Chemical and Microbiological Characteristics of Sardine Meal Fermented with *Aspergillus oryzae* IFO 4202

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Sardine moist meals were inoculated with *Aspergillus oryzae* IFO 4202 seed and fermented. Accompanying the growth of the fungus, the chemical composition of the meals significantly changed during incubation. The fermentation contributed to antioxidation of lipids and reduction of histamine. Glucose supplement during fermentation was effective in inhibiting the production of volatile basic nitrogenous compounds. Fermented meals maintained superior quality of the proteins, protecting amino acid residues during incubation. The initial bacterial cell counts in meals were 3×10^2 CFU/g and reached 10^8 CFU/g in 72-h incubation. The Gram-negative rod-shaped bacteria, *Pseudomonas* spp., *Moraxella* spp. and *Acinetobacter* spp., were predominant in the initial meals. Gram-positive bacteria, especially *Micrococcus* spp., gradually became predominant during incubation. Fermentation and glucose supplement enhanced this predominancy of *Micrococcus* spp. during incubation. Fermentation with *Aspergillus oryzae* in the presence of glucose was considered to be effective for improvement of the chemical, nutritional and microbiological quality of fish meals.

Keywords: sardine, fish meal, *Aspergillus oryzae*, fermentation, quality improvement, bacterial flora

Sardines are the most abundantly caught fatty fish in the world, and many have been utilized as fish meals or feedstuffs. There are some problems to solve in sardine meal processing. Because sardines contain a great amount of polyunsaturated fatty acids in the lipids, the sardine lipids are very oxidizable during processing and storage of fish meal, which sometimes leads to the deterioration of the nutritional value and flavor. It has also been reported that gizzerosine produced from histamine by drying at a high temperature during fish meal processing might induce chicken gizzard disease (Okazaki *et al.*, 1983; Toyama *et al.*, 1985).

Fermentation of fish meal with microorganisms was attempted because of concern with quality improvement as a feedstuff (Kato *et al.*, 1985, 1986; Hossain *et al.*, 1987a) and production of fish sauce (Hayakawa, 1994). Hossain *et al.* (1987a, b) and Rashid *et al.* (1992) have reported that the quality deterioration of fish meal induced by lipid oxidation could be reduced by the utilization of koji molds, *Aspergillus* species. Furthermore, Kunimoto *et al.* (1989, 1991) and Hoshino *et al.* (1991) studied the lipase in fish meal fermented with *Aspergillus oryzae* IFO 4202. Decomposition of trimethylamine and trimethylamine *N*-oxide by the *A. oryzae* during the fermentation have also been observed (Kunimoto *et al.*, 1992). Therefore, fermentation with fungi has been expected to contribute to improve fish meal quality.

A. oryzae is the most popular fungal species as koji mold

for various brewages in Japan. Koji fermentation is generally operated in an open atmosphere; therefore, koji is exposed to danger of contamination with various microorganisms other than koji molds. The contamination might lead to deterioration and putrefaction of the meals. Therefore, microbial regulation during the fermentation of meals is very important for safe fermentation and quality improvement of the meals.

Microflora of koji have been investigated in the koji of rice and soybean substrates. Predominant bacterial flora in the koji were reported to consist of *Micrococcus* spp. and lactic acid bacteria (Kubota *et al.*, 1976; Takema *et al.*, 1978). As for the bacterial flora of the koji based on fish meals, Kunimoto *et al.* (1995) observed that *Micrococcus* spp. had become dominant in the final stage of the fermentation.

In this paper, *A. oryzae* was inoculated on moist sardine meal; the changes in the chemical and microbiological characteristics of the meals during fermentation were investigated, and the general quality of the fermented sardine meals was evaluated.

Materials and Methods

Fungus *A. oryzae* IFO 4202 was obtained from the Institute for Fermentation, Osaka, Japan. The fungus was maintained on a malt-extract agar slant at 25°C for 7 days. A loopful of the spores was inoculated on sterilized wheat bran (moisture content, 50%) and incubated at 30°C to make mold seed.

Fish and preparation of meals Sardine *Sardinops melanostictus* was caught by a set-net in the coastal sea off Kamiiso-cho, Hokkaido, Japan. As soon as the fish were

caught, they were carried to our laboratory in an ice box. The fish were well washed to remove scales under running water and heated at 115°C for 10 min with an autoclave. The heated fish were then pressed to make the moisture content 50% after acetic acid was added at a concentration of 0.2% in the fish to pH 6.0. The pressed fish cakes were ground through a 3-mm dish of a chopper.

Fermentation of the meals Ten percent soluble starch, 1% K_2HPO_4 and 8% mold seed were added to the meals. The mixture was reground with the chopper to granular form. The fermentation of the granulated meal was started on a tray at RH (relative humidity) 92% and 30°C in a humidified chamber (IG-42M, Yamato, Tokyo) and then periodically stirred during incubation. When the temperature of the meal rose during the fermentation, at 20 h after the start, the atmospheric temperature was gradually lowered to keep the meal temperature at 30°C. After 30 h, 10% glucose was supplemented in the meal and the incubation was continued further. As a control, meal was prepared without the mold seed.

Chemical analyses of the meals Moisture content was determined by drying at 105°C. Water activity was measured by the method using Conway's units (Akiba, 1982). The pH was measured with a pH meter (model C-1, Horiba, Kyoto) for a meal homogenate with twice its volume of water.

Starch was collected by ethanol precipitation from meal solubilized by heating with 30% KOH and spectro-

photometrically determined by the anthrone-sulfuric acid method (Koehler, 1952). Also, the meal was homogenized with 80% ethanol 3 times, then defatted with chloroform, and the free sugar content was determined in the ethanol extract by the phenol-sulfuric acid method (Dubois *et al.*, 1956). D-Glucose (Wako Pure Chemical, Osaka) was used as a standard for both starch and free sugar determination. Glucosamine was determined by the method of Sakurai *et al.* (1977) using D-glucosamine hydrochloride (Sigma Chemical, St. Louis, MO) as a standard.

Free amino acids were determined by ninhydrin colorimetry in the 80% ethanol extracts and the content was expressed as leucine. Volatile basic nitrogen (VB-N) was measured by micro diffusion analysis (Ishizaka, 1969) using a Conway's unit for extracts with 5% trichloroacetic acid. Histamine was determined by the cotton acid succinate method (AOAC International, 1995a) using histamine dihydrochloride (Wako Pure Chemical) as a standard.

Lipid was extracted by the method of Folch *et al.* (1957). The peroxide value (POV) of the extracted lipid was determined according to the AOCS-AOAC method (AOAC International, 1995b). Thiobarbituric acid reactive substances (TBARS) in the meal were determined by the method of Uchiyama & Mihara (1978). Lipid composition was determined by thin layer chromatography. Lipid was spotted on a silica gel 60 plate (0.25 mm thick, Merck, Darmstadt, Germany) and developed with hexane-diethylether-acetic
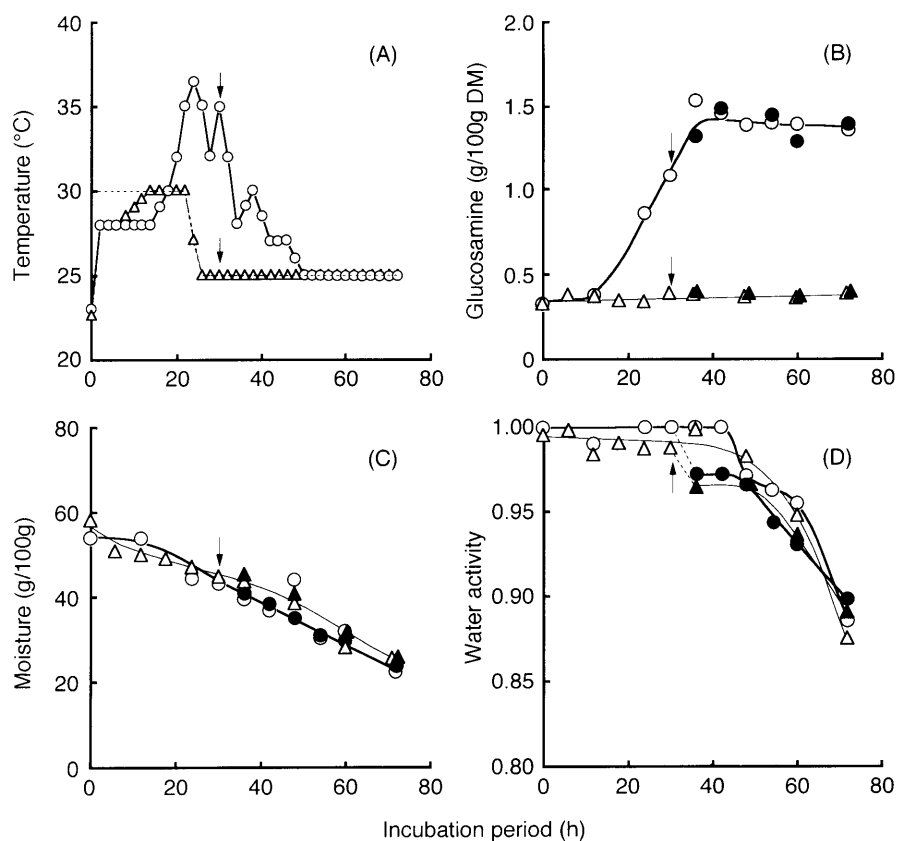

Fig. 1. Changes in the temperature (A), glucosamine (B) and moisture (C) contents, and water activity (D) of meals during fermentation with *Aspergillus oryzae* IFO 4202. Symbols: ○, fermented meal; ●, fermented meal supplemented with glucose; △, meal not inoculated with the fungus (control); ▲, control meal supplemented with glucose. Arrows indicate a supplementation point during the incubation. Dotted line in the (A) figure shows the incubation temperature. DM means dried matter.

acid (90 : 10 : 1). After the development, the plate was sprayed with 3% copper acetate-8% phosphoric acid and heated at 150°C for 20 min to visualize the lipids. Lipid composition was determined using a densitometer (CS-9000, Shimadzu, Kyoto) based on the reflection at 540 nm.

For nutritional evaluation, amino acid composition of meal protein was determined. The extraction residue of meals with 80% ethanol was defatted with diethylether and hydrolyzed with 4 N methane sulfonic acid at 115°C for 24 h. The hydrolysate was subjected to an amino acid analyzer (model 835, Hitachi, Tokyo). The essential amino acid index (EAAI), amino acid score (AA score) and protein efficiency ratio (C-PER) were calculated from the amino acid composition (Hatano et al., 1985; Resources Council, Sci. Technol. Agcy., 1986). Protein digestibility of fermented fish meal was determined by the method of Satterlee et al. (1979).

Microbial analyses of the meals Meals were periodi-

cally stirred, picked up, and homogenized with 9-fold volumes of sterile physiological saline. Viable cell count of bacteria was determined on standard plate count agar incubated at 30°C for 48 h after a serial dilution. Fifty strains of the colonized bacteria were randomly picked up and purified by repeated restreaking. Bacterial strains separated from the meals were identified to the genus level according to Kunimoto et al. (1995).

Results

Chemical characteristics of fermented meals The fermentation of sardine meal was started on a tray at RH 92% and 30°C. At 20 h after the start, the hyphae of the fungus had grown on the surface of the meals and the meal temperature had begun to rise. At 24 h after the start, the meals became sponge-like masses and the temperature reached 37°C. In order to inhibit putrefactive bacteria, the incubation tempera-
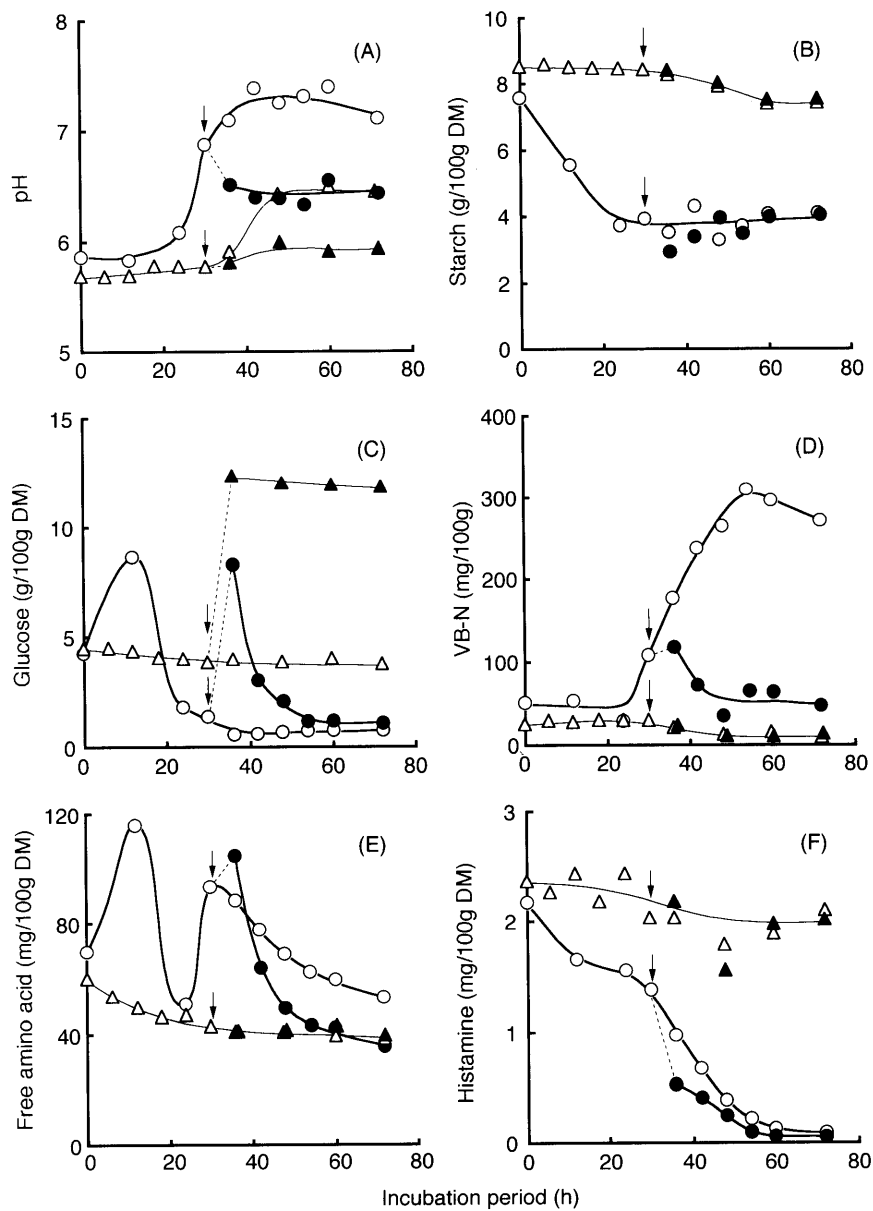

Fig. 2. Changes in pH (A), starch (B), glucose (C), volatile basic nitrogen (VB-N) (D), free amino acid (E) and histamine (F) contents of meals during fermentation with *Aspergillus oryzae* IFO 4202. Symbols are the same as in Fig. 1.

ture was lowered, and the massed meals were occasionally hand-ground with sterile gloves to keep the meal temperature below 30°C. In 40 h of incubation, no exothermic reaction was observed. For the control meal, no spontaneous temperature change was observed throughout the incubation (Fig. 1-A).

Glucosamine increased significantly 12 to 36 h into the fermentation. Because glucosamine constitutes the cell walls of the mold, the increasing content of glucosamine indicates the growth of the mold. The control meals did not produce glucosamine during the incubation (Fig. 1-B).

For both fermented and unfermented meals, the moisture contents were 50% at the start of incubation and were gradually decreased to become ca. 25% in 72 h of the incubation (Fig. 1-C). Water activity of both meals was 0.99 initially and was decreased following glucose addition and drying to 0.89 in 72 h (Fig. 1-D).

The pH value of the fermented meal was 5.9 initially, and then it began to increase after 24-h incubation, reaching 7.4 in 42 h of the incubation, and a strong ammonia-like odor was observed at this point. Supplement of glucose at the 30-h point reduced the pH value to 6.3, and a sweet koji-like flavor was observed in the meals. Also, the pH of the control meals gradually rose, and not an ammoniacal odor but a typical fishy odor of fish meal was observed (Fig. 2-A).

The starch content of meals decreased to less than 50% of the initial content in 24 h of the fermentation with fungus (Fig. 2-B). On the other hand, the glucose content of the fermented meals was changed drastically. In the first 12 h,

glucose had increased, and by 24 h it had significantly decreased; subsequently, it was gradually decreased. Supplemented glucose was also consumed rapidly in the fermented meals. In the control meals, the glucose content hardly changed during the incubation (Fig. 2-C).

Volatile basic nitrogen (VB-N) of the fermented meals significantly increased after 24-h incubation and reached a maximal content of more than 300 mg/100 g meal. By glucose supplement during the incubation, the VB-N was reduced effectively. No change was observed in the VB-N content of
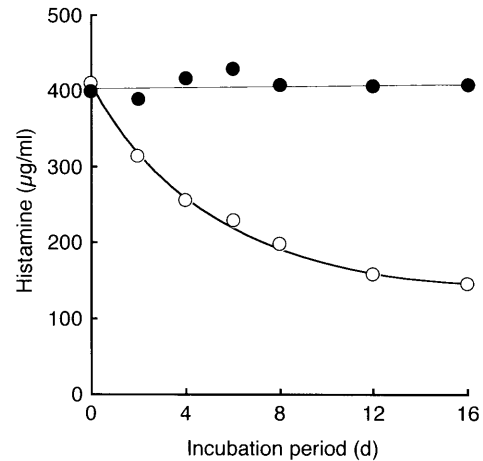

Fig. 3. Changes in histamine content in malt extract medium containing 400 μg/ml of hiatamine inoculated with *Aspergillus oryzae* IFO 4202 at 30°C. Symbols: ○, inoculated with the fungus; ●, not inoculated with the fungus.
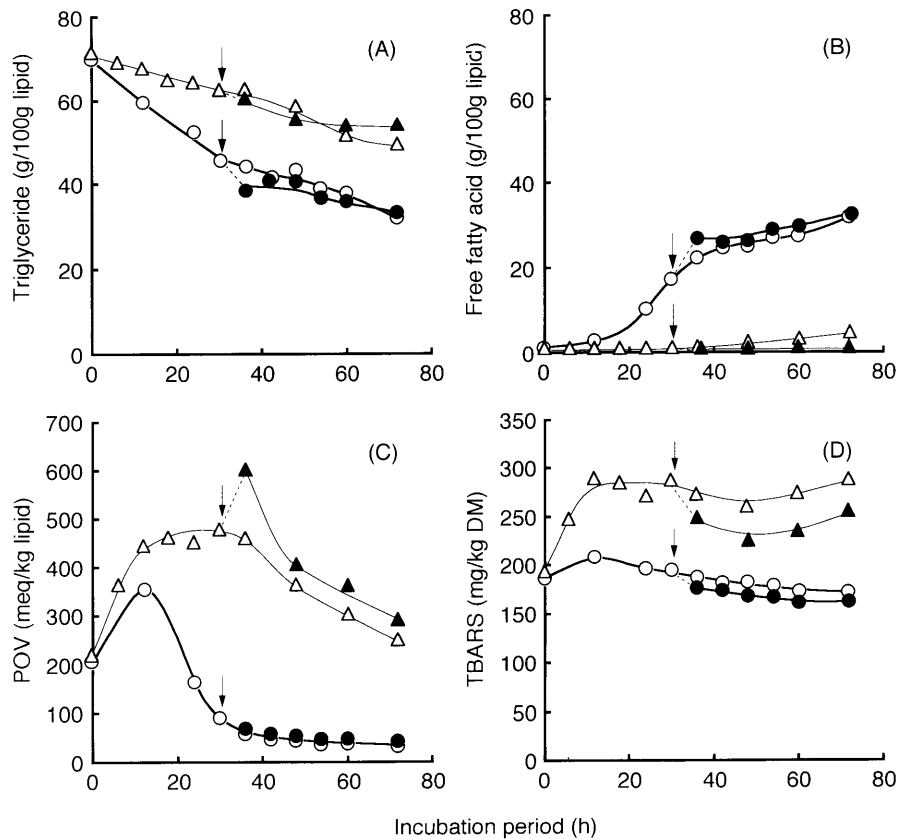

Fig. 4. Changes in triglyceride (A), free fatty acid (B) and peroxide (POV) (C) contents of the total lipids and thiobarbituric acid reactive substance (TBARS) (D) content of the meals during fermentation with *Aspergillus oryzae* IFO 4202. Symbols are the same as in Fig. 1.

the control meals (Fig. 2-D).

Free amino acid content (Fig. 2-E) markedly fluctuated in 30 h of fermentation and then decreased continuously. A slight decrease in amino acid was observed in the control meal during incubation.

Histamine in the meals was dramatically reduced by the fermentation with fungus. The glucose supplement somewhat enhanced the reduction. For the control meals, the histamine content did not change markedly from the initial level (Fig. 2-F).

We attempted to confirm the decomposition ability of histamine by *A. oryzae* IFO 4202 with a malt-extract medium containing histamine at a concentration of 400 μg/ml (Fig. 3). The fungus was inoculated in the medium and incubated at 30°C statically. An aliquot was periodically removed and the histamine content was determined. Histamine in the medium was reduced to less than 40% of the initial level during the incubation. No change was observed without the fungal inoculation.

The total lipids in the fermented meals decreased with elongation of the incubation period to 75% of the initial content. As for the lipid composition of the meals, free fatty acids increased and triglycerides decreased most significantly during the 12–24-h period of fermentation and then changed slowly. Free fatty acids reached more than 30% of total lipids and triglycerides decreased from the initial 70 to 30% lipids in 72 h of the fermentation. For the control meals, triglycerides did not decrease so much as for the fermented meal, and the free fatty acid content increased little during the incubation (Fig. 4-A, B). *A. oryzae* IFO 4202 was reported to have lipase activity (Kunimoto *et al.*, 1989, 1991; Hoshino *et al.*, 1991; Toida *et al.*, 1995). In the fermented meals, POV was reduced

and TBARS was not increased during fermentation with the fungus (Fig. 4-C, D).

Protein quality of fermented meals   The amino acid composition of the meal proteins changed in some ways during incubation. The amino acid composition and various nutritional properties of the proteins are shown in Table 1 for the fermented and unfermented control meals in 72 h of the incubation as an instance. In the fermented meals, glutamic acid, aspartic acid, lysine, and leucine were richer in composition. In the control meals, the loss of tryptophan was most significant during the incubation.

The EAAI of the fermented meals was 74–75 in 72 h of the incubation from 78 initially. For the control meals, the EAAI decreased to 63 with the glucose supplement and to 68 without glucose in 72 h of the incubation. The AA score remained 100 during incubation for the fermented meals and was reduced to 30 for glucose-supplemented control meal and 43 for the glucose non-supplemented control. For each control meal, the AA score was limited by tryptophan as a first limiting amino acid. C-PER was fairly decreased for the control meals. Enzymatic digestibility of the fermented meals was improved with fermentation to 96–97 in 72 h of fermentation from 87 initially. That of the control meals was not improved.

Microbiological characteristics of fermented meals   Changes in viable bacterial cell count of the meals during incubation are shown in Fig. 5. At the initial count, the bacterial cells in the meals were 3×10^2 CFU/g, then significantly increased after 24 h of incubation and reached 2.7×10^8 CFU/g in 72 h of incubation. For glucose, the supplemented meals at the 30-h-point viable cell count had somewhat lower levels than the non-supplemented meals. Bacterial count in

Table 1.   Amino acid composition and nutritional characteristics of meal proteins during fermentation with and without glucose supplement.

	Initial (0 h)	72 h-fermented		72 h-unfermented	
		G(−)[a]	G(+)[a]	G(−)[a]	G(+)[a]
Amino acid composition (mg/g protein)					
Aspartic acid	135.8	120.8	115.8	124.7	110.7
Threonine	61.4	55.5	53.0	55.1	49.6
Serine	53.0	48.1	45.6	45.9	43.1
Glutamic acid	184.8	150.8	145.6	171.4	150.1
Glycine	63.5	64.8	60.4	69.7	62.0
Alanine	80.4	70.7	67.4	78.1	69.7
Valine	79.0	71.0	69.3	74.3	65.1
Cystine/2	7.0	6.9	6.5	5.9	5.8
Methionine	39.5	30.1	27.3	33.2	29.2
Isoleucine	66.3	60.4	58.1	62.4	54.0
Leucine	113.9	95.7	92.4	103.5	91.5
Tyrosine	51.8	47.4	46.1	45.5	40.4
Phenylalanine	61.3	55.1	53.4	55.5	49.2
Tryptophan	13.0	12.0	11.8	4.7	3.3
Lysine	123.4	95.2	91.5	110.5	96.1
Histidine	38.5	34.5	32.7	39.9	54.5
Arginine	83.1	72.7	70.5	75.6	66.3
Proline	48.7	47.5	49.6	48.0	43.0
Nutritional characteristics					
EAAI	78	75	74	68	63
AA Score[b]	100	100	100	43 (Trp)	30 (Trp)
C-PER	2.9	2.9	2.9	1.3	0.4
Digestibility (%)	87	96	97	84	86

[a] G(−) and G(+) mean without and with glucose supplement during the incubation, respectively.
[b] AA Score was calculated by comparison to the FAO/WHO/UNU (1985) pattern for 2–5 year-old human. The first limiting amino acid was shown in parenthesis.
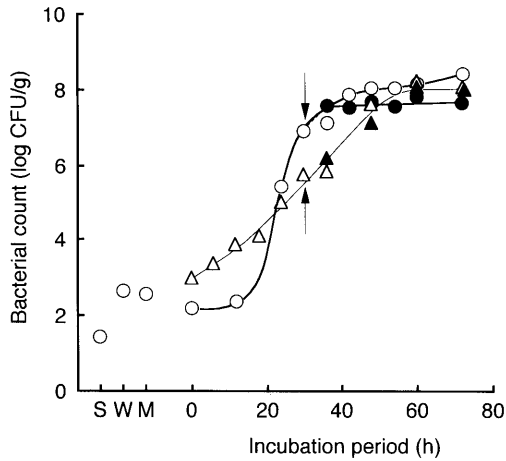
Fig. 5. Changes in viable bacterial cell count in the meals during fermentation with *Aspergillus oryzae* IFO 4202. Symbols are the same as in Fig. 1. S, W, and M means surface and whole body of fish, and heated and pressed meals, respectively. Bacterial count on the surface of fish represented as per cm².

the control meals was also increased as well as in the fermented meals.

During incubation, 1783 strains of bacteria separated from the body of the sardines and meals were identified regarding genera, and the resulting bacterial flora of the meals are shown in Fig. 6.

As for the microflora on fresh sardine surfaces, *Pseudomonas* spp. was predominant (>40%), then *Moraxella* spp. (20%) and *Acinetobacter* spp. (10%). These Gram-negative rod-shaped bacteria comprised 88% of the total bacteria. The microflora of the whole body of raw sardines and cooked meal at the initial state of fermentation were somewhat similar to those on the fish surface. These Gram-negative bacteria were universally detected in the surface and gills of fish and became the predominant genera on putrefactive fish (Gennari & Tomaselli, 1988; Ponce de Leon *et al.*, 1994).

During incubation, Gram-positive bacteria, especially *Micrococcus* spp., gradually became predominant in both fermented and control meals. Glucose supplement enhanced this tendency, and the ratio of *Micrococcus* spp. and other
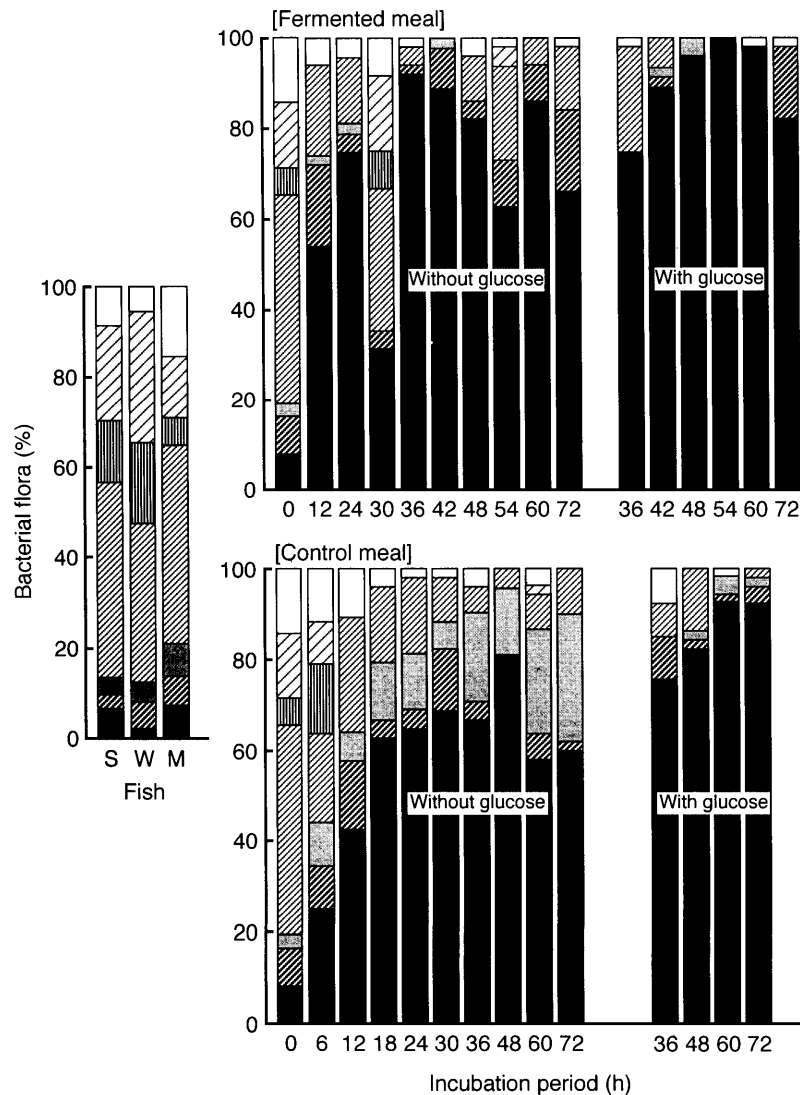

Fig. 6. Bacterial flora of the fermented and control meals during the incubation. S, W, and M means surface and whole body of fish, and heated and pressed meals, respectively. Columns: ■, *Micrococcus* spp.; ▨, *Staphylococcus* spp.; ▨, the other Gram-positive rods; ▨, *Pseudomonas* spp.; ▥, *Acinetobacter* spp.; ▨, *Moraxella* spp.; ☐, the other Gram-negative rods.

Gram-positive bacteria in total bacteria reached more than 90% in 50–60 h of the fermentation. *Bacillus* spp. was detected throughout the incubation in the control meals not supplemented with glucose.

Discussion

At 12 to 36 h into fermentation of sardine meals with *A. oryzae*, the fungus significantly grew as shown by the increasing glucosamine content. At the same time, rises in meal temperature and pH, increase in VB-N and free fatty acid content, decrease in starch, and changes in glucose content were observed. Starch added before fermentation was considered to be hydrolyzed to glucose, and it was consumed by the fungus as a carbon source to produce acids. Most of the glucose was consumed within 24 h, and then lipids or amino acids might be utilized as a carbon source by *A. oryzae*. Within 24 h into fermentation, free fatty acids were produced from triglycerides in the meal lipids by fungal lipase as reported by Kunimoto *et al.* (1989).

When nitrogenous compounds such as amines or amino acids were utilized for fungal growth as a carbon source, ammonia was produced and caused an increase in pH value. Glucose supplement at 30 h of fermentation led to pH reduction through consumption of ammonia and production of organic acid. Moreover, ammonia accumulated in the fermented meals might also be utilized as a nitrogen source to result in reduction of VB-N. Thus glucose supplement during fermentation could not only contribute fungal growth but improve the quality of the fermented meals by the inhibitional effects on the production and release of VB-N compounds.

Kunimoto *et al.* (1992) reported that *A. oryzae* could digest trimethylamine and trimethylamine oxide and utilize them as a nitrogen source in fermented fish meal. If carbohydrates were limited in the substrate, these amines could be utilized as a carbon source and ammonia would be released from them.

Accompanied by fermentation of the meals, bacterial contaminants grew together with the fungus. Because VB-N for control meals not inoculated with the fungus did not increase, most of the contaminated bacteria in the meals might not have strong ammonia-releasing activity. Increasing VB-N was considered to be attributed to utilization of nitrogen compounds as a carbon source by *A. oryzae*. On the other hand, considering the rise in pH of control meals also observed with bacterial growth, the production, consumption and assimilation of the basic compounds by bacterial contaminants should be characterized, compared with those properties of the fungus.

Gizzerosine has been thought to be made by the interaction between histamine in fish and proteins by heating during fish meal processing or by exothermic reaction during storage. Because the sardines used in this study were very fresh, histamine was present at a fairly low level in the meals. Some of the commercial meals had a higher level of histamine (Toyama *et al.*, 1981). The results in this study suggest that the fermentation with *A. oryzae* IFO 4202 might be effective for reduction of the histamine level. Capacity for histamine decomposition of the fungus should be evaluated in connec-

tion with histamine concentration and the level of histamine-producing contaminants.

As for the antioxidative effect of the fermentation, *A. oryzae* might have decomposing, digesting or reducing functionalities for oxidized lipids. Kato *et al.* (1985) reported the antioxidative effect of various fungi on fish oil. Hossain *et al.* (1987b) reported that scrap meals of mackerel fermented with *Aspergillus terreus* had a lower oxidation level. Rashid *et al.* (1992, 1993) suggested an antioxidant production by *Aspergillus sojae* and *A. oryzae*.

The oxidation of amino acids reduces the nutritional value of meals. It is known that tryptophan, methionine, cysteine, cystine, histidine and lysine residues can react with autoxidation products of lipid and be altered to unutilizable forms. The antioxidative effect of *A. oryzae* might contribute to inhibition of deterioration of tryptophan residues in meals during incubation. Thus the fermented fish meals could have higher nutritional proteins for their higher EAAI, AA score, PER and digestibility.

There are some reports that *Micrococcus* spp. became predominant during processing of rice koji and koji seed (Kubota *et al.*, 1976; Takema *et al.*, 1978). Kunimoto *et al.* (1995) observed that *Micrococcus* spp. had become predominant in the final stage of fish meal fermentation by *A. oryzae*, even though putrefactive bacteria such as *Pseudomonas* spp. and *Achromobacter* spp. had predominated in the early stage. Similar microflora were observed in the fermented fish meals in this study. It is considered preferable for good quality of koji that Gram-negative bacteria decreased and microflora mainly composed of *Micrococcus* spp. were formed during fermentation for koji processing. These phenomena might be attributed to complex factors of water activity, pH, chemical components, and competitive and symbiotic effects among *A. oryzae* and many kind of bacteria in the system (Kubota *et al.*, 1981; Narahara, 1988).

It was concluded that fermentation with *A. oryzae* in the presence of glucose might be a suitable process to improve the chemical, nutritional and microbiological quality of meals from easily deteriorated fish. Further study is needed for the control of fermentation and the evaluation of stability on long-term storage.

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