Studies on the Activities of Bacteria in Soy Sauce Brewing

Part V. The Effects of *Aspergillus sojae*, *Pediococcus soyae*, *Bacillus subtilis* and *Saccharomyces rouxii* in Purely Cultured Soy Sauce Brewing

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The soy koji mold *Aspergillus sojae* is purely cultured on the materials of soy koji, and soaked in the sterile concentrated sodium chloride solution (26%). To it, *Ped. soyae* and *Sacch. rouxii* are aseptically inoculated and their roles playing in soy sauce brewing are examined. *Bacillus subtilis* is added in the form of wheat bran culture into the purely cultured soy mash and its influences are observed.

The appropriate addition of the bran culture of *Bac. subtilis* favors for the development of necessary aroma, and eliminates the polypeptide-like turbidity in soy sauce. The growth of *Ped. soyae* in soy mash stimulates the formation of organic acids, and makes the mash acidic which is necessary for the sound fermentation, removes undesirable flavors, and adds the indispensable good flavors for soy sauce. But the rapid lowering of pH results in the depression of *Aspergillus* Alkaline Proteinase activities^{1,2,3)}, followed by its inactivation, thus the percentage of solubilization of the protein material decreases. The colour of soy sauce also becomes lighter. *Sacch. rouxii* produces ethanol, and cooperating with *Ped. soyae*, plays an important part for developing the soy sauce flavor. *Asp. sojae* is the most important microorganism for soy sauce brewing. Almost all the essential enzymes for soy sauce brewing originates in *Asp. sojae*, as a result, fundamental chemical components, aroma and flavor are developed by enzymes from this mold. The soy sauce brewed by *Asp. sojae* alone, however, is not equal to the one which is made with mixed organisms, but it is only the mother body of soy sauce. The solubilization ratio of the protein material has also reached to the maximum without the aid of other microorganisms. However, the production of organic acids and ethanol is insufficient, polypeptide-like turbidity in soy sauce has remained, and aroma and flavor are not as well as the normally brewed soy sauce.

By addition of bacterial and yeast cultures, attempts for improving the quality of soy sauce were frequently reported. But they were all dealing with open natural cultures, and no proper roles of individual microorganisms were extensively studied. In this experiment, the important four microorganisms relating to the soy sauce brewing were all purely cultured and added, and their roles were elucidated by

chemical analysis, by enzyme activities and by sensuous tasting. The four important microorganisms dealt with were; *Aspergillus sojae*^{4,5)}, *Pediococcus soyae*⁶⁾, *Bacillus subtilis*^{1,7,8)} and *Saccharomyces rouxii*⁹⁾. The other isolated microorganisms relating to soy sauce brewing;

- 1) K. Sakaguchi, This Bulletin, **22**, 345 (1958).
- 2) T. Mizunuma and N. Iguchi, This Bulletin, **22**, 35 (1957).
- 3) W. G. Crewther and F. G. Lennox, *Aust. J. Biol. Sci.*, **6**, 428 (1953).

- 4) K. Sakaguchi and K. Yamada, *J. Agr. Chem. Soc. Japan*, **20**, 64, 141 (1944).
- 5) K. Yamamoto, This Bulletin, **21**, 308, 313, 319 (1957).
- 6) K. Sakaguchi, This Bulletin, **22**, 353 (1958); **23**, 22 (1959).
- 7) Y. Ishimaru, *J. Agr. Chem. Soc. Japan*, **9**, 859, 953, 1143 (1933).
- 8) K. Matsumoto, *J. Soc. Brewing Japan*, **21** (1926), **22** (1927), **23** (1928), **24** (1929).
- 9) H. Onishi, This Bulletin, **21**, 137, 143, 151 (1957).

namely lactic acid bacteria growing in soy koji⁶⁾, *Brettanomyces*^{7,8)} and *Torulopsis*^{9,10,11)} appearing in the late stage of soy mash fermentation, were excluded in this study because of their meager influences on the brewing.

EXPERIMENTAL

Preparation of purely cultured soy mash. A large two necked Komagome type flask (Fig. 1) was used for the pure culture of soy koji. To it, 49.5g of defatted granular soy bean, 24.2g of parched and cracked wheat grain and 7.9g of wheat bran were added and autoclaved for forty minutes at 1.5 atm/cm². The defatted soy bean and wheat bran were pre-treated by spraying with boiling water. The sterilized materials were inoculated with *Aspergillus sojae* spores and incu-

FIG. 1. The Two Necked Komagome Type Flask for the Cultivation of Purely Cultured Soy Koji.

FIG. 2. The Bottle for the Brewing of Purely Cultured Soy Mash.

bated for three days at 30°C. After the whole materials were covered with mycelia and spores, the soy koji was aseptically transferred to the bottle illustrated in Fig. 2, and soaked with 180 ml of autoclaved brine containing 26.00% sodium chloride. The concentration of salt was too high in this case, and it should be corrected to about 24%. The salt used was the Arabian bay salt, and its sodium chloride content was about 91%. After the soaking, the bacterial contamination was inspected using eurocidin media¹²⁾. The bacteria-free bottles were incubated at 30°C for two and half months. Time to time the air was bubbled through the longer cotton tube into the bottle to stimulate the growth of yeast which was inoculated latter.

Inoculation of *Pediococcus sojæ*. *Ped. sojæ* dl 2⁶⁾ was subcultured in 5 ml of the broth, containing yeast extract (Difco) 0.3%, polypepton (Takeda) 0.5%, glucose 1.0%, dipotassium phosphate 0.5% and sodium chloride 7.0%. This culture was incubated anaerobically from three to six days at 30°C, then poured into the purely cultured soy mash three days after the soaking of soy koji.

Inoculation of *Saccharomyces rouxii*. *Sacch. rouxii* strain No. 7^{13,9)} isolated from the soy mash was subcultured in 5 ml of rice koji water (Bllg 10°) containing 7% sodium chloride for three or six days at 30°C. This culture was inoculated into the purely cultured soy mash on the same day as *Ped. sojæ*.

Addition of bran cultured *Bacillus subtilis*. Five grams of wheat bran, 1g of soy bean powder and 5 ml of water in 150 ml Fernbach flask was autoclaved at 1.5 atm/cm² for forty minutes. Strong proteinase producers, *Bac. subtilis* var. *Bioteucus* A-538 (Nagase Industries Co.,) *Bac. subtilis* strain Fn and H-127-H (Institute of Applied Microbiology, University of Tokyo) were inoculated and cultured respectively for three days at 30°C. The resulted each bacterial bran was added into the purely cultured soy mash at the time of soaking with brine.

Mixed culture of *Asp. sojae* and *Bac. subtilis* var. *Bioteucus* A-538. *Asp. sojae* was inoculated on the autoclaved soy koji materials, and after one day's culture, *Bac. subtilis* var. *Bioteucus* A-538 was inoculated. The incubation was continued for two days thereafter.

Brewing of the factory soy koji. The soy koji was supplied from the Seventh Factory, Noda Shoyu Co.. Four hundred grams of the soy koji was soaked

10) H. Onishi, read on the General Meeting of the Soc. Agr. Chem. Japan on April, 1957.

11) M. Mogi and H. Onishi, *J. Ferment. Technol.*, **33**, 396 (1955).

12) K. Sakaguchi, *J. Agr. Chem. Soc. Japan*, **28**, 758 (1954), *Rep. Noda Inst. Sci. Res.*, **2**, 25 (1958).

13) H. Onishi, unpublished.

with 700 ml of crude concentrated brine containing 22.60% sodium chloride. The mash was incubated for three months at 30°C in the same manner as the purely cultured soy mash.

Measurement of hydrogen ion concentration.

The pH Test Papers (Toyo Roshi Co.), Brom Cresol Purple and Brom Cresol Green were calibrated with a Beckman pH meter and were used.

Titration of total acid. One millilitre of soy sauce was diluted to 50 ml with distilled water, and titrated with N/20 sodium hydroxide to pH 7.0 using a Beckman pH meter Model G. The value was expressed by milligram of lactic acid per ml of soy sauce¹⁴⁾.

Measurement of proteinase activity. The method described in the previous paper¹⁾ was followed.

Assay of free amino nitrogen^{14,15)}. Formol nitrogen was titrated potentiometrically according to the method of Sakasai and Yokotsuka.

Assay of ammonium. The soy sauce was diluted 50 fold, and Conway's method of microdiffusion analysis¹⁶⁾ was applied.

Assay of polypeptide¹⁷⁾. To 10 ml of soy sauce, 25 ml of dist. water, 5 ml of 10% sodium tungstate solution and 10 ml of 1N sulfuric acid were added successively and polypeptides in soy sauce were precipitated in a refrigerator over night. The precipitate was collected by centrifugation, washed with 10% tungstic acid, and the nitrogen content of the material was measured by Kjeldahl's method.

Assay of glutamic acid. The soy sauce was diluted 1000 fold, and glutamic acid content was bioassayed using *Lact. arabinosus* as test organism^{18,19)}.

Measurement of α -amylase activity. The unpasteurized soy sauce was diluted ten fold with M/15 phosphate buffer pH 6.0, and used as an enzyme solution. Two millilitre of the enzyme solution was poured into 10 ml of 0.4% soluble starch solution (pH 6.0) and incubated at 30°C. A half millilitre of the mixed solution was taken out at five minutes intervals, and poured into 2 ml of the 2N I₂ solution. The time when the blue colour of starch-I₂ reaction has disappeared, was recorded.

Assay of reducing sugars. Fehling-Lehmann-Schoorl's method was followed.

Colour intensity. The soy sauce was diluted 12 fold, and measured by an electrophotometer (Erma) with the transmission light through 420 m μ filter. The value was given by the optical density.

RESULTS AND DISCUSSION

As shown in the Table, 10 lots and 18 bottles of purely cultured soy sauces were prepared and brewed for two and half months at 30°C, and their enzymic activities, chemical compositions, solubilization ratios of materials, flavors and aromas were examined. Since all microorganisms added were purely cultured, the roles of each of them were clearly shown in this table. The tested organisms were; *Asp. sojae* strain KS^{4,5)}, three strains of strong protease producers *Bacillus subtilis* var. *Biotecus* A-538 (Nagase Industries Co.), *Bac. subtilis* strain Fn and H-127-H (Institute of Applied Microbiology, University of Tokyo), *Pediococcus soyae*⁶⁾ strain dl 2 and *Saccharomyces rouxii* No. 7^{9,10,11)}.

Asp. sojae is the so called soy koji mold, and it is the most important microorganism for soy sauce making. The organism produces proteases, amylases, other important enzymes for soy sauce brewing, and also aromas and flavors. *Bacillus subtilis* grows only in the koji making stage. *Pediococcus soyae* is the only active bacteria in soy mash, and produces lactic acid. *Saccharomyces rouxii* also grows well in soy mash and ferments only glucose but no other sugars.

The combinations of microorganisms were; *Asp. sojae* koji alone, *Asp. sojae* koji plus *Bac. subtilis* brans, mix-cultured koji of *Asp. sojae* and *Bac. subtilis*, *Asp. sojae* mash plus *Ped. soyae* culture, *Asp. sojae* mash plus *Sacch. rouxii* culture, *Asp. sojae* mash plus *Sacch. rouxii* and *Ped. soyae* culture, *Asp. sojae* koji plus *Bac. sub. var. Biotecus* bran plus *Sacch. rouxii* culture plus *Ped. soyae* culture, and open cultured factory soy koji.

The tasting of the brewed soy sauce were entrusted to four members of tasting committee in Noda Shoyu Co.. The hydrogen ion con-

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15) M. S. Dunn and A. Loshakoff, *J. Biol. Chem.*, **113**, 395, 691 (1936).

16) E. J. Conway, *Microdiffusion Analysis and Volumetric Error*, p. 75 (1950).

17) S. Hori et al., *J. Agr. Chem. Soc. Japan*, **30**, 515 (1952).

18) T. Tsunoda, Z. Ishizuka et al., *ibid.*, **26**, 478 (1952)

19) E. E. Snell, *Nutrition Abstr. and Rev.*, **16**, 497 (1946-47).

centration of the soy mash was measured on seventh, thirteenth day, and two and half months after the soaking of soy koji. The proteinase activity of the soy mash juice was measured at three pH values, 3.5, 7.0 and 10.0 on the 7th day after the soaking, and two and half months later, at pH 7.0.

On the samples brewed for two and half months, the other properties of the products were examined, because, the fermentation and maturing were usually concluded in three months when the soy sauce brewing was conducted in the warmed chamber at 30°C, and the product was almost identical with the normal soy sauce brewed for a year under uncontrolled temperature.

Roles played by *Asp. sojae*. The soy koji mold *Asp. sojae* was undoubtedly the most important organism for soy sauce brewing. By its biochemical actions during koji making stage, and by its enzymatic actions in soy mash, the mother body of the soy sauce was produced. As shown on the table, the percentage of solubilized proteins (mainly soy bean protein) into the soy sauce (The value is called as utilizing ratio of total nitrogen.) reached to the maximum without the aid of other microorganisms. The proteinase activity of fresh soy mash juice also did not fall below other mixed cultured soy mash. This provides an evidence for the previous report¹⁾ that the proteinases in soy mash are almost *Aspergillus* origin.

Because of the lack of lactic acid bacteria, the pH of the mash was not rapidly lowered, and the proteinase activity was not so decreased even after two and half months incubation at 30°C.

The liberation of glutamic acid, α -amylase activity and solubilization ratio of starch in raw material were also normal.

It is apparent, however, that *Asp. sojae* is able to make *only the mother body* of soy sauce, and it is not the soy sauce in itself. The absence of lactic acid bacteria caused the acid insufficiency, and the pH was not lowered

below pH 5.5 even after two and half months incubation at 30°C. If the mash is open cultured, it will be easily rotten by salt tolerant *Bacilli*^{7,8)}, which is suppressed by the low pH value in the normal soy mash⁶⁾.

Although it was not a bad smell, soy sauce which was made with *Asp. sojae* alone had a different aroma and flavor from the normal one. It has a specific drug like odour, which was removed by the growth of *Ped. sojae* and *Sacch. rouxii*.

The absence of yeast resulted in the deficiency of ethanol, normal content of which was from 2.0% to 3.0% in the case of unpasteurized soy sauce.

A distinct fault of the purely cultured soy sauce which was made with *Asp. sojae* alone was the appearance of a large amount of polypeptide-like white precipitate in soy sauce. The precipitate appeared in soy sauce when it was refrigerated, heated, or even diluted by water. It is a troublesome character for an article of commerce.

Roles played by *Bacillus subtilis*. *Bac. subtilis* and other aerobic spore forming bacteria grow only in soy koji making stage, and their spores are surviving in soy mash¹⁾. These bacteria were formerly considered as a harmful contaminant in soy koji, but this experiment proved that the appropriate addition of such *Bacilli* which does not produce unfavorable flavor, was useful and even necessary for soy sauce brewing. A specific drug-like odour of soy sauce brewed with *Asp. sojae* was diminished by the addition of bran culture of *Bac. subtilis*, further, the polypeptide-like turbidity of soy sauce also disappeared.

This improvement of soy sauce flavour is no wonder because there exists usually 10^6 – 10^8 cells of *Bacilli* per gram of the open cultured normal soy koji. But both mixed culturing of *Bac. subtilis* var. *Bioteucus* A-538 with soy koji and the addition of its bacterial bran, gave soy sauce the so-called *Bacillus* odour. Therefore it is necessary to select strains and to regulate the excessive growth of *Bacilli*.

ANALITICAL AND TASTING DATA OF

Related microorganisms	NaCl concentration %	pH after			Total acid as lactic acid mg/ml	Proteinase activity				Total nitrogen in soy sauce 10mg/ml, %	Utilizing ratio of total nitrogen %	Amino N
		7 days	13 days	2.5 months		after 7 days		2.5 Months				Total N %
						pH 3.5	pH 7.0	pH 10.0	pH 7.0			
<i>Asp. sojae</i> .	20.34	6.6	7.0	5.5	7.4	0.1	4.5	5.1	4.0	1.53	77.7	56.9
	20.27	6.4	6.6	5.3	7.9	0.3	4.1	5.2	3.5	1.50	76.3	56.0
	20.43	6.6	6.4	5.3	7.2	0.1	3.5	3.9	3.1	1.47	74.5	55.6
<i>Asp. sojae</i> , <i>Bac. sub.</i> <i>var. Biotecus</i> .	20.36	6.6	6.4	5.3	6.4	0.0	5.2	3.8	5.0	1.54	77.8	56.6
	20.50	6.4	6.4	5.5	5.0	0.1	3.9	4.7	4.3	1.47	73.7	58.6
<i>Asp. sojae</i> , <i>Bac. sub.</i> Fn	20.30	6.6	7.0	5.7	4.1	0.1	4.8	3.9	2.8	1.50	76.0	57.5
	20.36	6.8	6.8	5.3	5.4	0.1	4.8	3.1	3.2	1.51	76.6	55.6
<i>Asp. sojae</i> , <i>Bac. sub.</i> H-127-H	20.59	6.8	6.8	5.5	4.5	0.0	2.6	2.6	2.3	1.46	73.1	57.3
	20.41	6.6	6.8	5.7	3.9	0.0	3.1	2.9	3.4	1.45	73.3	56.2
<i>Asp. sojae</i> , <i>Bac. sub. var.</i> <i>Biotecus</i> . (Mixed culture)	20.54	6.9	7.2	5.3	6.3	0.2	4.8	4.0	3.3	1.50	75.6	57.6
	20.46	6.9	7.2	5.3	7.2	0.6	5.6	4.2	4.2	1.54	77.5	54.7
<i>Asp. sojae</i> , <i>Sacch. rouxii</i> No. 7.	20.62	6.8	6.8	5.5	4.7	0.0	3.3	3.7	3.0	1.41	70.3	58.2
	20.52	6.8	7.0	5.5	5.0	0.1	3.4	3.5	3.0	1.46	73.5	57.5
<i>Asp. sojae</i> , <i>Ped. soyae</i> dl 2	20.10	6.4	4.9	4.7	11.7	0.0	3.6	5.1	0.6	1.30	66.5	58.4
	20.35	6.6	4.9	4.5	11.2	0.1	3.0	3.8	0.5	1.18	59.8	61.0
<i>Asp. sojae</i> , <i>Ped. soyae</i> dl 2, <i>Sacch. rouxii</i> No. 7.	20.50	6.4	5.6	4.7	10.5	0.2	2.9	3.4	0.5	1.30	65.3	54.9
<i>Asp. sojae</i> , <i>Bac. sub. var. Bio-</i> <i>tectus</i> , <i>Ped. soyae</i> , <i>Sacch. rouxii</i>	20.33	6.6	4.9	4.7	10.9	0.0	6.1	4.6	1.3	1.40	71.0	58.7
Factory soy koji.	17.78	6.0	5.6	4.5	12.2	2.0	6.3	5.4	1.7	1.37	—	58.7

By addition of *Bacillus subtilis*, no increase of proteinase activity was observed, and the solubilization ratio of protein in raw material (represented by the utilizing ratio of total nitrogen) also did not increase. But the appearance of protein-like turbidity in soy sauce was effectively inhibited by addition of *Bacillus subtilis*. This phenomenon probably is due to the breaking of certain peptide bonds which is resistant to the attack of *Aspergillus* proteinases, so even the small quantity of different *Bacillus* proteinase plays an important role.

The α -amylase activity was increased by the addition of this bacteria. The utilizing ratio of starch was, however, not improved.

Roles played by *Pediococcus soyae*. In this experimental brewing, the salt concentration of purely cultured soy mash was too high by accident, 2 or 3% higher than the normal concentrations. Even in these high salt concentrations at 20.2–20.6%, *Ped. soyae* grew well and the mash was rapidly acidified to pH 4.9 within ten days after the inoculation. The hydrogen ion concentration of the other lots were pH 7.0–6.4 on the same day, and no acid production was observed by other organisms. Their final hydrogen ion concentration were pH 5.3–5.5, and have not reached to the normal pH value of 4.8–4.5 without the aid of *Ped. soyae*. The total acidity was also paralleled

PURELY CULTURED SOY SAUCES

NH ₃ -N Total N %	Turbidity in Soy Sauce	Polypep- tide N ⁽¹⁷⁾ Total N %	Glutamic acid-N Total N %	α -amylase activity (Starch-I ₂) min.	Reducing sugars %	Alcohol %	Utilizing ratio of starch %	Color (420 m μ) Optical Density	Tasting
19.0	+	13.4	8.50	35	3.41	0.28	27.2	0.98	Mother body of soy sauce flavor, but not the soy sauce flavor itself. Lacked in acid taste. Special drug-like odour as phenol or related substances.
16.4	+	10.5	8.33	38	3.01	0.12	22.3	0.92	
18.3	±	11.3	8.16	35	2.97	0.13	17.7	0.89	
23.5	—	11.3	8.44	25	2.58	0.10	18.3	1.03	Drug-like odour was diminished.
24.3	—	11.4	8.84	25	3.61	0.13	26.3	0.96	
19.7	—	12.4	8.60	33	3.14	0.17	23.9	0.88	Moderate flavor.
19.5	—	10.1	9.59	34	2.68	0.15	20.4	0.95	A necessary flavor for the soy sauce was developed. Lacked in acid taste.
19.0	—	11.0	8.84	34	2.99	0.21	23.1	0.93	
19.2	—	11.0	8.69	34	3.08	0.23	24.1	0.83	Bacillus odour.
19.5	—	11.6	8.48	<10	2.67	0.07	19.0	1.01	
18.3	±	11.5	8.18	<10	2.61	0.07	18.8	1.02	Too strong flavor of yeast fermentation, but this flavor might be necessary for the soy sauce.
19.4	±	11.4	8.58	35	1.97	0.61	21.5	0.82	
18.7	±	12.0	8.36	40	1.86	0.64	21.3	0.82	Odd smells were almost removed.
23.4	±	11.5	8.00	90	2.28	0.15	17.9	0.66	
25.3	±	11.6	8.14	110	2.44	0.07	17.7	0.70	The essential flavor for the soy sauce was developed. Smells almost identical with soy sauce.
22.2	±	13.3	7.69	120	1.78	0.28	15.9	0.66	
23.7	±	10.9	7.71	70	1.67	0.25	14.9	0.72	Bacillus odour prevailed.
11.2	—	5.2	9.56	45	5.18	0.02	—	0.73	Yeast fermentation was insufficient.

with the hydrogen ion concentration.

One of the most important characteristics of this lactic acid bacterium is the ability to form essential aromas and flavors for soy sauce. Its growth clearly eliminated the drug-like smell which was pronounced in the purely cultured soy sauce of *Asp. sojae*, and aromas became mild and well-matured. This activity of improving soy sauce aroma and flavor was so distinguished that the necessity of *Ped. sojae* for soy sauce brewing was indisputable despite some disadvantages of this organism described later.

It was mentioned that the growth of this microorganism lowered pH of the mash below

pH 5.0, and protect the soy mash from spoilage by salt tolerant *Bacilli*. Another important effect of low pH value is to stimulate the growth of salt tolerant yeasts. Onishi¹⁰ reported that, in 18% salted media, *Sacch. rouxii* could grow only in the range of pH 5.0–4.0. (The value was measured by the pH Test Pater (Toyo Roshi Co.). Brom Cresol Green. When it was measured by a Beckman pH meter Model G, about 0.6 higher pH values were obtained in the case of highly salted solutions.) Without the growth of *Ped. sojae*, the growth and fermentation of yeasts is imperfect.

As stated above, *Ped. sojae* is indispensable

for the soy sauce brewing, but its too early growth in soy mash decreases the yield of soluble nitrogen in soy sauce. This micro-organism has only the weak proteinase and peptidase in the cell, but had neither extra-cellular proteolytic enzymes nor the co-operating ability with *Aspergillus* proteinases⁶⁾. On the other hand, the rapid growth of the lactic acid bacteria acidifies soy mash, and the optimal hydrogen ion concentration of *Aspergillus* alkaline proteinase^{1,2,3,5,20)} (pH 6.5–11.0) was soon missed. The proteinase activity of soy mash at pH 5.0 is about one tenth of that at pH 7.0. Moreover, the *Aspergillus* alkaline proteinase is rapidly inactivated because of the low pH of mash. The table shows that about eight tenth of the proteinase activity at pH 7.0 remained after two and half months brewing at 30°C in *Ped. soyae* omitted lots, while the lots with *Ped. soyae* kept only about two tenth of the activity. Accordingly, about 10% decrease of the solubilization ratio of total nitrogen in raw material was recognized on the lots.

The inactivation of *Asp. sojae* proteinases also caused the marked increase of the polypeptide-like precipitation in soy sauce, which was probably due to the imperfect digestion of protein.

The other actions played by *Ped. soyae* were; the increase of liberated ammonium, the inactivation of α -amylase of *Asp. sojae*, the decrease of the utilizing ratio of starch in raw material.

The colour intensity of soy sauce was markedly decreased by this organism, reaching to the light colour of the unpasteurized normal soy sauce.

In general, *Ped. soyae* is quite indispensable for forming the soy sauce aroma and flavour, and for conducting the sound brewing process in the open culture, but its acid production caused the inactivation of various enzymes of *Asp. sojae*. In order to overcome these unfavorable points, it is desirable to suppress the growth of *Ped. soyae* in the early stage of brewing, and after the enzymes of *Asp. sojae*

have played larger parts of their roles, namely two or three months later, *Ped. soyae* should grow vigorously to acidify the soy mash and to develop the good flavor to soy sauce. From this view, it is reasonable that when soy koji is prepared and soaked in the brine during winter time, the resulted soy mash is superior both in its solubilization ratio of materials and in its quality.

Roles played by *Saccharomyces rouxii*. The salt tolerant yeast *Saccharomyces rouxii* strain No. 7^{9,10,11)}, the former name of which was *Zygosaccharomyces major*, grows well in soy mash if it is sufficiently aerated. Although the yeast did not grow satisfactorily because of the insufficient aeration in this experiment, the effects of yeast growth is apparently distinguishable on the table.

The inoculation of the yeast resulted in the consumption of reducing sugars and the production of ethanol. A specific fermentation odour of yeast was not a bad smell in itself, but in this experiment, it was too intense and unfamiliar for soy sauce. The mixed inoculation of both *Ped. soyae* and *Sacch. rouxii*, however, have formed the most satisfactory aroma and flavour almost approximating to those of the normal soy sauce. That is, the yeast fermentation alone can not form the good flavor in soy mash, but the cooperation with *Ped. soyae* is quite necessary.

No other eminent activities of the yeast were observed in this experiment. Acids were not formed, and no influences on *Asp. sojae* enzymes were observed. The decolorizing activity for soy mash exists, but not so strong as that of *Ped. soyae*.

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²⁰⁾ N. Iguchi and K. Yamamoto, *J. Agr. Chem. Soc. Japan*, **29**, 387 (1955).