cells.

Since the Sake yeast cells washed out exhaustively with 0.5 per cent bicarbonate solution possesses only scanty, almost undetectable volume of their endogenous succinic acid which had been absorbed into the cells during cultivation, such bicarbonate-washed cells may be applied as a true former of the succinic acid from their endogenous substrate during 3 hours' incubation when tested. Then, the effect of pH upon this endogenous acid production was first investigated with such bicarbonate-treated cells which were harvested from 45 hours' culture in Kôji-juice with shaking and then washed five times with 0.5 per cent bicarbonate solution. The cells were incubated at 30° for 3 hours in the phosphate buffer of either pH 6.5 or pH 8.5, respectively, and the succinic acid then produced from their endogenous substrate was determined. Table 5 shows that the endogenous succinic acid production at pH 8.5 prevailed exceedingly over that at pH 6.5. Furthermore, the effect of inbibitors such as monoiodoacetate (MIA) or dinitrophenol (DNP) upon this endogenous ability of the bicarbonate-washed cells were also researched. At this test was employed the bicarbonate buffer for incubation of the cells. As seen from Table 6, this acid production from the endogenous substrate was inhibited thoroughly by MIA,

whilst much less by DNP. It
may be presumably supposed
that the endogenous substrate
contained in the bicarbonate-
washed yeast cells would be a
glycogen-like substance,since the
cellular ribonucleic acid which
has been once recognized by
Krampitz & Werkman (7) as an
endogenous respiration subst-

Table 5. Influence of pH on the
autoformtion of snccinic acid

pH	6.5	8.5
Succinic acid formed (μg/ml)	70	235

Dry cell weight (bicarbonate-washed) : ·
32 mg/ml.

Incubated in phosphate buffer of each
pH tested.

rate, would have been considerably removed away by the bicarbonate-treatment.

Table 6. Influence of inhibitor on the autoformation of succinic acid

Inhibitor	None	MIA $(10^{-3} \cdot M)$	DNP $(10^{-4} \cdot M)$
Succinic acid formed (μg/ml)	460	0	385
Inhibition rate(%)	—	100	27.2

Dry cell weight (bicarbonate-washed) : 32 mg/ml.
Incubated in 0.5% bicarbonate solution.

5. The endogenous respiration of the bicarbonate-washed yeast cells.

It may be probably assumed that the aerobic endogenous production of the
succinic acid is referable to the endogenous respiration of the cells. The latter
ability of the Sake yeast varied along with its growth periods, during which the
optimal pH for this endogenous respiration inclined more to the acidic side so
as to pH 5.6 from pH 7.2 (Table 7). Thus the yeast of 48 hours' growth respired

Table 7. Changes in optimal pH for autorespiration of the Sake
yeast during its growth

Culture period (hrs)	O_2 − uptake (Qo_2)			
	pH			
	4.4	5.6	7.2	8.4
12	2.0	4.0	4.6	3.3
24	6.0	7.9	6.9	6.7
48	8.0	9.2	7.2	5.6

Yeast is cultured at 30° in Kôji-juice with shaking, and washed thrice with
bicarbonate buffer.

its endogenous substrate more actively at pH 4.4 than at 7.2, though this endo-
genous respiration was considerably active in such a pH-range as between both
pH-values. It may be then probably deducible that the aged yeast cells become
more stable, even more active against an acidic environment. This endogenous
respiration of the yeast was also affected by several inhibitors such as DNP,
As_2O_3, MIA, or malonic acid. On investigation free malonic acid was used

without neutralization, where the phosphate buffer was not added to this inhibitor system alone in contrast to addition of this buffer to other systems. Among these inhibitors tested, MIA and malonic acid gave a strongly inhibitory effect upon this endogenous respiration, whereas As_2O_3 was a moderate inhibitor and DNP showed only a faint inhibition (Table 8). The contrasting relationship

Table 8. Influence of inhibitors on the autorespiration of the Sake yeast

	Inhibitor				
	None	DNP $(10^{-4}.M)$	As_2O_3 $(5.10^{-.3})$	Malonic acid $(4.10^{-2}.M)$	MIA $(10^{-3}.M)$
Xo_2(60 min) (μl)	420.5	348.6	175.2	81.1	38.7
Inhibition rate (%)	—	17.1	58.3	80.6	90.8

Bicarbonate-washed yeast cells (dry wt : 31.5 mg/ml.) are employed.

of the inhibitory effect of MIA to that of DNP agreed very well with the results of both these inhibitors in the case of the endogenous succinic acid production by this yeast. This means that, as preliminarily expected, both endogenous activities of the Sake yeast are related to each other and each endogenous substrate will be broken down through the same pathway, or at least in a few same points in its respective fate.

On the other hand, Krebs *et al.* (8) have detected no appreciable effect of the malonate upon the endogenous respiration of the dry-ice-treated yeast cells. The bicarbonate-washed cells of our yeast strain was also not affected by the malonate due to their permeability barriers, against which free malonic acid could overcome and showed a strong inhibition of their endogenous respiration occurring at pH 3.8. According to Krebs *et al.*, such a malonic acid inhibition occurring at the extremely acidic side of reaction is rather disputable, and therefore, notwithstanding a strong inhibition of this kind, it may not be concluded instantly that the succinic dehydrogenase system participates in the oxidation pathway of the concerned endogenous substrate.

6. Inhibitory effect of malonic acid upon oxidations of ethanol, acetic acid, and glucose by the bicarbonate-washed yeast cells.

Lynen *et al.*(8) have once investigated that the malonic acid does not inhibit the oxidation of ethanol to acetic acid by the yeast, in spite of its inhibitory effect upon the further oxidation of acetic acid. Since the intact cells were employed in their investigation, even free malonic acid may be disturbed in its penetration into the cell body by the cellular succinic acid previously absorbed to the cells during cultivation. Thus, from the stepwise inhibitory effect of the malonic acid upon the ethanol oxidation by such untreated cells as Lynen *et al.* employed, the stepwise oxidation process of the ethanol would not be instantly concluded, because a spare time for penetration of the malonic acid into the untreated cells

would have to be considered. Then, an inhibitory test of the malonic acid on the ethanol oxidation was carried out with the bicarbonate-washed yeast cells, as compared with its inhibitory effect upon the acetic acid oxidation, where according to Lynen *et al.* a large volume of the cells was used without addition of the phosphate buffer. The bicarbonate-washed cells so far tested did not show so clear-cut features of the stepwise inhibition of the ethanol oxidation by the malonic acid as Lynen *et al.* obtained (Fig. 2). If this inhibitory effect was

Fig. 2. Inhibitory effect of free malonic acid on the oxidations of ethanol and acetic acid. Contents of the vessel: yeast suspension (washed cell) 1.0 ml, substrates 0.5 ml, free malonic acid, or H_2O 0.5 ml, KOH 0.3 ml. $V_F=2$ 3 ml.
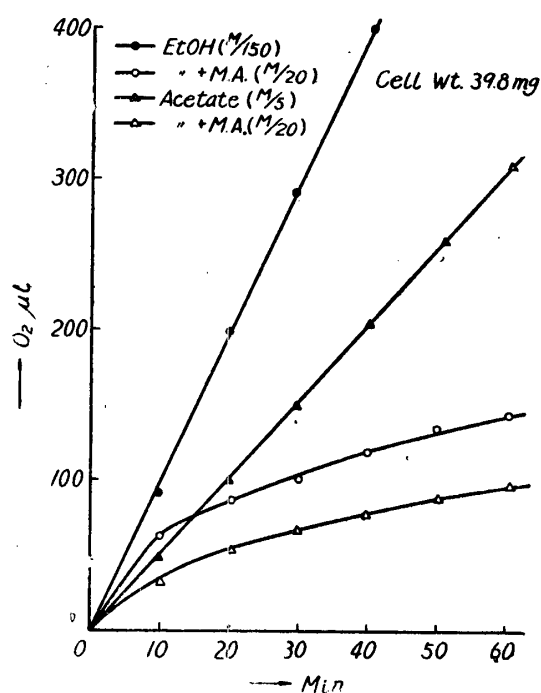
periodically traced, the inhibition rate of the malonic acid for the ethanol oxidation at the first 10 minutes was considerably smaller than that for the acetic acid oxidation, and soon after prevailed over the latter one along with elapsing time, as shown in Table 9. Since the yeast cells tested were free from

Table 9. Periodical changes in inhibitory effects of free
malonic acid on oxidations of ethanol and acetic acid by the Sake yeast

Substrate	Inhibition rate (%)							
	Time (min)							
	10	20	30	40	50	60	70	90
Ethanol (M/150)	27.2	58.2	68.6	72.1	71.9	76.6	76.4	82.0
Acetic acid (M/20)	40.9	52.5	61.6	66.0	65.9	70.1	71.6	75.1

Free malonic acid employed : 4.10^{-2}·M.

Dry cell weight (bicarbonate-washed) : 39.8 mg/ml.

the succinic acid, maybe a powerful competitive matter against the malonic acid, by exhaustive wash-treatments with the bicarbonate solution, this might

be presumably contributed to the stepwise effect of the malonic acid inhibition in accordance with Lynen's interpretation, though the oxidation process insensitive to the malonic acid have taken place only for a short period.

Inhibitory effect of the malonic acid upon the acetic acid oxidation also increased along with elapsing time, ranging in rate from 40.9 per cent at the first 10 minutes to 75.1 per cent at the last 10 minutes (after 90 minutes). It might be then supposed that this periodical increase of the inhibition rate for the acetic acid oxidation would occur due to need of free malonic acid for the time to penetrate into the cells. Other interpretation on this result would be, of course, also possible, but since this reaction occurred at the considerably acidic side, the penetration theory might be most significant above all.

The inhibitory effect of free malonic acid upon the ethanol oxidation when the phosphate buffer (pH 5.4) was used, was also observed. The results are given in Table 10, and it is interesting to note that its inhibition features are changeable due to concentrations of free malonic acid then added. When the concentrated solution of this acid (M/60 as final concentration) was used, it showed relatively stationary inhibition throughout the reaction, ranging in rate from the first 40.3 per cent to the last 55.4 per cent at 10 minutes intervals during 80 minutes' test. Reversely, when the dilute acid solution (M/960 as final concentration) was used,

Table 10. Influence of inhibitors on the oxidation of ethanol by the Sake yeast

Time (min) Xo₂(μl) Additions	10		20		30		60		80	
	Xo_2	IR (%)	Xo_2	IR (%)	Xo_2	IR (%)	Xo_2	IR (%)	Xo_2	IR (%)
None	218.2	0	375.6	0	475.0	0	695.3	0	824.2	0
Malonic acid (M/60)	127.2	40.3	214.6	42.9	258.3	45.6	329.0	52.7	367.5	55.4
Malonic acid (M/960)	121.3	43.1	268.8	28.4	391.8	17.5	615.5	10.9	813.0	1.4
Malonate (M/120)	201.8	5.3	373.4	0.6	468.5	1.4	697.4	0	812.7	0.5
DNP (10^{-4}.M)	53.4	75.0	136.6	63.6	209.1	56.0	415.4	40.3	519.5	37.0
As_2O_3 (5.10^{-3}.M)	78.2	63.1	167.3	54.9	232.6	51.0	336.6	51.6	383.1	53.5
MIA (10^{-3}.M)	4.0	98.1	8.9	97.6	8.9	96.0	15.8	97.3	15.8	98.1
H_2O	96.5	—	177.3	—	246.3	—	442.3	—	550.6	—

Dry cell weight (bicarbonate-washed) : 31.5 mg/ml.

Inhibition rate (abbreviation : IR) is calculated as Xo_2 (inhibitor) $\times$ 100/Xo^2 (none), which is accordingly associated by inhibitory effect on the autorespiration.

it inhibited at first the ethanol oxidation more intensely than did the former concentrated solution, and then this inhibitory effect decreased rapidly, which became alomst nullified after 80 minutes. Krebs *et al.* have once observed that relatively high concentrations of the malonic acid (0.0125 M) are required for inhibition of the acetic acid oxidation, on which however, its low concentrations (0.001 M) are ineffective. As they used also the phosphate buffer (pH 2.5) in their investigation, the results obtained by them might correspond to those by us, though the oxidized substrates were different from each other. In our case this might be interpreted as follows ; since the yeast cells were almost

thoroughly free from their endogenous succinic acid, the added malonic acid even at low concentrations would be able to penetrate easily into these washed cells, and casue an effective inhibition at first. But the phosphoric acid, a poly valent acid, would act in like manner with succinic or malonic acids, and therefore a competition in penetration into the cells would occur between them. Meanwhile the phosphoric acid might become to prevail over the malonic acid when low concentrations of the latter acid were employed, and cause resulting ineffectiveness of this acid.

Furthermore, some contradictory results on the malonic acid inhibition were obtained due to the presence or absence of the phosphate buffer in the reaction systems. When the phosphate buffer was present, then the ethanol oxidation by the yeast was inhibited considerably by the malonic acid (M/60) from the beginning of its process, and accordingly, the stepwise inhibitory effect of this acid was indiscernible, which would correspond to the stepwise oxidation of the ethanol, as previously mentioned. Another case was also seen in the acetic acid oxidation. The malonic acid (M/60) showed almost perfect inhibition of this oxidation in the presence of the phosphate buffer (Table 11), whereas this inhibitory effect in the absence of the phosphate buffer ranged only from the first 41 per cent to the last 75 per cent in 90 minutes' test as cited previously in Table 9. With these discrepancies may be partially concerned changes in pH

Table 11. Influence of inhibitors on oxidations of pyruvic acid, acetic acid, and glucose by the Sake yeast

Substrate	Additions (Time min. / Xo_2 μl)	10 Xo_2	10 IR (%)	20 Xo_2	20 IR (%)	30 Xo_2	30 IR (%)	60 Xo_2	60 IR (%)
Pyruvic acid	DNP (10⁻⁴.M)	20.0	70.9	35.3	74.1	42.0	78.1	78.3	78.9
	As_2O_3 (5.10⁻³.M)	28.3	58.8	45.5	66.6	57.6	70.0	111.1	70.1
	Malonic acid (M/60)	5.3	92.1	9.4	93.2			20.0	94.6
	None	68.7	0	136.0	0	192.0	0	371.1	0
Acetic acid	DNP (10⁻⁴.M)	15.7	78.7	34.3	75.4	46.0	78.0	103.2	74.2
	As_2O_3 (5.10⁻³.M)	26.9	63.6	48.8	65.1	53.7	74.4	109.5	72.6
	Malonic acid (M/60)	0	100	4.7	96.6			10.4	97.4
	None	73.7	0	139.7	0	209.5	0	400.4	0
Endogenous	None	58.3		99.0		149.8		265.5	
Glucose	DNP (10⁻⁴.M)	62.7	71,3	99.3	73.4	152.6	74.7		
	As_2O_3 (5.10⁻³.M)	140.6	33.3	223.4	44.6	316.2	47.7		
	Malonic acid (M/60)	180.6	14.6	259.8	35.6	328.1	45.7		
		(178.1)	(0)			(323.6)	(35.0)		
	None	210.9	0	403.2	0	604.3	0		
		(173.8)		(332.6)		(505.2)			
Endogenous	Malonic acid (M/60)	3.0		67.6		4.5			
	None	37.1				99.1			

Dry cell weight (bicarbonate-washed) : 29.5 gm/ml. In the case of glucose oxidation the manometer before measurement is shaken for 50 min., opening the cock. Abbreviation IR means inhibition rate. Figures in parentheses show inhibition rate, which is subtracted by inhibitory effect on the autorespiration.

of the reaction medium, caused due to whether the presence of the phosphate buffer in the reaction systems, but moreover, the significant effect of the phosphoric ion itself would have to be also considered. Further investigations on this inhibitory effect of the malonic acid will be required, as Krebs *et al*. have pointed out.

Among other inhibitors such as DNP, As₂O₃ and MIA inhibited also DNP the ethanol oxidation, indicating rather reversed aspects to those of the malonic acid inhibition, and this DNP inhibition decreased gradually along with the time. Whilst inhibition by As₂O₃ showed stationary rate in about 50 per cent till the last, MIA was a strong inhibitor of the ethanol oxidation by the bicarbonate-washed cells. Considering from the fact that MIA nullifies also completely the endogenous respiration of the yeast, it would be then supposed that the above inhibitory effect of this agent upon the ethanol oxidation may result from the lack of energy sufficient to activate the concerned enzyme systems preliminarily.

Both DNP and As₂O₃ inhibited in almost similar manner with each other the oxidations of either the acetic acid or the pyruvic acid by the bicarbonate-washed yeast cells, as shown in Table 11. Since such inhibition relationship of the oxidation of the acetic acid to that of the pyruvic acid agreed with each other, a common course would be present in each oxidation pathway.

Inhibition as to the glucose oxidation by the washed yeast showed different appearance from that in the oxidations of the above two acids. DNP inhibited this glucose oxidation in the rate of about 70 per cent throughout its process, whilst As₂O₃ inhibition of it was much weaker and, increasing a little at the last, attained only to the rate of 47.7 per cent after 30 minutes. Malonic acid did not affect this oxidation at the first 10 minutes when the endogenous respiration was already nullified by this acid, and then began to inhibit it, attaining to the rate of 36 per cent after 30 minutes, whereas, in contrast, the endogenous respiration was still almost eliminated at this time. Thus, in this oxidation process would be present some preliminary system insensitive to the malonic acid, and accordingly a stepwise oxidation process would occur in the glucose breakdown. If malonic acid refers to only one oxidation system, known as succinic dehydrogenase system, this system may participate undoubtedly in the glucose oxidation, and on the other hand, respond the endogenous yeast respiration, which is significantly blocked by the malonic acid as above noted. It is still doubtful whether such a simple interpretation is applicable for these facts.

DNP also inhibited a little an aerobic production of the succinic acid from the added glucose by the bicarbonate-washed cells. This inhibition rate was 26.4 per cent as shown in Table 12, and agreed nearly with that for the endogenous succinic acid production, but contradicted considerably larger rate for the glucose oxidation, i.e. 75 per cent. This indicates that with respect to the DNP inhibition the glucose oxidation would not correspond to the aerobic production

Table 12. Succinic acid production from various added substrates by the bicarbonate-washed cells in the presence of dinitrophenol (DNP)

Substrate	Succinic acid formed (μg per cup)*		
	without DNP	with DNP (10^{-4}.M)	Inhibition rate (%)
Glucose	1609	1183	26 4
Pyruvic acid	108	194	
Acetic acid	270	157	43.6
Citric acid	0	0	

Dry cell weight : 46.5 mg ml. Components of Warburg's vessel : yeast suspension 1 ml, phosphate buffer (pH 7.4) 1 ml, DNP or water 0.2 ml. Incubated 30°, 3 hours, in air.

* Figures in the Table show the volume subtracted by autoformation of succinic acid in the absence of DNP.

of the succinic acid from the added glucose. Quantities of the succinic acid produced from the added acetic acid by the yeast were too small to offer an interpretation as to a significance of this production, upon which however an inhibitory effect of DNP was to be appreciable.

On investigation of the inhibition test concerning the aerobic production of the succinic acid from either the endogenous or the added substrates as well as oxidations of these substrates by the bicarbonate washed yeast cells no clear-cut result was obtained by us to be useful in elucidation of these mechanisms, though some curious phenomena such as those in the malonic acid inhibition have been occasionally observed. Wash-treatment with the bicarbonate solution can rid the yeast cells of their endogenous succinic acid, whereas permeability barriers of the cells are not excluded by this procedure.

7. Anaerobic production of the succinic acid from the added glucose by the Moromi-yeast.

The Sake yeast cultured with shaking could not produce anaerobically any appreciable quantities of the succinic acid from the added glucose even when incubated in the bicarbonate buffer. Kleinzeller also once failed to observe an anaerobic production of the succinic acid by the yeast when the phosphate buffer was used. On the other hand, the yeast cells actually in the Sake-Moromi would be enriched in their anaerobic abilities, since they act in the deep layers of the Sake-Moromi. This might lead to a probable expectation that with such a yeast is induced a successful anaerobic production of the succinic acid from the added glucose even if the phosphate buffer is empolyed. Then, Sake-Moromi was filtered through a gauze, and the filtrate was centrifuged for about 3 minutes at 500 rpm to remove some heavier material like rice granules therein present. The supernatant was again centrifuged for 5 minutes at 1000 rpm, and the large part of the yeast cells was settled under white-colored layers of starch granules. Then, these starch layers were stripped off with a spatula. This procedure was repeated several times until the white-colored layers were not at all discerned.

The grey-colored layers of the yeast cells thus obtained indicated negative test of starch with iodine, and were observed microscopically to be homogeneous in yeast cells. Accordingly, it might be well recognized that this yeast fraction is sufficiently pure though mixture of several variants of the Sake yeast and capable of acting as a pure yeast. Thus it was designated "Sake-Moromi-yeast" (or simply "Moromi-yeast").

Sake-Moromi samples were collected periodically from two kinds of Sake batch characterized by the kind of added Moto (starter) at the Sake-brewery in 1952.

As preliminarily expected, the Moromi-yeast thus obtained was able to produce the succinic acid aerobically as well as anaerobically from the added glucose even when incubated in the phosphate buffer (pH 7.2). This ability of the yeast varied along with the fermentation stage of the Moromi, which corresponded to the growth phase of the yeast therein present. The results are shown in Table 13. The Moromi at the 6th day after its preparation indicated the so-called

Table 13. Succinic acid production from the added glucose by the Moromi-yeast at various stages of Moromi-fermentation process.

Sake-Moromi sample	Reaction under Moromi-stage(a) (day) / Changes in reaction constituents (b)	The anaerobic conditions (in N₂)				The aerobic conditions (in air)			
		6	11	17	24	6	11	17	24
Yamahai-Moto used (c)	Sugar consumed (mM/mg dry cell)	8.2	39.8	18.6	—	6.7	35.2	7.8	—
	Succinic acid formed (mM/mg dry cell)	0.05	0.10	0.93	0	0.05	0.26	0.56	0
Sokujô-Moto used (d)	Sugar consumed (mM/mg dry cell)	8.3	34.2	25.9	—	6.2	30.7	10.8	—
	Succinic acid formed (mM/mg dry cell)	0.16	0.26	0.57	0.05	0.29	0.70	0.14	0.05

(a) The day elapsing after completion of Moromi-preparation.
(b) Figures in the Table show the volume subtracted by the endogenous consumption or formation.
(c) Moromi-preparation added with Yamahai-Moto (naturally acidified Moto) as a starter.
(d) Moromi-preparation added with Sokujô-Moto (Moto acidified by preliminary addition of lactic acid) as a starter.

"Taka-awa" (high layers of foam formed by the vigorous fermentation of the Moromi) in its features, whilst that at the 11th day was in the decaying period of such Taka-awa stage. In the Moromi at the 17th day then had been accumulated more than 15 per cent ethanol and that at the 24th day was in an aging stage, where the fermentation had almost completely ceased and quantities of the ethanol then accumulated attained to about 18 per cent. Moromi-yeast at the 11th day regardless of the kinds of the Moto added for preparation of the Moromi was able to consume the glucose aerobically as well as anaerobically most actively during 3 hours' incubation. On the other hand,

anaerobic ability of this sugar consumption of the yeast seemed to be more active than its aerobic one throughout the Moromi-process, and indeed the Moromi-yeast at the 17th day could anaerobically consume the glucose twofold more than could aerobically. This indicated that anaerobic fermentability of such a Moromi-yeast, which however is less stable under aerobic conditions, is able to tolerate high concentrations of the ethanol. The Moromi-yeast obtained from either of both batches at this period showed also the highest ability of anaerobic production of the succinic acid from the added glucose among the yeasts in each series and the yeast obtained from the Moromi-batch added with Yamahai-Moto could aerobically produce the succinic acid most actively, whereas that from the corresponding Moromi-batch added with Sokujô-Moto could less actively. In the yeast series of the latter Moromi younger yeast, e.g. the Moromi-yeast at the 11th day, showed the highest aerobic production ability of the succinic acid.

As generally seen, it may be probably assumed that the yeast in the Moromi prepared by adding Sokujô-Moto grows more rapidly than does that in the Moromi by adding Yamahai-Moto, and ripening of the yeast, at the process of which the yeast can acquire not only the tolerance against high concentrations of ethanol but also aerobic and anaerobic production abilities of the succinic acid from the added glucose, occurs much sooner in the former Moromi than in the latter one, so that the succinic acid production ability of the former Moromi-yeast varies with an acute peak, as shown in Table 13.

The Moromi-yeasts obtained from several Sake-batches in 1954 were again researched on their anaerobic acid production ability from the added glucose and also the added glutamate in the presence or absence of 10 per cent ethanol. Lactic acid other than succinic acid then produced when the Moromi-yeast was incubated at 30° for 3 hours in the phosphate buffer (pH 5.5) with the substrate was colorimetrically estimated with p-hydroxydiphenyl. The yeast was harvested periodically from various fermentation stages of the Moromi-batch prepared by adding Sokujô-Moto. In this case, too, every Moromi-yeast then obtained could produce anaerobically appreciable quantities of the succinic acid from the added glucose, and more abundant ones of this acid also from the added glutamate (with glucose) when absent from 10 per cent ethanol (Table 14). When the yeast cell capable of producing the succinic acid more actively was employed and incubated in the presence of 10 per cent ethanol, this acid, to be rather curiously, was accumulated much more abundantly than in the absence of the ethanol, whereas the lactic acid production by such a yeast was not affected, if not at all, by its presence. This was not observed in too ripe yeast cells, e.g. the yeast obtained from the Moromi at the 17th day or later date. It may be then supposed that the shunt of the glucose breakdown by the yeast would be somewhat deviated due to the presence of 10 per cent ethanol and tend to form the succinic acid. Further

Table 14. Changes in anaerobic abilities of the Moromi-yeast during the Moromi-fermentation process

Moromi-batch	Days after inoculation process (Day)	Anaerobic activities of the Moromi-yeast.									
		$Q\,^{N_2*}_{CO_2}$ (glucose)		Lactic acid formed from (μg/mg dry cell)				Succinic acid formed from (μg/mg dry cell)			
		In the absence of 10% ethanol	Inhibition rate by the presence of 10% ethanol	None	Glucose	Glucose +L-glutamate	Glucose in the presence of 10% ethanol	None	Glucose	Glucose +L-glutamate	Glucose in the presence of 10% ethanol
A	11	96	16.5	2.6	8.0	4.4	3.7	0	38.0	115.0	101.0
	17	23	13.5	0.4	4.2	2.4	2.5	0	0.6	0.9	1.6
B	9	65	—	0	3.0	2.6	2.4	9.8	28.9	83.7	33.4
	14	111	19.7	0	1.7	1.8	2.7	2.6	16.3	58.1	≒0
	20	90	—	0	1.3	1.4	1.4	0	9.1	5.0	≒0

Moromi-batches, from which samples are collected, are prepared by adding the Sokujô-Moto. The phosphate buffer of pH 5.5 is employed for investigation of the anaerobic activities of the Moromi-yeast tested.

* $Q^{N_2}_{CO_2}$ (glucose) is subtracted by the endogenous one.

investigations will be required for confirmation of the evidence.

As compared with the lactic acid production ability of the Moromi-yeast, its anaerobic production ability of the succinic acid seemed to be less tolerable against high concentrations of ethanol such as 15 per cent or more, which had accumulated in the Moromi-process, since the yeast at the 20th day showed considerable ability of the former acid production whilst almost negligible one of the latter acid production.   The tolerance of the former lactic acid production ability of the yeast against the ethanol corresponded quite well to that of its fermentability in respects of intensity and continuity.   These facts may account for the nullified but rather inhibitory effect of 10 per cent ethanol upon the succinic acid production by too ripe yeast, i.e. the yeast less capable of producing this acid by damage due to high concentrations of ethanol accumulated in the Moromi.   Lactic acid was not produced from the added glutamate but only from the added glucose by action of the yeast, whilst both these substrates were responsible for the succinic acid production.   This may be also the case in the actual Moromi-process, and the results above obtained may well account for accumulation statues of both succinic and lactic acids during the fermentation process of the Moromi, which have been frequently observed by several investigators (1).

Anaerobic production ability of the succinic acid of the Moromi-yeast was also confirmed by direct isolation of this acid from the reacted mixture in large scale.   The Moromi-yeast was collected from a batch in 1954, and washed twice with distilled water.   Scale in quantities of the reactants was fifty times greater

than that of the above ordinary test in the Warburg vessel. The results thus obtained are shown in Table 15, and appreciable quantities of the succinic acid were isolated from both added substrates. The reacted mixture in each case

Table 15. Isolation of the succinic acid anaerobically produced from the added substrates by the Moromi-yeast

Glucose added (g)	L-Glutamate added (g)	Succinic acid isolated* (mg)	Succinic acid estimated**(mg)
0	0	0	0
4.5	0	11.0	13.2
4.5	0.74	42.0	48.2

Anaerobic reaction (30°, 3 hours incubation of the Moromi-yeast in substrate-phosphate buffer) is carried ont 50 times greater than ordinary test in Warburg's vessel. The Moromi-yeast is prepared from Moromi-batch (Sokujô-Moto used) of 14 days old.

* Succinic acid is actually isolated from the reacted mixture in large scale by ether extraction ; MP of the crystals shows 185°.

** Succinic acid when formed in Warburg's vessel of ordinary small scale, is determined by the dehydrogenase method and multiplied by 50.

contained less impurities, contrasted to the aerobic test as previously cited, and therefore the yields of this acid from either of both substrates corresponded well to each estimation value obtained at the ordinary test in small scale. Ratio between the yields from both substrates, though less in amounts of these yields, also agreed well with the ratio obtained at the previous aerobic test. The test without any addition of the substrate showed no formation of the succinic acid.

8. Anaerobic production of the succinic acid from the added glucose by the laboratory Sake yeast.

From preliminary supposition that the Sake yeast in the Moromi-process would be enriched in anaerobic abilities in as much as its habitation in deep layers of the Moromi-components, it was seen successfully that such a Moromi-yeast, if ecologically harvested without using any culture medium, shows considerable activity to produce anaerobically the succinic acid from either the added glucose or the added glutamate. Correspondingly it might be also presumably assumed that if even the ordinary laboratory Sake yeast permits itself in the stationary culture in considerably high layers of the cultural solution, then such a yeast cell may also indicate this anaerobic activity. Such attempts were done as to our Sake yeast strain (Kyôkai, No. 6), and in contrast to Kleinzeller's results, led to success in anaerobic production of the succinic acid from the added glucose even when this laboratory yeast so cultured was incubated in the phosphate buffer. In a long tube was admitted about 60 ml of the cultural solution, the height of which in layers attained to 13 cm. Kôji-juice, or peptone-glucose-salts solution with or without yeast extracts (20 minutes boiled extracts of the dry baker's yeast with twofold volume of water) were used

as cultural solution, respectively. The yeast strain was inoculated into each medium and then incubated at 30° for 2 days long. The harvested yeast crops were washed twice with distilled water, and then supplied to the test. The results are given in Tables 16 and 17. Each yeast cell cultured in such high layers of the cultural solution could produce anaerobically the succinic acid from the added

Table 16. Anaerobic abilities of the Sake yeast strain (Kyôkai No. 6), as cultured at 30° for 45 hours in the deep layers of various media

Cells harvested from cultures in	Anaerobic activities of the yeast						
	* $Q_{CO_2}^{N_2}$ (glucose)	Succinic acid formed from (μg/mg dry cell)			Lactic acid formed from (μg/mg dry cell)		
		None	Glucose	Glucose+ L-glutamate	None	Glucose	Glucose+ L-glutamate
Kôji-juice	319	3.2	44.0	131.0	1.3	4.6	5.1
Peptone-medium	235		33.0	45.0		4.9	4.7
Peptone-yeast ext-medium	298		58.0	136.0		6.8	6.8

Each medium contains 0.05% N. ''Yeast ext.'' is prepared by 20 min. extraction from baker's yeast and added in 2% to the peptone-medium. Acid production from the added substrates is carried out in the phosphate buffer of pH 7.0.

* $Q_{CO_2}^{N_2}$ (glucose) is subtracted by the endogenous one.

Table 17. Acid formation abilities of the Sake yeast from the added glucose under aerobic or anaerobic conditions

Cells harvested from 45 hours culture(30°) in	Under aerobic conditions (in air)		Under anaerobic conditions (in N_2)		
	Succinic acid formed (μg/mg dry cell)	Lactic acid formed (μg/mg dry cell)	$Q_{CO_2}^{N_2}$ (glucose)	Succinic acid formed (μg/mg dry cell)	Lactic acid formed (μg/mg dry cell)
Kôji-juice	27.2	1.3	238	20.3	4.2
Petone-yeast ext-medium	42.0	1.8	278	30.8	7.7

Figures shown in the Table are subtracted by the endogenous ones. On test of acids formation, 50 mg of glucose per vessel is used under aerobic conditions, while 100 mg of glucose per vessel under anaerobic conditions, as added substrate. In both cases pH of phosphate buffer is 7.3.

glucose as much as could aerobically, whereas it produced a larger volume of the lactic acid anaerobically than did aerobically. Glutamate was an available substrate for anaerobic and aerobic production of the succinic acid, but unavailable for the lactic acid production, as previously mentioned. When the yeast cells cultured in the peptone-glucose-salts medium without yeast extracts were employed, they produced much less quantities of the succinic acid from the glutamate as compared with activities of other yeast cells cultured in either Kôji-juice or peptone-glucose-salts medium with yeast extracts. Furthermore, when incubated at 30° for 3 hours in the phosphate buffer with tyrosine plus glucose, the cells grown on the peptone-medium without yeast extracts were

able to produce only inappreciable quantities of tyrosol, whereas other yeast cells could produce it in remarkable quantities (Table 18). Therefore, it might be probable that such a peptone-cultured yeast would be deficient in a certain

Table 18. Tyrosol formation by the Sake yeast from the added tyrosine

Cells harvested from 45 hours culture (30°) in	Tyrosol formed (μg/mg dry cell)
Kôji-juice	6.16
Petone-medium	1.53
Peptone-yeast ext.-medium	4.32

Tyrosol formation is done by incubating aerobically (in air) the yeast at 30° for 3 hours in Warburg's vessel. Components of the vessel : yeast suspension 1 ml, phosphate buffer (pH 5.5) 1 ml, M/10 glucose 0.5 ml, L-tyrosine 5.0 mg. The formed tyrosol in ether extract is estimated colorimetrically with Millon's reagent.

Figures shown in the Table are subtracted by the endogenous ones.

cofactor responsible for the amino acid breakdown, which was supplied extra-cellularly from vitamin-like substance contained in sufficient volume in Kôji-juice or yeast extracts. This presumption will be demonstrated by further investigations in the future. pH of the phosphate buffer ranging from pH 5.5 to pH 7.4, in which the cells were incubated for the reaction, was indifferent to the anaerobic production of the succinic acid from the glucose by them, whilst at more acidic pH, e.g. pH 3.8, this acid was produced more abundantly, as shown in Table 19. This was rather uncontemplated, since under aerobic conditions

Table 19. Effect of pH on acids production by the Sake yeast
under anaerobic conditions

pH of the phosphate buffer employed for the yeast incubation	Succinic acid formed from (μg/mg dry cell)			
	Glucose	Glucose+ L-glutamate	Glucose	Glucose+ L-glutamate
7.0*	27.8	78.9	—	—
5.5*	24.6	—	—	—
5.5**	40.5	157.0	3.9	3.7
3.8*	82.5	172.0	3.4	2.9

Figures shown in the Table are subtracted by the endogenous ones. Cells are harvested from(*) 48 hours,(**) 45 hours culture in Kôji-juice (30°).

the reverse was the case as previously observed. Accordingly, to say that acidic *milieu* is suitable to an anaerobic production of the succinic acid by this yeast may have to be still retained.

9. Anaerobic pathway to form the succinic acid from the glucose by the Sake yeast.

It would be now generally accepted that the glucose is aerobically broken down by the yeast through TCA cycle, though Krebs and his school have postu-

lated that this cycle would have to be significant only for the yeast growth. When the glucose is served as a respiration substrate to supply energy to the yeast cells, it was then supposed by them that some other cyclic pathway except TCA cycle would be activated for its aerobic breakdown. The contradiction of this kind is the cause of controversy between Krebs and other several investigators, which seems to be continued rather in irresolvable manner(10). However, our present thesis on the anaerobic pathway to form the succinic acid from the glucose by the yeast has not yet been touched by these investigators, who have engaged themselves in this controversy.

Our Sake yeast strain requires pantothenate for its growth, which in turn is well known to be able to convert into coenzyme A within the yeast cells and thus activate TCA cycle. Coenzyme A reacts at least at two points of TCA cycle, and indeed at the condensing enzyme system and also at the decarboxylation system of α-ketoglutarate. Thus, if the succinic acid is formed via TCA cycle, supplement of the yeast nutrition with the pantothenate would cause a singnificant effect upon the succinic acid formation from either the glucose or the glutamate, since the succinic acid may be apparently formed through decarboxylation of α-ketoglutarate in TCA cycle, which, on the other hand, is also a deaminated product from the glutamate (Fig. 3). Conversely, if not

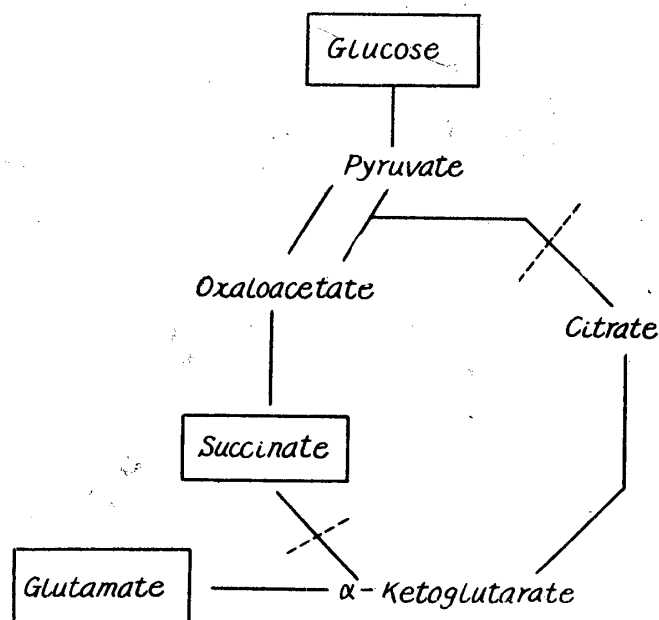...... Reaction blocked by pantothenate deficiency.

Fig. 3. Blocking effect of pantothenate
deficiency upon TCA-cycle.

the case, there would occur insignificant effect of the pantothenate deficiency upon the succinic acid formation from the glucose. Then the pantothenate

deficiency might serve to draw out a considerably definite clue to clarify our concerned thesis.

Novelli and Lipmann(11) have once observed that the pantothenate deficiency is not responsible for the fermentability of the yeast, but to aerobic breakdown of the glucose by it, where they used baker's yeast cultured with shaking. When our strain, Kôkai No. 6, was grown on the pantothenate-deficient medium (supply of 15μg of Ca-pantothenate to L of the defined basal medium), it showed a typical *diauxie* in features of its growth curve, and the yeast cells of 30-40 hours' growth indicated less abliity to ferment the glucose anaerobically $(Q_{CO_2}^{N_2})$ than that of the cells grown on the pantothenate-sufficient medium (500 μg of Ca-pantothenate to L of the defined basal medium) (Fig. 4). Then the former deficient yeast facilitated again its growth, approaching to the growth level of
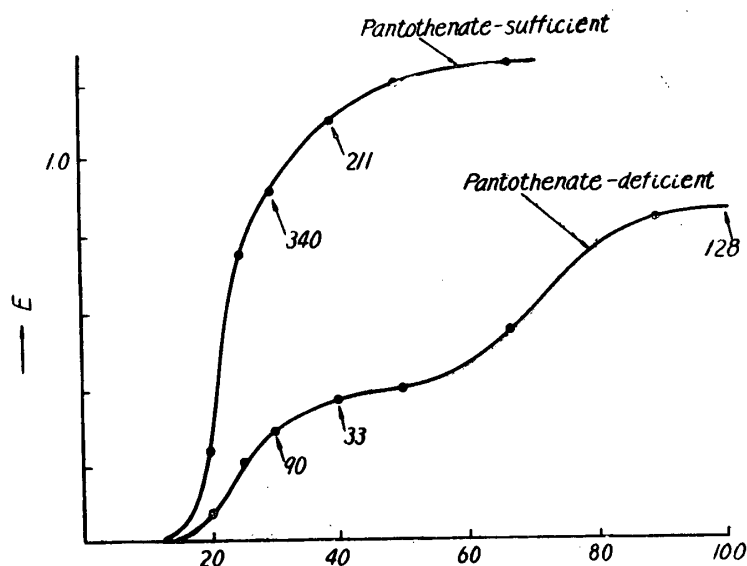

Fig. 4. Effect of pantothenate deficiency upon the growth of Sake yeast.

Figure ($\uparrow$) shows $Q_{CO_2}^{N_2}$.

the latter sufficient yeast, and also simultaneously intensified its anaerobic fermentability. At this growth test, the Sake yeast was cultured with resting in considerably deep layers of each concerned medium as above noted. This different cultivation method adopted by us from that of Novelli and Lipmann would cause such a discrepancy in effect of the pantothenate deficiency upon the yeast fermentability.

In Table 20 are given several effects of the pantothenate deficiency upon various abilities of the Sake yeast, where both sufficient and deficient cells, respectively, were cultured at 30° for 40 hours. As seen obviously from the Table, this vitamin deficiency displayed a significant rôle for the anaerobic

Table 20. Effect of pantothenate deficiency of the medium on anaerobic abilities of the cultured Sake yeast

Cells	Incubation period for growth (hrs)	$Q^{N_2}_{CO_2}$ (glucose) *	* $Q^{O_2}_{CO_2}$ (glucose)	Succinic acid formed from (μg/mg dry cell) None	Glucose	Glucose + L-glutamate	Lactic acid formed from(μg/mg dry cell) None	Glucose	Glucose + L-glutamate
Pantothenate-sufficient	30	340	—	4.5	28.3	116.0	0.05	0 80	0.63
Pantothenate-deficient	30	90	—	1.5	19.3	22.2	0	0.22	0.21
Pantothenate-sufficient	40	211	16.5	0	41.5	131.5	0.14	1.00	0.91
Pantothenate-deficient	40	33	1.8	0	21.3	18.4	0.03	0.82	0.45

Cells are grown at 30° in the deep layers of culture solution. Deficient cells are obtained from the culture in the medium added with 15μg/L of Ca-pantothenate, while suffiicent cells with 500 μg/L. Basal medium : glucose 55g, maltose 5 g, vitamin-free casein hydrolysate 0.05% (as N), KH_2PO_4 1g, $MgSO_4$ 0.5g, $CaCl_2$ 1g, biotin 10 μg, inositol 2mg, thiamine 1mg, nicotinamide 0.4 mg, pyridoxine 1 mg, distil. water 1L.

* The values are subtracted by the endogenous ones.

production of the succinic acid from the added glutamate, whilst on the contrary this rôle was scarcely observed on that from the added glucose. In the latter case quantities of this acid produced by the deficient cells, however, considerably less than those by the sufficient cells, but the differences between them agreed rather well with those between fermentabilities of both cells. From these results it might be reasonably pointed out that anaerobic formation of the succinic acid from the glucose by the yeast did scarcely occur via TCA cycle, because, if this were the case, quantities of this acid produced from the glucose would be affected as seriously by the pantothenate deficiency as occurred in the glutamate breakdown. Consequently, the deficient cells seemed almost lack in coenzyme A, and therefore it might be supposed that they are nearly eliminated in their activity of anaerobic decarboxylation of α-ketoglutarate, on which Krebs *et al.* have once observed by dry ice-treated yeast cells. The technique of Krebs *et al.* to destroy permeability barriers of the yeast membrane was applied by us to both pantothenate-sufficient and -deficient cells. Both these yeast cells harvested from 40 hours' culture were left over a night in an ice box, then warmed at 30° for 40 minutes, and suddenly cooled with direct addition of dry ice to them, simultaneously incubated in a dry ice bath. Table 21 indicates that the deficient cells showed only 3.7 per cent in the anaerobic decarboxylation activity of this α-keto acid, as compared with that of the sufficient cells. This nullified activity of the former cells corresponded very well to their activity of the succinic acid production from the glutamate. This means apparently that the deficient yeast cells can scarcely form succinic acid from α-ketoglutarate and accordingly also from any tricarboxylic acid such as citric, isocitric, *cis*-aconitic acids, etc.

Table 21. Effect of pantothenate deficiency of the culture medium on anaerobic decarboxylation of α-ketoglutarate by the dry ice-treated cells of the Sake yeast

Cells	$Q_{CO_2}^{N_2}$		
	Dry ice-treated		Untreated
	α-ketoglutarate	Glucose	Glucose
Pantothenate-sufficient	8.27	26.9	211.0
Pantothenate-deficient	0.29 (3.5%)	4.7 (17.3%)	32.5 (15,4%)

The cells tested are harvested from the cultures of 40 hours growth. According to Krebs *et al.*, the cells are warmed, frozen within dry ice, and thawed ("dry ice-treated"). Anaerobic decarboxylation is done at 30° in phosphate buffer(pH 5.5), where M/70 glucose and M/140 α-ketoglutarate in final concentrations are employed as substrate. Figures in parentheses show the relative decarboxylation rate of the deficient cells, as compared with activities of the sufficient cells.

Thus it is now eveident that the succinic acid is scarcely formed anaerobically from the glucose via TCA cycle by the Sake yeast. This conclusion seems to agree well with Krebs' concept on the aerobic mechanism of the glucose breakdown by the yeast, but it is inconsistent to extend this conclusion, which has been drawn out from the results obtained through the anaerobic experiments, to an aerobic region.

If the succinic acid is not formed anaerobically via TCA cycle, then it may be assumed that this acid is formed either by direct carboxylation of pyruvate (Szent-Gyorgyi's pathway) or by dehydrogenated condensation of a certain C_2-compound maybe derived from the pyruvate (Thunberg-Wieland scheme). The condensation mechanism of the latter C_2-compound is still obscure and it has been frequently supposed that this is a system concerned with coenzyme A (e.g. Krebs *et al.*). However, this would not be the case, since anaerobic formation of the succinic acid from the glucose by the yeast so far tested is not affected by the pantothenate deficiency. The former carboxylation system of the pyruvate such as the reaction system of Wood-Werkman(12) or the malic enzyme system(13) is well defined by several investigators, and known to be not concerned with coenzyme A, and also to take place in yeast metabolism. In our case it would be supposed consistently that such a carboxylation system is a possible intermediate pathway to form anaerobically the succinic acid from the glucose by the Sake yeast, and the fact that anaerobic decarboxylation of the pyruvate was not affected by the pantothenate deficiency as shown in Table 22, is rather suggestible. As we obtained abundant quantities of the succinic acid produced by the Moromi-yeast in the presence of 10 pen cent ethanol in contrast to those in its absence, the condensation mechanism of the C_2-compound to form the succinic acid by the yeast will yet remain in our investigation scheme.

In addition, it is interesting to note that alcohol-tolerance of the fermentability of the yeast grown on the perfectly defined medium, in which the yeast crops are

Table 22. Effect of pantothenate deficiency of culture medium on anaerobic decarboxylation of pyruvate by dry ice-treated cells of the Sake yeast

Cells	$Q^{N_2}_{CO_2}$			
	Dry ice-treated		Untreated	
	Pyruvate	Glucose	Pyruvate	Glucose
Pantothenate-sufficient	30.8	13.2	1.4	58.5
Pantothenate-deficient	47.0	1.2	9.0	22.2

Cells of 45 hours old are employed in both cases, where the final concentrations of each substrate are 10^{-2}·M.

harvested as much as in Kôji-juice, is considerably smaller, as compared with that of the Moromi-yeast (see Table 14). It is then presumed that a certain factor influencing this tolerance would be contained in Sake-Moromi.
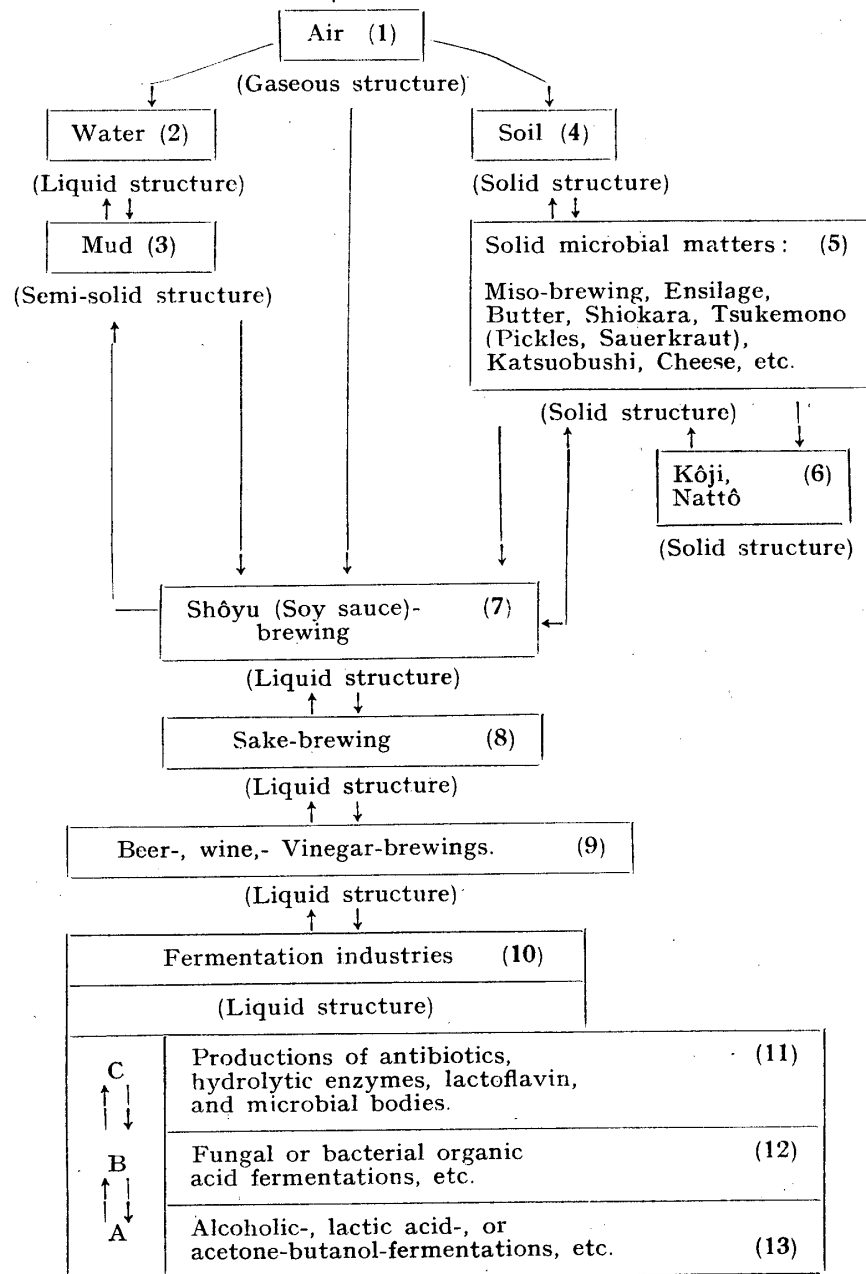
IV. Discussion

Sake yeast is a top-fermenter, and grows more readily than does a bottom-fermenting beer yeast because of its more intense respiratory ability inducing to facilitate the nitrogen assimilation. A considerable formation ability of the succinic acid from the glucose during the fermentation also seems to be one of the characteristic properties of the top-fermenting yeast. This means that simultaneous pyruvate shunt other than chief pathway of ethanol formation would occur more actively in such a yeast, and partially proceed either to TCA cycle under aerobic conditions, or to Szent-Gyorgyi's dicarboxylic acid pathway under anaerobic conditions. Recently, Swim and Krampitz (14) clarified that glucose is aerobically broken down via TCA cycle in *E. coli*, and then postulated that the succinic acid accumulation would be accounted for from dismutation of malic and fumaric acids systems and also an inhibitory effect of oxaloacetic acid upon succinic dehydrogenase system. This dismutation has been also observed more recently in Tubercle bacteria by Kususe *et al.*(15). The succinic acid accumulation occurring during the fermentation by the Sake yeast may be considered to be due to such a formation mechanism.

Glutamic acid is a good source of the succinic acid, and therefore the latter acid contained in Sake wine may be derived by action of the yeast partially from this amino acid present in Sake-Moromi though considerable quantities of this amino acid remain still in the Sake wine. When the Moromi-yeast becomes to intensify its formation ability of the succinic acid from the glucose and the ethanol is accumulated in considerable quantities near about 10 per cent, then the succinic acid will be chiefly produced from the glucose derived from rice starch by Kôji-amylase, and not contributory to the glutamic acid therein present, which may be thereafter accumulated during the later process of the Moromi-fermentation and thus remain to be left alone in the Sake wine.

Ecological considerations on the microbial actions. On the other hand, comparing with the ordinary laboratory yeast, the so-called Moromi-yeast possesses high alcohol-tolerance in its fermentability and also more powerful anaerobic ability to form the succinic acid from either the glucose or the glutamic acid. From these dominant abilities of the Moromi-yeast it may be well apprehensible why Sake wine is rich in ethanol (about 18 per cent or more) as well as in succinic acid. However, it is still obscure by what means the Moromi-yeast can require such dominant characters, and knowledge on the concerned environments may offer a key to elucidate this problem. In respect of the microbial *milieu* it may be roughly differentiated into two categories, that is, viable environment and non-viable one. The former is pathogenic, while the latter is non-pathogenic. In the latter, to which chiefly belong our research subjects, are environmental conditions rather static and vary themselves essentially by actions of the microbes therein habiting. This would make elucidation of the facts more easily and also possible to systematize the concerned subjects according to their structure, other various environmental conditions, and actions of habitants, etc., as shown in the following diagram (Fig. 5), which indicates development of the matters from simple systems to fully complex ones. Based only on such a point of view as a special regard on the interaction between the mirobes and the concerned environmental conditions, ecological aspects of microbiology would disclose their true features, and become a beneficial means for occupation of an application territory. If such ecological aspects are not considered, then in the laboratory microbiology are treated only isolated systems under voluntarily artificial conditions, which alone tend always to lead to a false interpretation of the outer microbial world. If the concerned isolated systems are not at all, but ecological aspects alone are investigated, then it is inevitable to fall into a simply phenomenal treatment and an essential part of the microbiology remains obscure. Both ecological and isolated treatments would have to be united to each other, and then they may become more effective upon elucidation of the microbial facts. The yeast acts in a broad territory of the microbial matters, as seen from the diagram, and possesses various dominant, well-discernible characters such as fermentability, alcohol- and acid-tolerances, etc. Furthermore, it can be easily separated from other fungal or bacterial bodies by centrifugation due to its characteristic weight. These physical and physiological properties of the yeast may be adapted well to an ecological research, with which many difficulties are associated due to their complexity. On the other hand, the yeast in isolated system has been researched from various points and accordingly the knowledge on the isolated yeast thus estabilshed may serve to elucidate an ecological part of the yeast action. Consequently, we have adopted the yeast for the ecological research of the microbial action. At present we are dealing with some actions of the yeast in

Fig. 5. Systematic diagram of microbial matters in
non-viable environments.

| Air (1) |

(Gaseous structure)

| Water (2) |

(Liquid structure)
↑ ↓

| Mud (3) |

(Semi-solid structure)

| Soil (4) |

(Solid structure)
↑ ↓

| Solid microbial matters : (5) Miso-brewing, Ensilage, Butter, Shiokara, Tsukemono (Pickles, Sauerkraut), Katsuobushi, Cheese, etc. |

(Solid structure)

| Kôji, (6) Nattô |

(Solid structure)

| Shôyu (Soy sauce)- (7) brewing |

(Liquid structure)
↑ ↓

| Sake-brewing (8) |

(Liquid structure)
↑ ↓

| Beer-, wine,- Vinegar-brewings. (9) |

(Liquid structure)
↑ ↓

| Fermentation industries (10) |

(Liquid structure)

C ↑↓	Productions of antibiotics, hydrolytic enzymes, lactoflavin, and microbial bodies. (11)
B ↑↓	Fungal or bacterial organic acid fermentations, etc. (12)
A	Alcoholic-, lactic acid-, or acetone-butanol-fermentations, etc. (13)

(1). . . . Complex species (fungal spores, bacteria, yeast). Non-specific microbial actions and substrates.

(2). . . . Complex species (bacteria). Complex substrates. Chiefly non-specific but sometimes specific microbial actions (and substrates).

(3). . . . Complex species (bacteria). Complex substrates. Some times specific (methane fermentation), or non-specific microbial actions (and substrates).

(4). . . . Complex species (fungi, bacteria, yeasts, *Actinomyces*). Complex substrates. Usually non-specific microbial actions and substrates. Often characteristic

microbial actions (N$_2$-fixation, denitrification, desulfurication, etc.).

(5).... Complex species (fungi, bacteria, yeasts). Complex substrates. Sometimes specific (lactic acid fermentation by *Lactobacillus sp.*, or weak alcoholic fermentation by *Saccharomyces, sp., Zygosaccharomyces sp*),. and usually non-specific microbial actions (especially fungal action).

(6).... Single species (*Aspergillus sp.*, or *Rhizopus sp.*, *Bacillus Natto*). Complex substrates. Non-specific microbial actions.

(7).... Complex species (*Bacillus subtilis-mesentericus* group, *Lactobacillus sp., Zygosaccharomyces sp.*, and *Saccharomyces sp.*). Complex substrates, or periodically a single substrate (sugar). Periodically specific microbial actions (lactic acid-, or alcoholic fermentation).

(8).... At first complex species (bacteria, especially *Lactobacillus sp.*, and yeasts), but later a single species (*Saccharomyces sake*).
    A single substrate (sugar) throughout the process.

(9).... A single species (*Saccharomyces cerevisiae, Saccharomyces ellipsoideus*, or *Acetobacter sp.*). A single substrate (sugar or ethanol).

(10).... A single species.

(11).... A single species, but complex substrates. Action due to respiration.

(12).... A single species, and a single substrate (sugar). Action due to aerobic fermentation.

(13).... A single species, and a single substrate (sugar). Action due to anaerobic fermentation.

the fermentation process of Sake-Moromi(3), and of Shôyu-Moromi(16) and also in the silaged process(17). Yet only a few data on the ecological aspects of the yeast actions occurring in such matters have been obtained even though somewhat available harvestry might be expected, a part of which is presented in this report.

V. Summary

1. A strain of the Sake yeast, Kyôkai No. 6, was able to produce aerobically well discernible quantities of the succinic acid from the added glucose when incubated at 30° for 3 hours in the phosphate buffer. The yeast cells from the shake culture possessed this ability markedly.

2. The endogenous succinic acid was easily obtained from the yeast cells by wash-treatment with 0.5 per cent bicarbonate solution, and in turn, the cells thoroughly washed out easily absorbed this acid when soaked in a concentrated solution of the succinic acid. Then the succinic acid seemed to be able to unite loosely with the yeast cell body. This may also account for the reason why an abundant volume of the endogenous succinic acid is accumulated when the cells are incubated in the bicarbonate buffer.

3. The inhibition test showed that the aerobic production of the endogenous succinic acid by the bicarbonate-washed yeast cells coresponds well to their endogenous respiration. The latter ability of the yeast was inhibited perfectly by free malonic acid.

4. Effects of free malonic acid or other inhibitors upon oxidations of ethanol,

acetic acid and glucose, and also upon the aerobic production of the succinic acid from these various substrates by the washed yeast cells were observed. Of the inhibitors tested, free malonic acid could inhibit them in various manners such as Krebs *et al.* have once pointed out.

5. The "Moromi-yeast", the yeast fraction directly separated from Sake-Moromi by differential centrifugation, could produce anaerobically the succinic acid from either the added glucose or the added glutamate when incubated at 30° for 3 hours in the phosphate buffer.

6. The cell crops of the Sake yeast strain, Kyôkai No. 6, harvested from its culture in deep layers of the cultural solution showed also this anaerobic ability remarkably. The cells grown on the peptone-glucose medium without yeast extracts showed only a faint activity of such anaerobic production of the succinic acid from the added glutamate, and also of tyrosol from the added tyrosine. Thus a certain factor responsible for the amino acid breakdown is deficient in such cells. On the other hand, the lactic acid was produced abundantly from the added glucose by this yeast strain under anaerobic conditions, whilst scantly under aerobic conditions.

7. Pantothenate deficiency was not responsible for the anaerobic production of the succinic acid from the added glucose, but for that from the added glutamate by this yeast stratin. This vitamin deficiency was also concerned with anaerobic decarboxylation of α-ketoglutaric acid by this yeast. It was concluded that the anaerobic pathway to form the succinic acid from the glucose by the Sake yeast is indifferent to TCA cycle.

8. Ecological pattern of the microbial matters in non-viable environments was systematized, and the significance of the yeast in this system was also discussed.

Addendum

Several microbial matters cited in the above diagram (Fig. 5) such as Shôyu, Miso, Sake, etc., belong to foodstuff or alcoholic beverage used especially in Japan. Because of such a speciality, a simple explanation concerning these microbial matters is given as follows.

"**Natto**" is prepared from boiled soybean, grown with *Bacillus Natto* (a bacterial species belonging to *Bacillus mensentericus-subtilis* group). Bacterial growth is carried out in a small and closed chamber keeping the temperature (about 37°) nearly constant and full humidity. Incubated for about 30 hours, until fertile mucilage with a good flavor is sufficiently produced by bacterial

action. Natto is widely favored by the Japanese as a nutritive foodstuff.

"Sake" is a fermentation product of boiled rice saccharified with rice-Kôji, and accordingly it is prepared from the mixture of boiled rice and rice-Kôji in water. Rice-Kôji is prepared also from boiled rice, grown with *Aspergillus oryzae*. The yeast is fertilized primarily in Moto after its inoculation, which is thus a kind of starter and of the same composition but much smaller e.g. about 1/20 scale, as compared with Sake -Moromi, the chief fermenter. "Sokujô-Moto" is a starter acidified with preliminary addition of lactic acid while "Yamahai-Moto" by preliminary action of natural lactic acid bacteria (*Lactobacillus sp.*, or *Leuconostoc sp.*). It takes about 30 days for fermentation of the Sake-Moromi, including its aging. *Saccharomyces sake* is only a dominant organism in the Sake-Moromi.

"Shôyu" is a filtered liquid of the aged Shôyu-Moromi, which is composed of Shôyu-Kôji and concentrated salt water (about 18 per cent in concentration). Shôyu-Kôji is prepared from a boiled mixture of soybean and wheat bran, grown with *Aspergillus sojae*. The aged Shôyu-Moromi contains about 2 per cent nitrogenous matters. It takes about a year for its aging during which occurs an alcoholic fermentation at least once in summer. *Bacillus mesentericus-subtilis* group, *Lactobacillus sp.*, and the yeast (*Zygosaccharomyces sp.*) are dominant microbes living therein. These organisms are osmophilic.

"Miso" is a salted product of the mixture of boiled soybean and rice-Kôji, laid under heavy weights. It takes usually three months or more for its aging. Lactic acid bacteria (sometimes spore-former), and several species of the yeast (mainly *Zygosaccharomyces sp.*) are dominant microbes living therein.

"Tsukemono" is a salted product of fresh green vegetables laid under heavy weights. Rice bran often with rice-Kôji is usually used for a medium. The yeast (maybe *Zygosaccharsmyces sp.*) and lactic acid bacteria (sometimes spore-former) are dominant microbes living therein.

"Shiokara" is a salted product of fish viscera, to which is often added rice-Kôji, then laid under heavy weights. The yeast (mainly *Zygssaccharomyces sp.* and *Debaryomyces sp.*) and bacteria are dominant microbes living therein. It takes about 10 days or more for its aging.

"Katsuobushi" is prepared from the dried and smoked meat of *Katsuwonus vagans* (Lesson), which is then inoculated with fungi. Fungal growth covers the whole fish meat due to several times inoculation. Aged a few months till the fertilized body is dried up and tightened. Several species of both *Aspergillus* and *Penicillium* are dominant.

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