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Development of koji by culturing *Aspergillus oryzae* on nori (*Pyropia yezoensis*)

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Koji is a traditional fermentation culture medium, based on *Aspergillus oryzae*, which is commonly used in the manufacture process of Japanese fermented products such as soy sauce, miso, and sake, and promote enzymatic degradation. Koji is usually prepared by culturing a mold on cereals such as wheat flour, soybean, or rice, but that cultured on seaweeds has not been developed yet. This study prepared the koji by culturing *A. oryzae* on seaweed nori (dried piece of *Pyropia yezoensis*), and, then, characterized on this nori koji. The nori koji contained 0.85 μg *N*-acetyl-glucosamine, estimated as 6.1 μg mold cells, per gram dry matter and showed various kind of enzymatic activities in glycosidase, protease, and phosphatase as well as traditional soy sauce koji and rice koji. The suitability of these characteristics for degradation of nori was tested on nori sauce culture with and without the addition of the nori koji. After 167 days of culture, the fermentation tank with the nori koji showed over 74% recovery of supernatant while that without the nori koji had less than 57% recovery. The supernatant of culture mashes contained more than two times larger quantity of total nitrogen compounds in nori koji test group against control group. The present study prepared koji on seaweed nori for the first time and demonstrated its advantages to shorten the culture period and increase taste value in nori sauce manufacture. Development of seaweed koji enables a method to prepare cereal allergen free fermented sauces from seaweeds.

[**Key words:** *Aspergillus oryzae*; Fermentation; Gluten free; Koji; Low allergen risk; Nori; *Pyropia yezoensis*; Seaweed sauce]

Despite the long history of fermentation technology, fermented food items produced from seaweeds have not been developed (1). Seaweed sauce was recently developed by fermentation from nori (laver or dried sheet product of *Pyropia yezoensis*) (2). This nori sauce is reported to have a unique taste and characteristics: rich in functional compounds such as taurine and vitamins, and is expected to be a new nutritional source for humans. Furthermore, the nori sauce has application as a low allergy risk seasoning because it does not contain cereal components such as soybean and wheat. Nori sauce usually needs more than one year to complete the degradation and fermentation process if it is manufactured by simply added with salt. For improving the manufacturing process, it is expected that the culture period of nori sauce can be shortened by use of koji which is commonly used in manufacture process of soy sauce and miso seasoning. However, traditional kojis contain cereals such as soybean and wheat as components, and if the traditional kojis are used for nori sauce manufacture, the advantageous characteristics of nori sauce as a low allergen risk food will be lost. So, the authors considered developing a koji from seaweed nori for the quick manufacture of nori sauce without contamination of cereal allergens. A few studies have reported on solid state mold culture of seaweeds (3,4). Suraiya et al. (4) reported culture of red mold *Monascus* spp. on *Saccharina japonica* and *Undaria pinnatifida*. However, the objective of this study was to promote extraction from seaweeds for obtaining bio-functional materials, and not to prepare koji which could be used in the manufacture process of fermented products.

The present study attempted to prepare a koji by culturing *Aspergillus oryzae* on nori. The obtained koji was characterized for its microbial composition and enzyme activities. Then, nori sauce fermentation was conducted with and without the nori koji to demonstrate the promotion of degradation of solid material by the nori koji.

MATERIALS AND METHODS

Nori Three types of nori (*Pyropia yezoensis,* harvested in Saga) were purchased from Inoue-nori Co., Ltd. (Tokyo, Japan). Dried sheet nori (protein content 33.9% and carbohydrate content 54.4% on a dry weight basis) was cut into 3 × 13 mm pieces using a Shredder Machine (Fujiteck Co., Ltd., Okayama, Japan) and used for measurement of water activity and preparation of koji. Dried and fresh types of iroochi-nori (i.e., discolored nori) were used for the sauce fermentation test. The dried iroochi-nori (protein content 15.9% and carbohydrate content 72.7%) was milled by Drying Crusher VTG modified type (EarthLink, Inc., Hyogo, Japan) to pass through a 2 mm mesh and used. The fresh iroochi-nori (moisture 91.5%, protein content 14.4% and carbohydrate content 77.5% on a dry weight basis), which had been preserved in a refrigerator until use, was minced using a Mincer BX-1 to pass through a plate with 5.5 mm pore size (Watanabe Kikaikogyo K.K., Aichi, Japan).

Measurement of water activity Shredded nori was added with distilled water at different moisture levels (10–90% (w/w)), mixed well, and their water activity of nori was measured by LabMaster-aw evc-1 (Novasina, Lachen, Switzerland). Data are shown as the mean of duplicate measurements.

Preparation of nori koji Three types of commercial products (40 g of Three-dia, 40 g of Takarakin, and 20 g of Diamond-C) containing *A. oryzae* strains were

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purchased from Higuchi Moyashi Co., Ltd. (Osaka, Japan), mixed together, and used as a seed culture mixture. Six kilograms of shredded nori was added with 0.84 g of the seed culture mixture and six liters of tap water, mixed well, scattered to a tray, wrapped with filter cloth and gently aerated with a blower conditioned at 30.5 °C and >90% humidity using the NFT Automatic Koji-making Machine (Nagata Brewing Machinery Co., Ltd., Fukuoka, Japan). The culture was monitored its temperature and mixed by hand two times (the 1st mixing and 2nd mixing) for cooling down to keep the temperature less than 45 °C. The temperature of the conditioned air was changed to 25.5 °C after the 2nd mixing and continued incubation for three days from the start.

Microbial analysis of nori koji Nori koji was serially diluted with sterile 0.85% sodium chloride solution and the number of viable microorganisms including bacteria and yeast was counted on Plate Count Agar with BCP plates (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). Mold colonies on agar plates were visually discriminated from bacteria and yeast colonies by their hyphal appearance and separately counted. *N*-Acetylglucosamine (GlcNAc) was measured as follows: Shredded nori and nori koji were degraded to a fine powder by Vibrating Sample Mill TI-10 (C.M.T. Co., Ltd., Tokyo, Japan). Duplicate 0.1 g subsamples were transferred to microcentrifuge tubes and rinsed with 1.5 ml of 50 mM phosphate buffer (pH 7.0) three times by centrifuging at 20,000 × *g* for 20 min and eliminating the supernatant. The pellet was suspended in 1.5 ml of 50 mM phosphate buffer (pH 7.0), added with 1 mg of yatalase (Takara Bio, Inc., Shiga, Japan), incubated at 37 °C for 1 h with moderate shaking. Then, the supernatant was collected, weighed, and measured for GlcNAc by the colorimetric method of Reissing et al. (5). Growth quantity of mold was estimated using a parameter that 1 mg mold contains 139 μg GlcNAc (6). Microbial composition in the nori koji was analyzed by MiSeq sequencing: Genomic DNA (gDNA) was extracted by Fast DNA SPIN Kit for soil (MP Biomedicals, LLC., Solon, OH, USA) from a 0.5 g subsample of the seed culture mixture and nori koji. Gene amplification and sequencing was carried out at Bioengineering Lab (Atsugi, Japan) using an MiSeq Genome Sequencer (Illumina, San Diego, CA, USA). For mold gene amplification from the extracted gDNA, first PCR was performed using a primer set targeting internal transcribed spacer region of the 18S rRNA gene (ITS1, approximately 300 bp): ITS1-F_KYO1 (5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-CTHGGTCATTTAGAGGAASTAA-3′) and ITS2_KYO2 (5′-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-TTYRCTRCGTTCTTCATC-3′) (7). The PCR program for amplifying the ITS1 region was as follows: initial denaturation at 94 °C for 2 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 60 s, and a final extension at 72 °C for 5 min. For bacterial gene amplification, first PCR was performed using a primer set targeting the V3 to V4 regions of 16S rRNA gene (V3/V4, approximately 450 bp): 341f (5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-CCTACGGGNGGCWGCAG-3′) and 805r (5′-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-GACTACHVGGGTATCTAATCC-3′). The PCR program for amplifying 16S rRNA region was as follows: initial denaturation at 94 °C for 2 min, 25 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. The PCR product was used as a template in the second PCR. For the second PCR, a primer set F (5′-AATGATACGGCGACCACCGAGATCTACAC-Index2-ACACTCTTTCCCTACACGACGC-3′) and R (5′-CAAGCAGAAGACGGCATACGAGAT-Index1-GTGACTGGAGTTCAGACGTGTG-3′). The Index2 tag sequence included 8 nucleotides designed for sample identification barcoding. The Index1 primer sequence was ACTGATCG. The second PCR program was as follows: initial denaturation at 94 °C for 2 min, 10 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. The PCR products were purified with the AMPure bead kit (Agencourt, Beverly, MA, USA). Quality and product size were assessed on a Fragment Analyzer and dsDNA 915 Reagent kit (Advanced Analytical Technologies, Inc., Ankeny, IA, USA). Paired-end sequence reads were quality filtered by a command line tool package FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/): Only the sequence reads that had identical sequences in the head region to the primer sequence were collected. After the primer sequence was trimmed, the sequence reads with a quality score of less than 20 and length less than 40 bp (for ITS1) or 150 bp (for V3/V4), were removed. The quality filtered sequence reads were merged using a script FLASH with the following parameters: sequence length after the merge was 320 bp for ITS1 or 420 bp for V3/V4, read length 280 bp, and overlap sequences were at least 10 bp. Chimeric sequences were identified using UCHIME (8) algorithm. The remaining sequences were stored into operational taxonomic units (OTUs) with a criterion of 97% sequence identity. The taxonomic assignment of OTUs was performed using the Workflow script implemented in the QIIME package (9,10) with default parameters. The identities of phylotypes were further analyzed by comparison of sequences against the DNA Data Bank of Japan (DDBJ) using a BLAST analysis. The OTUs assigned to unclassified were removed.

Enzyme activities of koji Enzyme activities were measured for soy sauce koji, rice koji, and nori koji. The soy sauce koji and rice koji are both commercial products distributed by Maruhide Shoyu Co., Ltd. (Saga, Japan). The soy sauce koji is a fresh product prepared by culturing *A. oryzae* on a mixture of defatted soy and wheat flour (1:1). The rice koji is a two weeks-old product prepared by culturing *A. oryzae* on rice. A seed culture mixture containing the commercial *A. oryzae* products (Tree-dia:Takarakin:Diamond-C = 2:2:1, This starter composition is commonly used at the Maruhide Shoyu Co., Ltd. based on a veteran koji master's experience) was used for preparing the soy sauce koji and rice koji. The nori koji prepared in the present study were stocked for 6 months at −30 °C and measured for enzyme activities.

Subsamples of the koji were mixed with 10−30 volumes of 50 mM sodium acetate buffer (pH 6.0) and stirred with a blender. The stirred solution was centrifuged (8000 × *g*, 15 min), the supernatant was dialyzed against the same buffer for 20 h, and the dialyzate was centrifuged at 8000 × *g* for 15 min, and the supernatant was used as the enzyme solution.

Glucomannan, β-1,4-mannan, and β-1,3-xylan were prepared from *Amorphophallus konjac* (konjac powder), *Codium fragile* (green seaweed), and *Caulerpa racemosa* (green seaweed) according to the methods of Sugiyama et al. (11), Love and Percival (12), Iriki et al. (13), respectively. Starch, agarose, and carboxymethyl cellulose were purchased from Wako Pure Chemical Industries (Osaka, Japan).

For measuring the polysaccharide-degrading enzyme activity, 0.4 ml of enzyme solution was added to 1 ml of each substrate (starch, glucomannan, β-1,4-mannan, β-1,3-xylan, agarose, carboxymethyl cellulose) and 0.6 ml of 200 mM sodium acetate buffer (pH 6.0). The mixture was incubated at 37 °C for an appropriate period of time, and the resulting reducing sugar was measured by the Somogyi-Nelson method (14). One unit of the enzyme was defined as the amount of enzyme reducing a sugar equivalent to 1 μmol of each constituent sugar (glucose, mannose, xylose, or galactose) per minute. For measuring glycosidase activities, 0.1 ml of enzyme solution was added to 0.1 ml of 6 mM of various *p*-nitrophenyl glycosides and 0.1 ml of 200 mM sodium acetate buffer (pH 6.0). Each mixture was incubated at 37 °C for a suitable period and then the reaction stopped by adding 1 ml of 2% sodium carbonate solution. The mixture was measured at 400 nm spectrophotometrically. One unit of glycosidase activity was defined as the amount of enzyme producing 1.0 μmol of *p*-nitrophenol liberated from each *p*-nitrophenyl glycoside. For measuring protease activity, 0.2 ml of enzyme solution was added to 1.3 ml of 1% casein in 100 mM phosphate buffer (pH 6.8). The mixture was incubated at 37 °C for 20 min and then the reaction was stopped by addition of 1.5 ml of 5% trichloroacetic acid solution. The mixture was centrifuged at 8000 × *g* for 10 min and supernatant was determined by the Lowry method. One unit of protease activity was defined as the amount of enzyme producing the value responding to 1.0 μmol of tyrosine. Data was shown as mean ± SD based on triplicate measurements.

Sauce fermentation test Sauce fermentation test was conducted with or without adding nori koji (n = 3). Test sauce culture with dried nori was prepared with 5 g of nori powder, 3.5 g of nori koji, 4 g of sodium chloride, 36 g of drinking water. Test sauce culture with fresh nori was prepared with 31 g of fresh minced nori, 3.5 g of nori koji, 4 g of sodium chloride, 10 g of drinking water. Control sauce cultures were prepared as the test culture but replacing 3.5 g of nori koji with 2.5 g of shredded nori and 1 g of drinking water. The salt concentration was set at an 8.2% (w/w) level for preparing the nori sauce to reduce the salty taste of the final product based on the result in previous studies (2,15). The cultures were mixed well using a sterile spatula and incubated at 23 °C, and thereafter mixed on day 5, day 8, day 13, day 20, day 27, and day 40 to promote fermentation. Subsamples were taken periodically from triplicate sauce cultures and determined for the supernatant fraction ratio% and total nitrogen compounds in the supernatant. Supernatant fraction ratio% was calculated on a weight basis after a two-gram subsample was transferred to a 2 ml-volume centrifuge tube, centrifuged at 20,000 × *g* for 20 min, and the pellet weighed after removing the supernatant. Total nitrogen compounds of the supernatant were measured by the Kjeldahl method. Organic acids were measured by a high performance liquid chromatography system (JASCO Corporation, Tokyo, Japan) according to the method of the previous study (15). Briefly, the organic acids were separated with ion exclusion chromatography columns (8 mm × 300 mm × 2 columns, Shodex RSpac KC-811, Showa Denko K.K., Tokyo, Japan), derivatized with bromothymol blue, and detected with UV-detector (445 nm). Data are shown as the mean ± standard deviation (SD) based on triplicate measurements.

Statistical analysis A one-way ANOVA with ad hoc Student−Newman−Keuls test was conducted to compare the effect of koji type on each kind of enzyme activities.

Nucleotide sequence accession numbers Datasets of 18S rRNA gene (ITS1 region) and 16S rRNA gene (V3/V4 region) reads sequenced in this study have been deposited in DDBJ/EMBL/Genbank under the accession number DRA006947.

RESULTS

Water activity of nori at different moisture levels Relationship between moisture content and water activity of nori is shown in Fig. 1. The nori shows 0.952 and 0.962 of water activity at 50% and 60% (w/w) of moisture levels, respectively.

Culture of koji mold on nori Nori koji culture containing the seed culture mixture and water at a 50% moisture level were incubated and monitored for its product temperature (Fig. 2). The product temperature started to increase from 32 °C (17 h after start) to 38 °C (19.5 h), and the first mixing was conducted to cool down to 34 °C (19.7 h). The product temperature increased again to 44 °C (25 h), and the second mixing was conducted to

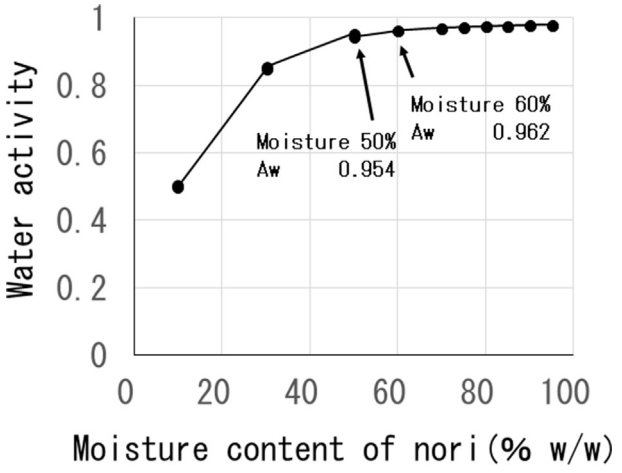

FIG. 1. Relationship between moisture and water activity of nori.

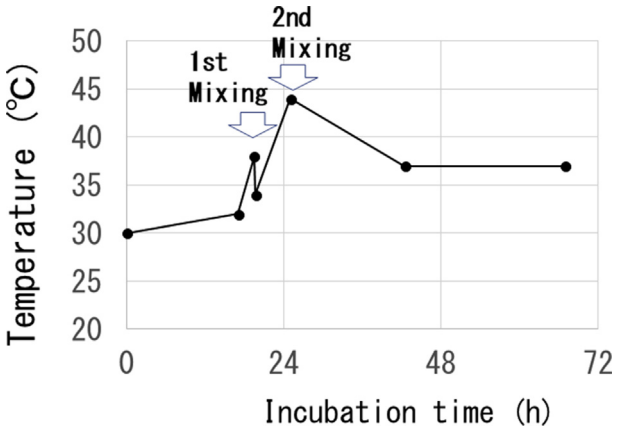

FIG. 2. Product temperature record during culture of koji mold on nori.

cool down. The product temperature just after the 2nd mixing was not measured but decreased to 37 °C at 42.5 h after start. The last period 42.5 h–67 h was cultured at 37 °C, and vigorous growth of yellow-brown colored hyphae was observed (Fig. 3). The final product of nori koji showed a moisture value 28.6%, which corresponds to water activity value 0.82 based on the result from Fig. 1.

Microorganims in nori koji The seed culture mixture contained 1.6×10^8 colony forming units (cfu)/g of mold (*A. oryzae*), and bacteria and yeast colonies were not counted (less than 10^5 cfu/g). The nori koji contained 2.0×10^7 cfu/g of mold, and 1.3×10^7 cfu/g of bacteria and yeast. All of the mold colonies formed on agar plates prepared for microbial counting had a homogeneous yellow-

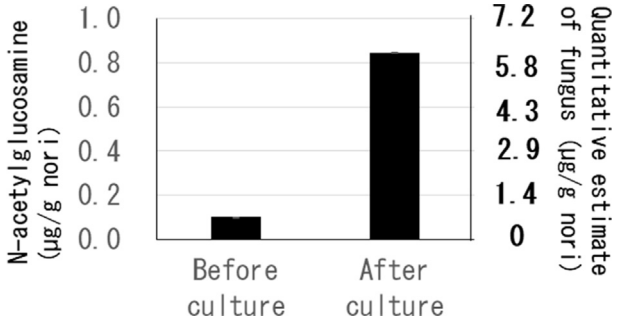

FIG. 4. Measurement of *N*-acetylglucoamine (GlcNAc) and quantitative estimate of koji mold grown on nori. Data are shown as mean (bar:SD) of duplicate measurements. Quantity of mold was estimated on the hypothesis that 1 mg of dry mold contains 139 μg of GlcNAc.

brownish color and an appearance between that of the seed culture mixture and the nori koji. Growth of mold was quantified as 0.85 μg GlcNAc/g koji or estimated as 6.1 μg cell/g koji (Fig. 4). MiSeq sequencing analysis obtained 38,341 (for ITS1 of the seed culture mix), 82,341 (for ITS1 of the nori koji), 41,523 (for V3/V4 of the seed culture mix), and 54,330 (for V3/V4 of the nori koji) reads and 29,299 (for ITS1 of the seed culture mix), 62,824 (for ITS1 of the nori koji), 1337 (for V3/V4 of the seed culture mix), and 40,566 (for V3/V4 of the nori koji) reads after the quality control and chimera check, respectively. Results of phylogenetic assignment are shown in Table 1 based on the ITS1 region sequences and Table 2 based on the V3/V4 region sequences. The figures of OUTs shown on Tables 1 and 2 do not involve the reads assigned to chloroplast of *P. yezoensis* and unclassified. As for the seed culture mixture, total of 29,299 of ITS1 reads were banded into three OTUs at 97% similarity, and 29,297 (>99.9%) of the 29,299 reads were grouped to OUT-denovo 8, which was assigned to a *A. oryzae/flavus* group. The sequence similarity of the OUT-denovo 8 was 100% to *A. oryzae*, accession number MH279453. As for nori koji, total of 62,824 of ITS1 reads were banded into ten OTUs at 97% similarity and 62,812 (>99.9%) out of the 62,824 OTUs were also grouped to the OUT-denovo 8. As for bacterial composition, total of 101 reads of V3/V4 in the seed culture mixture were banded into one OTU-denovo 165, which was assigned to *Acinetobacter brisouii*-related species with 96.3% similarity. As for nori koji, total of 30,765 reads of V3/V4 were banded into seven OTUs: OTU-denovo 275, -denovo 179, -denovo 253, -denovo 46, -denovo 98, -denovo 36, and -denovo 31 were assigned to *Staphylococcus gallinarum* (21,973 reads, 70.8%), *Bacillus amyloliquefaciens* (6510 reads, 21.1%), *S. gallinarum*-related (735 reads, 2.4%), *Staphylococcus epidermidis* (631 reads, 2.1%), *Staplococcus warneri* type A (622 reads, 2.0%), *Bacillus altitudinis* (194 reads, 0.6%), and *S. warneri* type B (100 reads, 0.01%), respectively.

FIG. 3. Appearance of nori before (left) and after (right) culture of koji mold.

TABLE 1. Distribution and abundance patterns of dominant 18S-ITS1 rRNA OTUs associated with seed culture mixture and nori koji.

OTU ID[a]	Taxonomic classification[b] (acc. no. of the most neighbor sequence, similarity %)	Seed culture mixture	Nori koji
Denovo 8	*Aspergillus oryzae* (MH279453, 100)/*flavus*	29297	62812
Denovo 0	*Meyerozyma caribbica* (KY104222, 100)	0	3
Denovo 5	*Aspergillus oryzae*/*flavus*	1	2
Denovo 1	*Aspergillus oryzae*/*flavus*	0	1
Denovo 2	*Aspergillus oryzae*/*flavus*	0	1
Denovo 3	*Aspergillus oryzae*/*flavus*	0	1
Denovo 4	*Aspergillus oryzae*/*flavus*	0	1
Denovo 6	*Aspergillus oryzae*/*flavus*	1	0
Denovo 7	*Aspergillus oryzae*/*flavus*	0	1
Denovo 9	*Aspergillus oryzae*/*flavus*	0	1
Denovo 10	*Aspergillus oryzae*/*flavus*	0	1

[a] The OTUs assigned to unclassified were removed and the remaining OTUs were banded into eleven denovos at 97% sequence identity.
[b] The taxonomic assignment of OTUs was performed using the Workflow script implemented in the QIIME package. The OUTs-denovo 8 and 0 were further assigned by BLAST search on the DDBJ database.

TABLE 2. Distribution and abundance patterns of dominant 16S rRNA OTUs associated with seed culture mixture and nori koji.

OTU ID[a]	Taxonomic classification[b] (acc. no. of the most neighbor sequence, similarity %)	Seed culture mixture	Nori koji
Denovo 275	*Staphylococcus gallinarum* (D83366, 100)	0	21973
Denovo 179	*Bacillus amyloliquefaciens* (HQ231913, 100)	0	6510
Denovo 253	*Staphylococcus gallinarum*-related (D83366, 96.3)	0	735
Denovo 46	*Staphylococcus epidermidis* (D83363, 98.8)	0	631
Denovo 98	*Staphylococcus warneri* Type A (L27603, 98.8)	0	622
Denovo 36	*Bacillus altitudinis* (AJ831842, 99.8)	0	194
Denovo 31	*Staphylococcus warneri* Type B (L27603, 97.9)	0	100
Denovo 165	*Acinetobacter brisouii*-related (DQ832256, 96.3)	101	0

[a] The OTUs assigned to chloroplast of *Porphyra yezoensis* and unclassified were removed and the remaining OTUs were banded into eight denovos at 97% sequence identity.
[b] The OTUs were taxonomically assignment by the Workflow script implemented in the QIIME package and further assigned by BLAST search on the DDBJ database.

Enzyme activities of nori koji Enzyme activities contained in various koji samples were measured and shown in Tables 3–5. Soy sauce koji, rice koji, and nori koji commonly showed high activities in amylase (432, 232, and 111 units, respectively), protease (56.2, 17.1, and 23.0 units), β-D-glucosidase EC 3.2.1.21 (2.11, 0.257, and 1.23 units), α-D-galactosidase EC 3.2.1.22 (7.43, 2.61, and 6.83 units), and phosphotase EC 3.1.3 (2.08, 4.79, and 1.80 units). Nori koji showed relatively high activity in β-1,4-mannanase EC 3.2.1.78 (1.35 units) and significantly higher activity ($P < 0.05$) in α-D-mannosidase EC 3.2.1.24 (0.065 ± 0.002 units) than those of soy sauce koji (0.018 ± 0.001 units) and rice koji (0.010 ± 0.000 units). The nori koji also showed enzyme activities in α-D-glucosidase EC 3.2.1.20 (0.069 units), β-D-galactosidase EC 3.2.1.23 (0.141 units), β-D-mannosidase EC 3.2.1.25 (0.068 units), β-D-xylosidase EC 3.2.1.37 (0.060 units), and α-L-arabinosidase EC 3.2.1.55 (0.015 units). Enzyme activities in β-1,3-xylanase EC 3.2.1.32, agarase, and α-L-fucosidase EC 3.2.1.51 were not detected for the three kinds of koji.

Sauce fermentation test with koji Sauce fermentation test was conducted for 167 days to demonstrate the promotion effect by koji on the degradation of nori. As the fermentation period proceeded, solid material of nori sauce culture prepared with nori koji was remarkably degraded and the viscosity of culture mashes was decreased. The supernatant fraction collected after centrifugation increased and its ratio against the total material increased from 1.4% at the start to 72.7% on day 121, and 74.4% at day 167 for dried nori, and from 7% at the start to 74.6% on day 121, and 74.3% at day 167 for fresh nori, respectively (Fig. 5). Degradation of nori was also

TABLE 3. Comparison of degrading enzyme activities for polysaccharides and protein in soy sauce koji, rice koji and nori koji (units[a]/g of koji).

	Amylase (starch)	β-1,4-Mannanase		β-1,3-Xylanase (β-1,3-xylan)	Agarase (agarose)	CMCase (carboxymethyl cellulose)	Protease (casein)
		Glucomannan	β-1,4-Mannan				
Soy sauce koji	432 ± 7[b]	5.96 ± 0.06[c]	1.14 ± 0.27[b]	ND	ND	2.82 ± 0.44[b]	56.2 ± 0.5[b]
Rice koji	232 ± 31[c]	ND[d]	ND[c]	ND	ND	ND[d]	17.1 ± 0.8[d]
Nori koji	111 ± 17[d]	6.87 ± 0.13[b]	1.35 ± 0.33[b]	ND	ND	1.01 ± 0.12[c]	23.0 ± 1.3[c]

Data are shown as mean ± SD of triplicate measurements. ND, not detected (<0.01 units).
[a] One unit is activity to increase 1 μmol of constituent sugars or tyrosine per minute.
[b,c,d] Different characters mean significant difference among the koji samples (Student–Newman–Keuls test, $P < 0.05$).

TABLE 4. Comparison of glycosidase activities in soy sauce koji, rice koji and nori koji (units[a]/g of koji).

	PNP-α-D-glucoside	PNP-β-D-glucoside	PNP-α-D-galactoside	PNP-β-D-galactoside	PNP-α-D-mannnoside
Soy sauce koji	0.408 ± 0.022[b]	2.11 ± 0.01[b]	7.43 ± 0.11[b]	0.516 ± 0.014[b]	0.018 ± 0.001[c]
Rice koji	0.337 ± 0.005[c]	0.257 ± 0.006[d]	2.61 ± 0.05[d]	0.089 ± 0.002[d]	0.010 ± 0.000[d]
Nori koji	0.069 ± 0.005[d]	1.23 ± 0.03[c]	6.83 ± 0.07[c]	0.141 ± 0.003[c]	0.065 ± 0.002[b]

Data are shown as mean ± SD of triplicate measurements.
[a] One unit is activity to increase 1 μmol of *p*-nitrophenol per minute.
[b,c,d] Different characters mean significant difference among the koji samples (Student–Newman–Keuls test, $P < 0.05$).

TABLE 5. Comparison of other kinds of glycosidase and phosphatase activities in soy sauce koji, rice koji and nori koji (units[a]/g of koji).

	PNP-β-D-mannnoside	PNP-β-D-xyloside	PNP-α-L-fucoside	PNP-α-L-arabinoside	PNP-phosphate
Soy sauce koji	0.161 ± 0.004[b]	0.629 ± 0.010[b]	ND	0.132 ± 0.001[b]	2.08 ± 0.04[c]
Rice koji	0.011 ± 0.001[d]	0.009 ± 0.001[d]	ND	0.008 ± 0.000[d]	4.79 ± 0.07[b]
Nori koji	0.068 ± 0.001[c]	0.060 ± 0.001[c]	ND	0.015 ± 0.000[c]	1.80 ± 0.08[d]

Data are shown as mean ± SD of triplicate measurements. ND, not detected (<0.008 units).
[a] One unit is activity to increase 1 μmol of *p*-nitrophenol per minute.
[b,c,d] Different characters mean significant difference among the koji samples (Student–Newman–Keuls test, $P < 0.05$).

observed probably due to an involvement of enzyme activities released from the dominant microorganisms but very slow for the case of sauce culture prepared without nori koji, and the supernatant fraction ratio% on day 167 was only 53.9% for dried nori and 56.4% for fresh nori, respectively. Supernatant fraction of nori sauce culture was analyzed for total nitrogen compounds and organic acid contents to study the effect on taste elements by use of nori koji. The supernatant prepared with and without nori koji on day 167 contained 0.34 ± 0.03 (mean ± SD) g and 0.15 ± 0.01 g N/100 ml, respectively, for the dried nori test (Fig. 6). The supernatant prepared with and without nori koji on day 167 contained 0.28 ± 0.01 g and 0.09 ± 0.01 g N/100 ml, respectively, for the fresh nori test. Significant increase ($P < 0.05$) in total nitrogen compounds of the supernatant fraction was observed by use of nori koji. Organic acid contents are shown in Fig. 7. Citric acid, lactic acid, and acetic acid were the major components in nori sauces. Lactic acid and acetic acid were significantly ($P < 0.05$) increased by the use of nori koji: 14.1 ± 3.3 and 4.3 ± 2.5 mg/100 ml (without nori koji, dried nori), 59.0 ± 0.9 and 55.2 ± 11.8 mg/100 ml (with nori koji, dried nori), 74.5 ± 0.0 and 15.0 ± 7.4 mg/100 ml (without nori koji, fresh nori), 125.4 ± 0.3 and 45.2 ± 20.4 mg/100 ml (with nori koji, fresh nori), respectively.

DISCUSSION

There are several points to be considered for performing solid state culture of koji mold successfully on nori. Firstly, the dried sheet product of nori was used because it was desalted by washing during its manufacture process and would not inhibit the growth of the mold. Secondly, the nori was shredded and converted into small pieces. This helped to form compact three-dimensional masses of nori and kept an aerobic condition, which is suitable for solid state

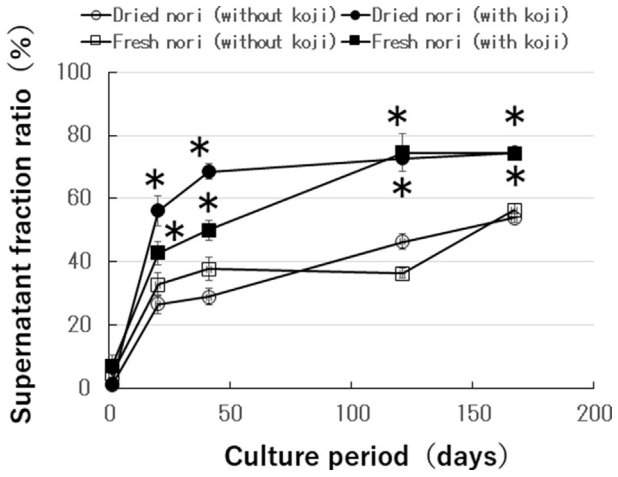

FIG. 5. Results of nori sauce fermentation test with or without addition of nori koji. Subsample of culture mash was centrifuged and the supernatant fraction ratio% was calculated from ratio of supernatant against total on a weight basis. Data are shown as mean (bar: SD) of triplicate tests. Asterisks show significant difference ($P < 0.05$) against the control sample prepared without koji.

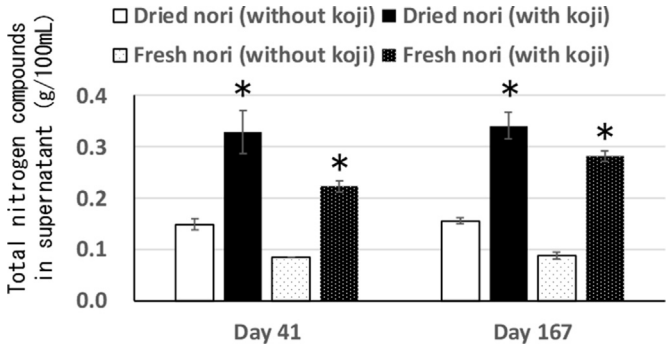

FIG. 6. Results of nori sauce fermentation test with or without addition of nori koji. Total nitrogen compounds contained in supernatant was analyzed and compared after 41 and 167 days of culture. Data are shown as mean (bar: SD) of triplicate tests. Asterisks show significant difference ($P < 0.05$) against the control sample prepared without koji.

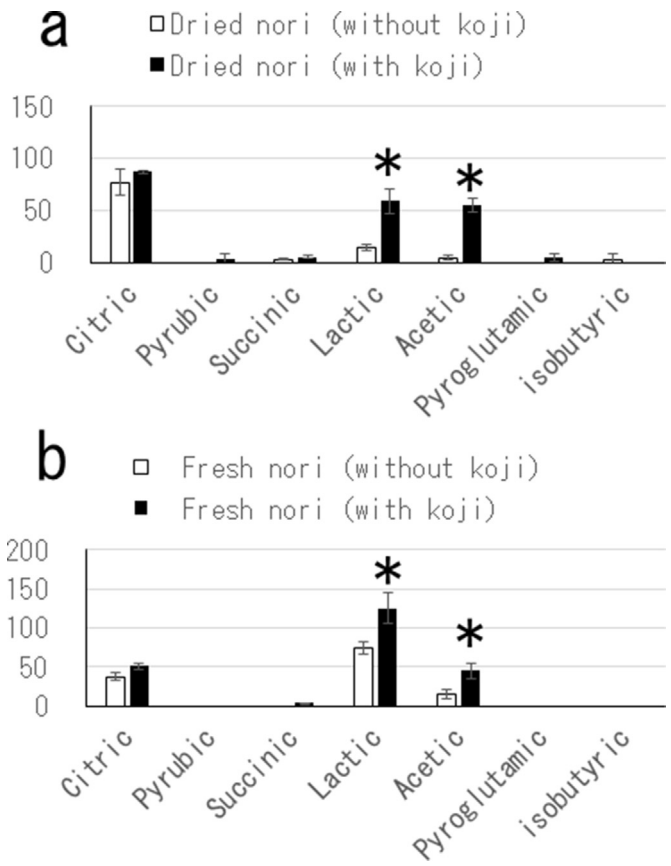

FIG. 7. Comparison of organic acids contained in nori sauces (six months-old) prepared with or without addition of nori koji using dried nori (a) and fresh nori (b). Data are shown as mean (bar: SD) of triplicate tests. Asterisks show significant difference ($P < 0.05$) against the control sample prepared without koji.

mold culture. Third point is the moisture condition. The moisture condition of nori should be determined considering the water activity value. For preparing rice koji, the rice is added water at a 30–35% (w/w) moisture level to make a water activity value of approximately 0.96–0.98 (3,16,17). The present study clarified that nori will have suitable water activity values for koji preparation in the range of 0.954 and 0.962 at a moisture level of 50–60% (Fig. 1). Solid state culture of nori was performed at a 50% moisture level in the present study based on a sense feeling from finger touch by a veteran koji master who has been working over 40 years for a soy sauce manufacturer. It was interesting observation that the koji master's decision based on his finger touch feeling coincided with the suitable water activity value, but not the moisture value, for koji preparation. Product temperature was controlled to avoid excess of 45 °C because a higher temperature will increase the growth risk of bacilli. Final product of the cultured nori koji formed a mass aggregation, which was formed by hyphal growth of the koji mold similar to traditional soy sauce koji and rice koji.

The present study used a mixture of three types of *A. oryzae* products as a seed culture because there was no information on which kind of mold strain is most suitable for solid state culture on nori. Based on an information given by the manufacturer, these commercial *A. oryzae* products are mixtures of single or multiple strains having different characteristics, respectively, and the seed culture mixture used in the present study contained five kinds of strains in total. These five kinds of mold strains were banded to one group of OUT-denovo 8, while the dominant mold contained in the nori koji were also grouped to the OUT-denovo 8 (Table 2). The OUT-denovo 8 was a phylogenic group including *A. oryzae* and *A. flavus*. Appearance of mold colonies formed on agar plates prepared for counting microbial numbers were also similar between the seed culture mixture and the nori koji, suggesting some of the seed culture strains (*A. oryzae*) had made successful growth in the nori koji. The dominant mold was not characterized at a strain level in the present study. As for the bacterial composition, the seed culture mixture contained negligible number of viable bacteria (Table 1), while MiSeq sequencing analysis based on 39,817 reads clarified that the nori koji contained seven dominant bacterial groups and had a small diversity. The dominant bacteria in the nori koji were all the Bacillales group such as *S. gallinarum*, *B. amyloliquefaciens*, *S. epidermidis, S. warneri* and *B. altitudinis* (Table 2). There is the potential of food risks from contaminant bacterial elements, however, pathogenic risk of these species for human beings to our knowledge is not known. Dominancy of *Bacillus* and *Staphylococcus* group bacteria is commonly observed also in culture mashes of soy (18,19) and fish sauces (20). Further technical improvements to decrease the viable bacterial number contained in the nori koji is favorable for reducing the food risk from microbial elements.

Comparison of enzyme activities clarified the unique characteristics of nori koji (Tables 3–5). The nori koji showed enzyme activities to wide range of sugars such as amylase, β-1,4-mannanase, carboxymethyl cellulase, β-D-glucosidase, α-D-galactosidase, β-D-galactosidase, α-D-mannosidase, β-D-mannosidase, β-D-xylosidase, and α-L-arabinosidase. Especially, relatively higher enzyme activity in β-1,4-mannanase is regarded advantageous for degrading nori fronds. The nori koji and rice koji measured for enzyme activities were six months- and two weeks-old products, respectively, and therefore the enzyme activities shown in Tables 3–5 may be underestimated values for the cases of nori koji and rice koji.

A remarkable degrading effect was observed for nori in the sauce fermentation test with nori koji (Fig. 5). The nori sauce mash remarkably lost its viscosity when the supernatant fraction ratio marked over a 70% level and seemed suitable for recovery by squeezing treatment. It was expected that fermentation period necessary before the squeezing recovery will be shortened to 4–6 months from 1.5 years by use of nori koji, although multidimensional perspective is necessary from a viewpoint of taste element to determine the suitable recovery period. Soy sauce koji also showed a remarkable degrading effect for nori, and the rice koji showed a medium degrading effect based on unpublished observation in our laboratory while use of nori koji had advantages in that it does not contain any cereal allergens (21).

Furthermore, nori koji has advantages to increase taste elements of the nori sauce products (Figs. 6 and 7). Taste of the nori sauce products was not precisely evaluated in the present study. However, improvement of taste element was clearly estimated by increase of total nitrogen value of nori sauces by use of the nori koji: dried nori sauce 0.15 g N/100 ml (without nori koji) to 0.34 g N/100 ml (with nori koji), fresh nori sauce 0.07 g N/100 ml (without nori koji) to 0.28 g N/100 ml (with nori koji). Improvement of taste by use of the nori koji was also suggested based on a preliminary sensory evaluation by authors. On the other hand, the nori sauce products showed a higher point in sourness taste based on the evaluation by a taste sensing system, but the taste difference due to the use of nori koji was not clear (unpublished observation).

Since fresh nori contains moisture at a higher level and protein at a lower level compared to soybean, the nori sauces prepared from low quality nori in the present study contained limited quantity of total nitrogen compounds in the range of 0.09–0.34 g N/100 ml regardless of the use of nori koji (Fig. 6). This value is low when compared to soy sauce products (usually 1.5 g N/100 ml or more). Major objective of this study is to develop and characterize nori koji and to confirm the advantage of nori koji use and developing a perfect product with good taste is out of scope. However, it is not difficult to obtain a tasty nori sauce product with total nitrogen value larger than 1.5 g N/100 ml by using high quality nori as a raw material as reported in a previous study (2). As a conclusion, development of seaweed koji enables an effective way to prepare fermented seaweed sauce without contamination by cereal allergens.

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