Preservation of Raw Fish and Meat[†]

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The sugar-yeast (SY) addition method, which was developed for the production of fermented protein foods, was applied to the preservation of raw fish and meat and compared with the NaCl and ethanol addition methods. The preservation mixture, consisting of minced raw meat of fish or livestock, 10% glucose, and 10^6 yeast cells/g, was incubated at 12°C. Glucose was consumed quickly and about 2–4% ethanol was produced after 3–5 days of preservation. About 10^5 cells/g of contaminating bacteria present in the preservation mixture increased to about 10^6 cells/g after 3 day of preservation, and then the cell number of bacteria showed a tendency to decrease. The preservation mixture did not putrefy. The amount of eicosapentaenoic acid contained in sardines was preserved effectively by the SY addition method and by the ethanol addition method, but not well by the NaCl addition method. The freshness (*K* value) of sardines preserved by the SY addition method was maintained at the same level as that using the NaCl method. However, deterioration of freshness was observed in the ethanol addition method. The water holding capacity (WHC) of the NaCl addition method decreased, but WHC was well maintained by the SY and ethanol addition methods.

These days, low salt foods, especially fermented foods, are favored for health reasons such as the prevention of hypertension. Therefore, several kinds of production methods, such as the ethanol addition method^{1–6)} and yeast mass addition method,⁷⁾ were developed to produce low-salt fermented foods.

In a previous paper,⁸⁾ we reported a new method, the sugar-yeast (SY) addition method, which did not use a high concentration of NaCl for the production of fermented protein foods. This method used the bacteriostatic effect of ethanol produced from sugar sources by alcoholic fermentation of added yeast (*Saccharomyces cerevisiae*), and also amylolysis of koji mold.

The fermentation mixture, consisting of ground soybean koji of *Aspergillus*, a sugar source (glucose was mainly used), and yeast cells, was incubated at 22°C. Under these conditions, yeast grew well, ethanol was quickly produced, and glutamic acid and amino

acids were also quickly liberated. The number of contaminating bacteria increased to 10^6 cells/g at the beginning of fermentation, then gradually decreased. The fermentation mixture did not putrefy.

From this result, we thought that the SY addition method could be used as a preservation and processing method for raw foods in place of the NaCl addition method (salting). Therefore, we investigated the application of the SY addition method in preserving raw marine products and livestock meat. This paper deals with variations of the component content, such as eicosapentaenoic acid, and also biochemical properties such as freshness and the water holding capacity of raw meat during preservation.

Materials and Methods

Microorganism and materials. *Saccharomyces cerevisiae* (sake yeast, Kyokai No. 7) was used to preserve raw meat,

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and was cultivated as previously described.⁸⁾

Sardine, pork, and beef meats minced with a blender were mainly used as starting materials.

Preservation procedure. To measure microbial growth, the amount of eicosapentaenoic acid (EPA), and the freshness of the meat, raw sardine meat (100 g) without head and internal organs, or raw pork meat (100 g), was minced with a blender, and 10% glucose (powder, 10 g) and a suspension of yeast cells (10^8 cells/ml water, 1 ml) were added and mixed well. The final concentration of yeast cells was adjusted to 10^6 cells per g of raw material. The preservation was allowed to proceed in a container with a lid at 12°C.

To measure the water holding capacity (WHC), a block (500 mg) of white sardine flesh or of pork meat was soaked for 3 days in a 5 ml solution containing glucose and yeast cells. In the NaCl addition method, NaCl was added at a concentration of 10% (w/w) in place of the yeast cells and 10% glucose, and in the ethanol addition method, 10% ethanol was used.

Analytical method. Viable cells, glucose, ethanol, and amino acids were measured as previously described.⁸⁾

Measurement of EPA: The fermentation mixture (5 g) was suspended in 5 ml of water. Then 25 ml of chloroform was added and mixed well. As an internal standard, nonadecanoic acid (5 mg) was usually added to the chloroform mixture. After extraction, a 0.2 ml chloroform layer was taken and mixed with 0.2 ml phenyl trimethyl ammonium hydroxide as a methylation reagent, and 2 μ l of the mixture was gas chromatographed. The analytical conditions were as follows: apparatus-Shimadzu GC-7A with a flame ionization detector; column-glass column (3 mm $\times$ 3 m) packed with 5% Shinchrom E-71 on 80/100 mesh Shimalite (AW) (Shimadzu); column temperature, 200°C; injection port temperature, 270°C; N₂ (carrier gas) flow rate, 50 ml/min; and integrator-Shimadzu C-R1A.

Measurement of freshness: The freshness of the sardines was measured by the *K* value.⁹⁾ A sample for determination of *K* value was prepared as follows. Five grams of preserved meat was ground with 25 ml of 5% cold perchloric acid solution by hand in a mortar. The extract was centrifuged, and the supernatant was neutralized to pH 6.5 with 1 M KHCO₃, then centrifuged again. The supernatant was used for analysis. The amounts of hypoxanthine, inosine, and nucleotides (adenine- and hypoxanthine-nucleotides) were measured by high performance liquid chromatography (Hitach Co., Ltd.) on a Shim-pack CLC-ODS column (6 $\times$ 150 mm). Elution was done with 24 mM 2-dimethylamino ethanol and 16 mM citric acid solution at 40°C. The elution was monitored at 250 nm.

Measurement of water holding capacity (WHC): The amount of water of 500 mg white flesh (*M*) was measured by dryness at 105°C. White flesh (500 mg) soaked in a solution of each additive was held between 10 sheets of filter paper (Toyo filter paper No. 4) previously weighted

(*A*₁) and pressed with a presser (Riken Co., Ltd.) at 10 kg/cm² for 1 min. Residual white flesh was then rapidly peeled off and the filter paper, including water, was weighted (*A*₂). WHC was calculated by the following formula:

$$\text{WHC (\%)} = \left(1 - \frac{A_2 - A_1}{M} \right) \times 100.$$

Results and Discussion

Course of the SY addition method

Using sardine, pork, and beef meat, the course of alcoholic fermentation and the cell numbers of yeast and contaminating bacteria in the SY addition method were compared to those in the control experiment without additions (Table I).

In the control experiment using each meat, the cell number of contaminating bacteria increased to about 10^9 cells/g after 5 days of preservation, and a putrefactive odor, methyl mercaptan, and H₂S were detected.

By contrast, in the SY addition method using each meat, the glucose was consumed quickly and 3–4% ethanol was formed after 5 days of preservation. The cell numbers of yeast and bacteria increased to about 10^8 and 10^6 cells/g, respectively; but putrefactive odor was scarcely detected.

Effects of additives on the preservation of EPA

Meat of sardines contains a large amount of EPA and higher unsaturated fatty acids.^{11–13)} EPA is known as a precursor of prostaglandins and has a preventive function related to thrombosis¹⁴⁾ and other disorders.^{15–20)} Therefore, the preservation of EPA was investigated under conditions containing each additive (Fig. 1).

EPA was quickly decomposed in the control experiment (Fig. 1(A)). In the NaCl addition method (Fig. 1(C)), the viable cell numbers of contaminating bacteria decreased gradually, and putrefaction was not observed, but EPA was quickly decomposed in the same way as in the control experiment. On the other hand, the ethanol addition method (Fig. 1(B)) did not cause bacterial growth or putrefactive

Table 1. GROWTH OF MICROORGANISMS AND FORMATION OF ETHANOL IN A MIXTURE OF THE CONTROL EXPERIMENT AND SY ADDITION METHOD

Each control experiment contained only minced meat (100g). The SY addition mixture consisted of minced meat (100g), 10% glucose and 10^6 yeast cells/g of preservation mixture. Preservation was carried out at 12°C for 5 days. Other conditions were the same as those given under Materials and Methods.

Material and preservation method	Incubation time (days)	Cell number (cells/g)		Glucose (%)	Ethanol (%)
		Bacteria	Yeast		
Sardine					
Control experiment	0	6.8×10^4	0	0.3	0
	1	5.2×10^6	0	0.2	0
	3	1.1×10^8	0	0.2	0.1
	5	8.8×10^8	0	0.2	0
SY addition method	0	5.3×10^4	2.3×10^6	10.7	0
	1	6.8×10^5	1.7×10^7	7.6	0.7
	3	1.3×10^6	9.8×10^7	3.4	2.6
	5	7.4×10^5	9.6×10^7	0.5	3.8
Pork meat					
Control experiment	0	9.3×10^4	0	0.3	0
	1	7.5×10^6	0	0.3	0.1
	3	2.1×10^8	0	0.2	0.1
	5	8.4×10^8	0	0.2	0
SY addition method	0	8.4×10^4	2.3×10^6	10.7	0
	1	9.2×10^5	2.4×10^7	7.3	0.6
	3	2.5×10^6	7.4×10^7	3.8	2.4
	5	8.4×10^5	9.6×10^7	0.8	3.5
Beef meat					
Control experiment	0	4.8×10^4	0	0.3	0
	1	4.4×10^6	0	0.2	0
	3	1.5×10^8	0	0.2	0.1
	5	7.6×10^8	0	0.3	0.1
SY addition method	0	5.3×10^4	2.3×10^6	10.7	0
	1	6.3×10^5	2.6×10^7	7.4	0.6
	3	1.6×10^6	6.9×10^7	3.5	2.6
	5	7.9×10^5	9.6×10^7	0.7	3.6

preservation, and it repressed the decomposition of EPA.

Moreover, in the SY addition method (Fig. 1(D)), alcoholic fermentation proceeded, bacterial growth was repressed as described above, and the decomposition of EPA was repressed in the same way as the ethanol addition method.

Effects of components of the SY addition method on the preservation of EPA

The effects of the glucose and yeast components in the SY addition method on the preservation of EPA were investigated (Fig. 2).

Omission of either glucose or yeast caused a

decrease in EPA content and an increase in the number of bacterial cells; and preservation mixtures putrefied. Therefore, both glucose and yeast were required to preserve EPA by the SY addition method.

What is generally known as phytochemical reduction during alcoholic fermentation by yeast keeps the inside of fermentation mixture in a remarkably reduced state.²¹⁻²⁵⁾ Therefore, it was thought that EPA was not decomposed in the SY addition method.

Moreover, a substrate, glucose, and the product, ethanol, in the SY addition method also function as reductants. Therefore, it is thought that the amount of decomposed EPA in this method is less than that in the NaCl

addition method (Fig. 1(C)). So, this method might effectively function not only to prevent the oxidative degradation of higher unsaturated fatty acids, but also of lipids and vitamins in meat.

Effects of component concentration in the SY addition method on the preservation of EPA

Figure 3 shows the effects of the initial

concentration of yeast cells on the preservation of EPA. The yeast cell concentration influenced both the formation of ethanol and the preservation of EPA. The initial rates of glucose consumption and ethanol formation increased with an increase in the yeast cell concentration.

In mixtures with 10^5 cells/g or less, EPA was quickly decomposed, and the number of bacterial cells increased with a decrease in the initial yeast cell concentrations. About 10^8

Fig. 1. Effects of Additives on the Preservation of EPA.

The control experiment (A) contained only 100 g of sardine meat. Experiment (B), the ethanol addition method, contained 100 g sardine meat and 10 g ethanol. Experiment (C), the NaCl addition method, contained 100 g sardine meat and 10 g NaCl. Experiment (D), the SY addition method, contained 100 g sardine meat, 10 g glucose, and 10^6 yeast cells/g. Preservation was carried out at 12°C. Symbols: ○, EPA; △, glucose; ▲, ethanol; ■, yeast; □, bacteria.

Fig. 2. Effects of Components of the Preservation Mixture on the Preservation of EPA.

The complete system contained 100 g sardine meat, 10 g glucose, and 10^6 yeast cells/g. Preservation was done at 12°C.

Symbols: ○, EPA; △, glucose; ▲, ethanol; ■, yeast; □, bacteria.

Fig. 3. Effects of Initial Yeast Cell Concentration on Preservation of EPA.

Each preservation mixture contained 100 g sardine meat, 10 g glucose, and yeast cells as shown in Figure. Preservation was done at 12°C.

Symbols: ○, EPA; △, glucose; ▲, ethanol; ■, yeast; □, bacteria.

cells/g of bacteria were found in the mixture containing 10^5 cells/g yeast cells. This might be due to insufficient alcoholic fermentation to produce ethanol, which works as a reductant and as a bacteriostatic reagent. On the other hand, in a mixture with 10^6 cells/g yeast and more, alcoholic fermentation quickly proceeded, so that both the decomposition of EPA and the growth of bacteria were repressed.

Furthermore, the effects of glucose concentration were investigated (data not shown). With 10^6 cells/g yeast and less than 10% glucose, alcoholic fermentation proceeded quickly; however, decomposition of EPA and bacterial growth occurred, because alcoholic fermentation did not continue and ethanol concentration in the mixture was insufficient. This could have been due to a lack of glucose as the substrate. Using 10–15% glucose, alcoholic fermentation proceeded quickly so that both EPA decomposition and bacterial growth were repressed. Yet, with the addition of 20% glucose or more, alcoholic fermentation was retarded at the beginning of preservation, where the decomposition of some EPA and a slight growth of yeast and bacteria was observed. However, since an increase in the initial concentration of yeast cells brought about quick alcoholic fermentation, the decomposition of EPA and the growth of contaminated bacteria were repressed greatly.

The effects of preservation temperature on the SY addition method was also investigated (data not shown). Preservation at above 20°C caused bacterial growth (about 10^8 cells/ml) at the beginning of preservation, and a decrease of EPA content was observed. Below 12°C, neither decomposition of EPA nor bacterial growth was significant.

Consequently, for the satisfactory preservation of EPA, the addition of more than 10^6 cells/g yeast and 10–15% glucose was required, and the optimum temperature for incubation was less than 12°C. However, a shift of incubation temperature from 12°C to 22°C after accumulation of 3–4% alcohol did not stimulate further decomposition of EPA or growth of contaminating bacteria.

Removal of ethanol from the preserved-processed product

The conditions for removing ethanol from fermented meat mixtures was investigated, because removal of ethanol was needed in order to use the preserved-processed meat not only for manufacturing foods, but also as intermediate materials for food processing and for shipping.

As shown in Fig. 4, ethanol in the product

Fig. 4. Removal of Ethanol with Distillation.

Preservation mixture (100 g) containing about 4% ethanol was distilled under the conditions of reduced pressure (about 15 mmHg) and heating at 40 (○), 50 (◐), and 60°C (●).

Fig. 5. Effects of Additives on Freshness.

The control experiment contained only 100 g sardine meat. The ethanol addition method contained 100 g sardine meat and 10 g ethanol. The NaCl addition method contained 100 g sardine meat and 10 g NaCl. The SY addition method contained 100 g sardine meat, 10 g glucose, and 10^6 yeast cells/g. Preservation was done at 12°C.

Symbols: ○, control experiment; △, ethanol addition method; □, NaCl addition method; ●, SY addition method.

was almost completely removed by heating at 40–60°C for 30–40 min under reduced pressure. The product from which ethanol was removed can easily be shipped and used for food processing.

Preservation of the freshness of meat

Regarding the preservation of fish, the freshness is extremely important. Figure 5 shows the effects of the preservation method on the freshness of sardines. In both the control experiment and the ethanol addition method, freshness deteriorated greatly. However, in the SY and NaCl addition methods, freshness was lost only gradually, and the deterioration of freshness in the SY addition method was slightly less than that in the NaCl addition method.

Effects of additive concentration on freshness

Table II shows the effects of additive concentration on freshness with or without yeast. Without yeast, the increase of concentration of

each additive (glucose, NaCl, and ethanol) was only slightly effective for the preservation of freshness; the addition of NaCl being most effective. By adding 10^6 cells/g yeast cells to each preservation mixture described above, preservation of freshness was observed only in the glucose-added mixture, *i.e.*, in the SY addition method.

In the SY addition method, the effects of the initial concentration of yeast cells on freshness were investigated with 10% glucose (Table III). An increase in yeast cell concentration preserved freshness. But the addition of only

Table III. Effects of YEAST CELL CONCENTRATION ON FRESHNESS

The amount of added glucose was 10% of the preservation mixture. Preservation was done at 12°C for 2 days. Other conditions were the same as those given under Materials and Methods.

Yeast concentration (cells/g)	K value (%)
10^3	81
10^4	76
10^5	67
10^6	52
10^7	49
10^8	48

Table II. EFFECTS OF CONCENTRATION OF ADDITIVES AND YEAST CELLS ON FRESHNESS

The concentration of added yeast cells was 10^6 cells/g of preservation mixture. The concentration of each additive (glucose, NaCl, and ethanol) was weight percent per weight of sardine meat. Preservation was carried out at 12°C for 2 days. Other conditions were the same as those given under Materials and Methods.

Additive	Concentration (%)	K value (%)	
		Without yeast	With yeast
Control experiment		100	100
Glucose	2.5	89	64
	5	86	60
	10	84	52
	15	83	46
	20	84	44
NaCl	2.5	76	77
	5	69	68
	10	59	60
	15	59	58
	20	58	56
Ethanol	2.5	98	99
	5	96	95
	10	86	87
	15	88	88
	20	88	86

Fig. 6. Effects of Temperature and Addition of Yeast on Freshness.

The experiment systems without yeast cells (○) and with yeast cells (10^6 cells/g) (●) contained 100 g sardine meat and 10 g glucose. The temperature of preservation was changed as described in the Figure. Preservation was done at 12°C.

yeast did not affect the preservation of freshness.

Therefore, these findings indicate that alcoholic fermentation contributed to the preservation of freshness, although the reason was not evident. The decomposition of IMP to inosine might be inhibited by alcoholic fermentation, because a small amount of AMP, ADP, and ATP found in sardines disappeared, but the hypoxanthine, inosine, or IMP content scarcely changed during the preservation period.

The effects of temperature on the preservation of freshness by the SY addition method were investigated as well (Fig. 6). Low temperatures repressed the deterioration of freshness while at high temperatures (above 22°C), freshness deteriorated. Therefore, alcoholic fermentation was effective in preserving freshness at low temperature, although at high temperatures alcoholic fermentation proceeded actively, and freshness deteriorated. Accordingly, it was thought that at high temperatures, other factors, such as stimulation of nucleotidase activity, contributed to the deterioration of freshness.

Effects of preservation method on WHC

The texture of the foods was strongly

Fig. 7. Effects of Additives on Water Holding Capacity.

A 500-mg block of white sardine fish was soaked in 5 ml of solution containing each of the additives at 12°C for 3 days. Each additive (glucose, ethanol, or NaCl) concentration in the glucose (only glucose) addition method (○), ethanol addition method (△), NaCl addition method (□), and the SY addition method (●) was changed as shown in the Figure. In the SY addition method, the concentration of yeast was 10^6 cells/g and the glucose concentration was changed.

influenced by components within the foods. Water, protein, lipid content, and so on provide good examples of this. Water content was greatly influenced by dehydration which could occur with the addition of additives. Therefore, WHC, a factor influencing food texture, was investigated (Fig. 7).

The addition of more than 5% NaCl caused expansion of meat, and a decrease in WHC, while the addition of only glucose without yeast caused a slight increase in WHC. Little putrefaction was observed when only glucose was added. On the other hand, in the SY addition method, WHC scarcely varied, similar to the findings of the ethanol addition method. This method is effective for preserving WHC.

WHC was also influenced by yeast cell concentration and preservation temperature (Table IV). A decrease in initial yeast cell concentration and an increase in preservation temperature increased the WHC. The same result was obtained using livestock meat.

These results indicate that variations of EPA content and physical properties scarcely occurred in the SY addition method. Thus, this

Table IV. EFFECTS OF YEAST CELL CONCENTRATION AND TEMPERATURE ON WATER HOLDING CAPACITY

Exp. 1: The amount of added yeast cells was varied as indicated in the Table. Preservation was done at 12°C for 3 days. Exp. 2: The preservation temperature was changed as indicated in the table. The amount of added yeast cells was 10^6 cells/g. Preservation was done for 3 days. Other conditions were the same as those given under Materials and Methods.

Condition	WHC (%)
Exp. 1	
10^3 yeast cells/g	52
10^4	51
10^5	47
10^6	42
10^7	41
10^8	38
Exp. 2	
5°C	38
12	42
22	49
28	57
37	69

method was superior to the commonly used NaCl addition (salting) method for preserving raw foods.

Since it has already been reported that the cultivation of several kinds of yeast in fish extracts caused a decrease of fish odor,²⁵⁾ the flavor of products preserved by the SY addition method might be improved.

Recently, using ethanol, the production of "marine beef",²⁶⁾ a processing method for fish meat containing protein as a main component, was developed. Marine beef is now experimentally being used as a material to be cooked by itself or by mixing it with other meats or foods. Products obtained by the SY addition method might also be used in the same way as marine beef, because the meat is processed without NaCl, and the product is easily seasoned.

Moreover, pathogenic germs, food poisoning germs and parasitic worms might be sterilized by ethanol, which is produced by the alcoholic fermentation of yeast.

In this paper, we discuss the preservation of raw fish and meat by the SY addition method from the standpoint of application of the method, though in these days, the freezing method seems to be best for preservation of these raw foods. We envision materials preserved by this method being used as materials for new types of foods. Now, we are currently investigating variations of content of other components (lipid peroxide, vitamins, and amines), physical properties (deodorization and coloration), the removal method of ethanol from the product, and use of the product. Moreover, we are also investigating the subjects indicated above using whole fish and blocks of meat.

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