Food Sci. Biotechnol. Vol. 16, No. 3, pp. 355 ~ 360 (2007)

Food Science and Biotechnology

Characterization of Bacteria Isolated from the Traditional Steeping Process and Their Application as Starters for the Production of *Yugwa*, a Korean Traditional Snack

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Abstract During the traditional production process of *yugwa*, a Korean traditional oil-puffed snack, three bacterial isolates, 12, 37, and B1 from waxy rice slurry were characterized by using the molecular techniques, and the quality of the *yugwa* prepared by using three isolates was examined in relation to physical and sensory properties. Isolates 37 and B1 were identified as *Lactobacillus* sp. by 16S rDNA sequence analysis, while isolate 12 was as *Bacillus amyloliquefaciens* by *gyrA* sequence analysis. Waxy rice inoculated with isolate 12 demonstrated the lowest hardness and the highest peak and final viscosity. *Yugwa* prepared from isolate 12-inoculated waxy rice indicated the lowest hardness and the highest volume expansion, the key characteristics of *yugwa*. This result presents the possibility for the use of a starter in the commercial production of *yugwa* and other rice-based snacks.

Keywords: *yugwa*, waxy rice, starter culture, *Bacillus amyloliquefaciens*

Introduction

A Korean traditional oil-puffed snack, *yugwa* is made of waxy rice (*Oryza sativa*). The process of its traditional manufacturing involves water-steeping of waxy rice for 3-15 days, pulverizing, steaming, pounding, and kneading for aeration, molding, drying, deep-frying with two-stage operations, and coating with honey syrup (1). The lengthy water-steeping process is one of the most essential steps for *yugwa* preparation due to its role in hydration and biochemical changes via natural fermentation of the waxy rice grains (2, 3). Particularly, this process results in the leaching of soluble components, sufficient gelatinization on subsequent heating, and changes of texture and volume expansion, the most important characteristics of rice snacks and cracker products (2, 4-6). Nevertheless, water-steeping over a long period (15 days), especially in the case of commercial production, results in difficulties in quality control including hygienic problems caused by spoilage microorganisms as well as the high cost of *yugwa* production.

In our previous study, 16 tentatively identified bacterial strains during the steeping process were isolated on the selective media, and were characterized by the carbohydrate fermentation pattern using the API 50 CHL kit (3). It was found that lactic acid bacteria (LAB), particularly *Lactobacillus* and *Leuconostoc* predominated during the entire steeping process. This report was consistent with many other reports, all suggesting that the major microorganism involved in the fermentation of most cereal-based or plant-derived foods such as *fu-fu* and *gari* (cassava), *mesu* (bamboo shoot), *ogi* (sorghum), and *togwa* (cassava, maize, sorghum, millet) is lactic acid bacteria (7-12). In order to improve the problems of variation in organoleptic quality, as well as microbial stability and detoxification in various fermented foods, the use of starter culture would be valuable as an appropriate approach for the control and optimization of the fermentation process (13-16). However, the use of starter culture has not been attempted in *yugwa* production.

In an effort to shorten the water-steeping process and improve the quality of *yugwa*, bacterial isolates from the traditional steeping process were used for *yugwa* production. Three representative bacterial isolates were selected as starters among sixteen isolates considering steeping period (3, 6, and 9 days) and amylolytic enzyme activity. Three isolated strains from the traditional steeping process were characterized for utilization as a starter culture, and the quality of *yugwa* prepared by use of the starter culture was compared to that prepared by use of the traditional steeping process.

Materials and Methods

Materials and bacterial starters Bacterial isolates to be inoculated into steeping medium containing milled waxy rice (*Shinsunchalbyeo* cultivar) were chosen as starters for *yugwa* preparation. Bacterial isolates were obtained from steeping slurry at different steeping days as previously reported (3). *Lactobacillus amylophilus* KFRI 00238 (LA) and *Bacillus amyloliquefaciens* IMSNU 11009 (BA) as commercial strains were obtained from the Korea Food Research Institute (Korea) and Seoul National University (Korea).

Single colonies of isolates 12, 37, and B1 were taken from a MRS agar plate, inoculated into MRS broth and grown in a 30°C incubator for 48 hr. After taking single colony and liquid-culturing, bacterial starters resulting in suspension of isolates 12, 37, and B1 were inoculated into

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Received July 12, 2006; accepted August 13, 2006

steeping medium with 0.1%(w/v) (final bacterial count ca. 1.0×10^6 CFU/mL). Commercial bacterial strains were inoculated with 0.01%(w/v) (final bacterial count 1.0×10^6 CFU/mL).

Enzyme assays Amylolytic enzyme activities of isolated strains were assayed as described by Yamamoto *et al.* (17) with a slight modification (3). In the α-amylase assay, 1.0 mL of sample and 2.0 mL of 0.5% potato starch solution (pH 5.9) were prepared and incubated for 10 min at 40°C. A 0.3 mL aliquot of the reaction mixture was then combined with 0.1 mL of 0.13% iodine in 0.1 N hydrochloric acid. After the addition of 10 mL of distilled water, the color intensity was measured with a spectrophotometer at 660 nm. When the blue color intensity decreases by half in 10 min under the specified condition, the activity is defined as one unit activity of α-amylase. To assay β-amylase activity, 1.0 mL of sample and 2.0 mL of 0.5% potato starch solution (pH 5.9) were incubated as described in α-amylase assay. Then, the amount of reducing sugar produced was estimated by AOAC method (18) with glucose as a standard. One unit of β-amylase was defined as the enzyme amount that produces reducing sugar equivalent to 10 μM glucose under the assay conditions.

16S rDNA sequence analysis Genomic DNA in isolates 12, 37, and B1 was extracted by using a slight modification of the guanidine thiocyanate-phenol-chloroform method (19). A fragment of the 16S rDNA gene was amplified by polymerase chain reaction (PCR) performed in the GenAmp™ PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The PCR product was purified using a Wizard PCR prep (Promega Corp., Madison, WI, USA) subsequent to DNA gel electrophoresis. The 16S rDNA primers 27f (5' AGAGTTTGATCMTGGCTCAG 3') and 1522r (5' AAGGAGGTGWTCCARCC 3') were used for amplification of the 16S rDNA and sequencing of the PCR product. Sequencing was performed with an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems) and % similarity was analyzed by comparing various bacterial strains. The phylogenetic tree was constructed by the neighbor-joining method (20).

***GyrA* sequence analysis** The *gyrA* was amplified by PCR using primers p-gyrA-f (5' CAGTCAGGAAATGC GTACGTCCTT 3') and p-gyrA-r (5' CAAGGTAATGCT CCAGGCATTGCT 3'). The PCR product was purified using a Wizard PCR prep (Promega Corp.) after DNA gel electrophoresis. Sequencing was performed with an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems) and % similarity was analyzed by comparing various bacterial strains.

Preparation of *yugwa* Milled waxy rice was steeped for 15 days in water at 25°C for the control and for 24 hr in water at 25°C inoculated by bacterial starters, isolated strains 12, 37, and B1 respectively. Next, the steeped waxy rice was washed, drained, and crushed by rollers (KyungChang Machine Works, Daegu, Korea) into fine powder. After steaming at 100°C for 30 min, the resulting powder was kneaded at 250 rpm for 5 min using a screw kneader (KyungChang Machine Works). The kneaded dough was pressed into a 5 mm thick sheet by means of a rolling machine and dried by hot air at 60-65°C for 2 hr. With a cutting machine (KyungChang Machine Works), the dried sheet was divided into small pieces, with an average dimension of 30×8.5×3.0 mm and these segments were then dried again by hot air at 60-65°C for 2 hr. Following that, the pieces (called *bandegi*) were fried twice consecutively using two-stage deep frying. The frying time and temperature were 1.5 min at 100±5°C in the first stage and 1.5 min at 165±5°C in the second stage. These fried *yugwa* were used for physical and sensory tests without coating with honey or roasted cereal grits on the surface.

Physicochemical, microbial, and sensory tests The titratable acidity and reducing sugar content in the steeping medium were measured by the AOAC method (18). The titratable acidity was expressed as a percentage of lactic acid [(amount of 0.1 N NaOH/weight of sample titrated) ×0.009×100]. Total bacterial count on each strain was determined by counting colony forming unit per mL.

The hardness of steeped waxy rice and *yugwa* were measured using a Texture analyzer (TA-X2; Stable Micro Systems Ltd., Godalming, UK) and the results were expressed as maximum force (*g*) from texturegram. The steeped waxy rice was washed, drained, and crushed by mill (GM-307; Gold Mill, Daegu, Korea) for 1 min, and then the diameter and specific surface of the steeped waxy rice flour were analyzed using a particle size analyzer (1064L; CILAS, Orleans, France) (21). Pasting properties of the steeped waxy rice flour (40 mesh) by mill (GM-307; Gold Mill) were determined by a RVA (Rapid Visco Analyzer-4D; Newport Scientific Pty. Ltd., Warriewood, Australia). Peak viscosity and final viscosity were generated from a RVA viscogram and expressed as Rapid Visco Unit (RVU) (22).

The volume expansion of *yugwa* was evaluated by means of computerized image analysis. A computer vision system, consisting of a 0.66 in. one-chip color CCD camera (TMC-74; PULNiX America Inc., Sunnyvale, CA, USA) and a personal computer equipped with a color frame grabber (Oculus-TCX; Coreco Inc., St. Laurent, Quebec, Canada), was used to measure the dimensions of the *yugwa*. A color image was acquired through a lens system with an 11- to 110-mm zoom lens and a close-up lens of diopter +2. The sensory properties of *yugwa* were evaluated by panelists trained over a period of 2 weeks using 15-unstructured line scales. Hardness and expansion among various sensory attributes were evaluated and presented in this study.

Statistical analysis The analysis of variance and Duncan's multiple range test were performed to indicate the differences among treatments (SAS Institute Inc., Cary, NC, USA).

Results and Discussion

Characterization of bacterial isolates Sixteen bacterial isolates from the traditional steeping process were divided into 4 groups on the basis of steeping period, which were 3, 6, 9, and 12 days as previously reported (3). They were

characterized and identified by carbohydrate fermentation pattern. The representative strain from each group was selected considering α- or β-amylase activity as follows; isolate 12 with high β-amylase activity at 3 steeping days, isolate 37 with high α-amylase activity at 6 steeping days, and isolate B1 with high α- and β-amylase activities at 9 steeping days (Fig. 1).

The 16S rDNA sequences of 3 representative isolates, 12 (722 bp), 37 (697 bp), and B1 (697 bp), and the *gyrA* sequence of isolate 12 (938 bp) were determined. The phylogenetic tree was constructed from evolutionary distances by the neighbor-joining method as shown in Fig. 2. Isolates 37 and B1 were closely related to *Lactobacillus plantarum* JCM 1149T (D 79210) and *Lactobacillus pentosus* JCM 1588T (D 79211) phylogenetically, and they demonstrated high 16S rDNA sequence homology values (100%). On the other hand, isolate 12 was closely related to *B. amyloliquefaciens* ATCC 23350T (X 60605) phylogenetically, and it indicated high 16S rDNA sequence homology values (99.9%). Additionally, *gyrA* sequence analysis was performed for isolate 12, which presented the lowest hardness and the highest volume expansion, and it also indicated 99.9% *gyrA* sequence homology with *B. amyloliquefaciens* DJ-5. From the 16S rDNA and *gyrA* sequence analysis results, isolate 12 was considered as *B. amyloliquefaciens* and isolates 37 and B1 were considered as *Lactobacillus* sp.

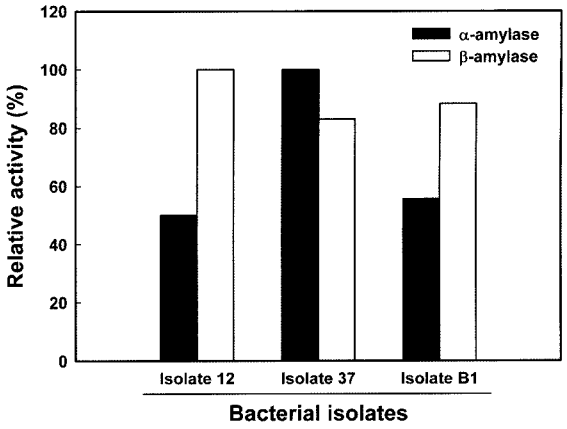

Fig. 1. Relative amylolytic enzyme activities of isolated strains cultured in tryptic soy broth.

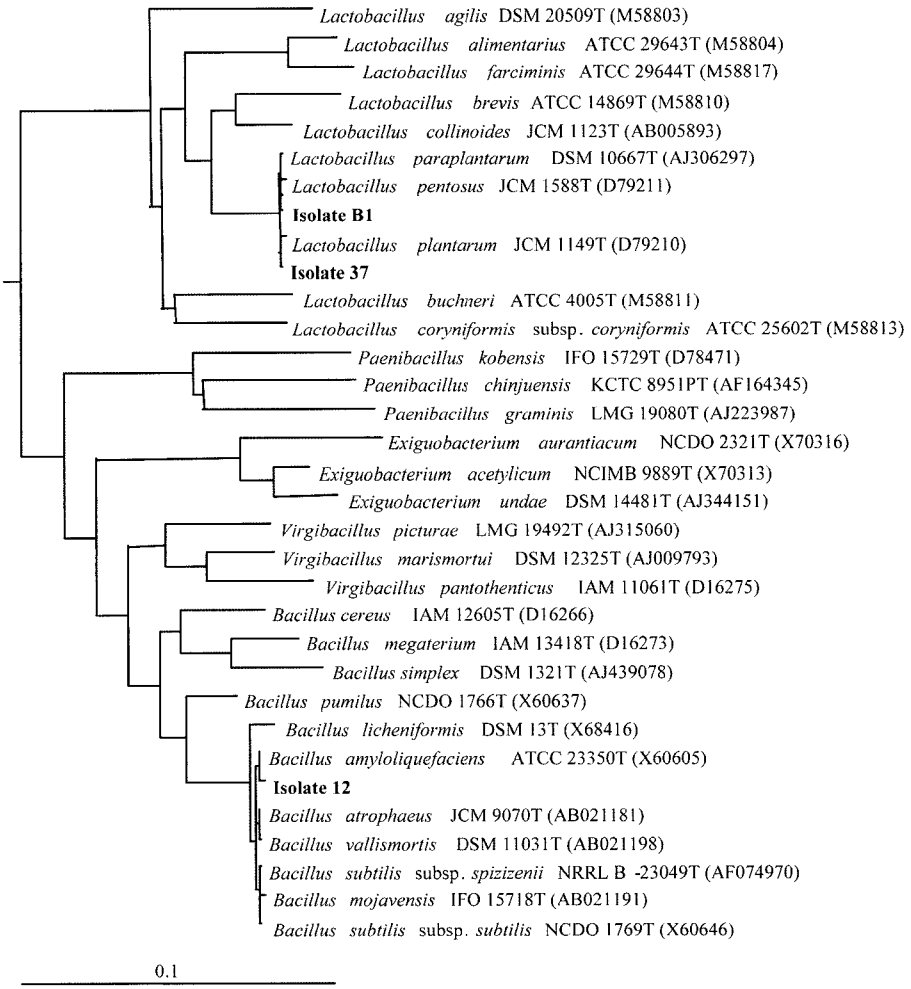

Fig. 2. Phylogenetic tree based on the 16S rDNA sequence. The tree was inferred by the neighbor-joining method. Scale bar indicates 0.1 nucleotide substitution per nucleotide position. T, type strain.

The phylogenetic relatedness of bacteria is mainly determined by comparative sequence analysis of 16S ribosomal ribonucleic acid according to Woese's report (23). Isolates 37 and B1 were identified as *Lactobacillus* sp. since they were more closely related to *L. plantarum* and *L. pentosus* by 16S rDNA sequence analysis. Isolates 37 and B1 demonstrated 100% similarity with *L. plantarum* and *L. pentosus*. These results were similar to those of the carbohydrate fermentation pattern showing that isolates 37 and B1 were identified as *L. plantarum* with 99.0 and 60.0% similarity, respectively, by API 50 CHL results.

There have been many studies reported on the dominance and beneficial effects of LAB in the fermentation process of starchy food products (7-10, 12). LAB, including *Lactobacillus*, plays an essential role in food technology as it utilizes fermentable carbohydrates as an energy source to degrade into lactate (homofermentatives) or other products such as acetate, ethanol, carbon dioxide, formate, and succinate (heterofermentatives). LAB can also improve the aroma and texture of food and inhibit the growth of spoilage bacteria (24). These advantages can be applied to improve the quality of *yugwa*.

The 16S rDNA sequence analysis is currently very efficient at identifying the organism by genus and species. Nevertheless, it often shows limitation for the discrimination of closely related taxa (25, 26). In order to discriminate *Bacillus subtilis* and *Bacillus cereus* groups, not only the 16S rDNA region but also *gyrA* genes are necessary as reported by Chun and Bae (27) and Chen and Tsen (28). In the present study, isolate 12, which is tentatively identified as *Lactobacillus* by carbohydrate fermentation pattern result turned out as *Bacillus* sp. from 16S rDNA and *gyrA* gene analysis (99.9 and 99.9% similarity with *B. amyloliquefaciens* ATCC 23350T and *B. amyloliquefaciens* DJ-5, respectively). Genus *Bacillus* and *Lactobacillus* are common fermenting bacteria and the dominant enzyme-producing microorganisms in applied and industrial microbiology (29, 30).

Chemical and microbial properties of steeping medium

The changes in titratable acidity, reducing sugar, and total microbial counts during the steeping process are shown in Table 1. The titratable acidity, expressed as a percentage of lactic acid, of the steeping medium treated with three isolates, 12, 37, B1 and two commercial strains, LA and BA, increased from 0.01 to 0.48-0.91% in parallel with an increase in steeping time from 0 to 48 hr. After 48 hr of steeping, titratable acidity of the steeping medium treated with or without 5 starters did not show any significant differences.

The concentration of reducing sugar of the isolates 12, 37, and LA groups increased rapidly from 0.31-0.42 to 1.44-1.82 mg/mL until 8 hr of steeping, and thereafter decreased gradually until 48 hr of steeping. The reducing sugar concentrations of the control, isolate B1 and BA groups increased until 8 hr of steeping, and thereafter decreased until 24 hr of steeping; however, at 48 hr of steeping, the concentration of reducing sugar tended to increase slightly. These differences in reducing sugar concentration among 6 groups were not statistically significant.

Total bacterial count in steeping medium increased until 24 hr of steeping, thereafter remaining at a similar level until 48 hr of steeping. Statistical differences in total bacterial count among all groups were not shown to be significant except for the BA group at 24 hr of steeping time.

L. plantarum is a type of amylolytic LAB that is expected to increase the availability of energy source, to contribute to rapid pH decrease, and to impart favorable rheological properties (31). Although no significant differences were found in titratable acidity among the groups, isolates 37, B1, and LA each identified as LAB, showed only slightly higher values than others due to an increase in organic acid production from LAB dominated in the steeping medium. Reducing sugar in the steeping medium also did not display any significant differences, but it tended to increase until early 8 hr of steeping time had passed and then decrease gradually. This phenomenon is supposed to be a result of the action of amylolytic enzyme secreted from *Lactobacillus* or *Bacillus* at the early stage of steeping. After 8 hr of steeping, reducing sugars degraded from starch were likely to be reused by

Table 1. Changes in titratable acidity, reducing sugar, and total bacterial count by treatment of different bacterial starters in the steeping medium[1)]

Treatment	Titratable acidity[2,3)] (%)			Reducing sugar[4)] (mg/mL)			Total bacterial count (log CFU/mL)		
	8 hr	24 hr	48 hr	8 hr	24 hr	48 hr	8 hr	24 hr	48 hr
Control[5)]	0.06^{NS}	0.26^{bc}	0.65^{ab}	1.51^{NS}	0.91^{NS}	1.40^{NS}	7.08^{NS}	8.46^{b}	9.18^{NS}
Isolate 12	0.06	0.26^{bc}	0.67^{ab}	1.82	1.50	1.09	7.08	8.66^{b}	9.34
Isolate 37	0.06	0.57^{a}	0.90^{a}	1.44	1.20	0.96	7.77	9.11^{b}	9.11
Isolate B1	0.06	0.38^{b}	0.80^{ab}	1.57	1.17	1.41	7.00	8.57^{b}	9.40
LA	0.06	0.27^{bc}	0.91^{a}	1.61	1.29	0.93	5.83	8.64^{b}	8.53
BA	0.06	0.23^{c}	0.48^{b}	2.01	0.98	1.38	7.85	9.57^{a}	9.36

[1)]The same letter in the same column indicates no significant differences ($p<0.05$). NS, not significantly different among 6 groups ($p<0.05$).
[2)]A percentage of lactic acid [(mL of 0.1 N NaOH/weight of sample titrated)×0.009×100].
[3)]Titratable acidity (%) at the beginning was 0.01 for all groups.
[4)]The concentration of reducing sugar (mg/mL) at the beginning was 0.31 (control), 0.42 (isolate 12), 0.31 (isolate 37), 0.33 (isolate B1), 0.37 (LA), and 0.38 (BA), respectively.
[5)]Steeping medium without treatment of starter was used as a control.

these strains. The control group without starter inoculum showed the same tendency as the other 5 starter groups, since indigenous enzymes in waxy rice could degrade carbohydrates and also natural fermentation could occur partially.

Physical properties of steeped waxy rice The hardness, diameter, specific surface, and pasting properties as physical characteristics of steeped waxy rice are shown in Table 2. The hardnesses of the isolate 12 and B1 groups were 2,463 and 2,480 g/cm^2, respectively, which were significantly lower than those of the control. Generally, isolates 12 and 37, as well as the B1 groups indicated lower hardness compared to that of the LA and BA groups. The isolate 12 group presented the highest value in the peak and final viscosity as pasting properties followed by isolate 37, and the B1 and LA groups.

The isolate 12 group displaying significantly low hardness also indicated the highest values in peak and final viscosity as presented in Table 2. This result could be explained as an action of the proteolytic enzymes, because isolate 12 was identified with *Bacillus,* which is known to be a major source of proteolytic enzymes among bacterial strains (32-34). These enzymes may degrade the protein matrix that holds starch granules together, thus facilitates their swelling and consequently increases hot paste viscosity (35). The reason that the BA group showed a different tendency with the isolate 12 would be considered as a result of the difference between subspecies.

Physical and sensory properties of *yugwa* The hardness and volume expansion as physical and sensory properties of *yugwa,* the final product comprised of waxy rice steeped in steeping medium treated with isolated starters, are shown in Table 3. The degree of hardness of the *yugwa* did not indicate any significant differences among all groups, with the isolate 12 group presenting the lowest value in hardness. All groups indicated significantly different volume expansion. In particular, the isolate 12 group showed the greatest volume expansion. Hardness and volume expansion, which are the main textural properties of *yugwa*, were selected to aid in the investigation of sensory characteristics. The isolate 12 group demonstrated the lowest hardness and the highest volume expansion followed by the isolate 37 group.

Chinnaswamy and Hanna (36) reported that hardness, an important textural parameter in snacks, is closely related to volume expansion and even distribution of air cells. Hardness in *yugwa* tended to decrease as the number of steeping days increased (1, 37). *Yugwa* prepared from isolate 12-inoculated waxy rice (24 hr steeping) demonstrated the lowest hardness and the highest volume expansion in regards to physical and sensory properties. Conversely, *yugwa* made by the traditional method (15 day-steeping of waxy rice) showed higher hardness and lower volume expansion in both properties than *yugwa* prepared from isolate 12-inoculated waxy rice, despite its lengthy steeping period. This result suggested that steeping period did not affect hardness and volume expansion in *yugwa* prepared from isolate 12-inoculated waxy rice. Finally, desirable physical properties of isolate 12-inoculated waxy rice as shown above had an effect on the physical and sensory properties of the final product, *yugwa,* prepared from isolate 12-inoculated waxy rice.

Table 2. Physical properties of steeped waxy rice treated with different bacterial starters[1)]

Treatment	Hardness (g/cm^2)	Diameter (μm)	Specific surface (cm^2/g)	Pasting property	
				Peak viscosity (RVU)	Final viscosity (RVU)
Control[2)]	2,811±429.0^a	50±2.8^a	7,947±353^b	230.6^f	98.5^c
Isolate 12	2,463±365.5^b	46±1.8^{ab}	8,022±92^b	283.0^a	122.2^a
Isolate 37	2,620±378.4^{ab}	48±5.2^{ab}	8,046±540^b	268.6^b	106.8^b
Isolate B1	2,480±426.0^b	43±2.7^b	8,490±189^{ab}	266.2^c	107.5^b
LA	2,756±390.6^a	45±0.9^{ab}	8,387±122^{ab}	258.0^d	106.0^b
BA	2,801±408.7^a	44±1.0^b	8,671±33^a	234.0^e	99.8^c

[1)]The same letter in the same column indicates no significant differences (p<0.05).
[2)]Waxy rice steeped for 24 hr without treatment of starter was used as a control.

Table 3. Physical and sensory properties of *yugwa* made from steeped waxy rice treated with different bacterial starters[1)]

Treatment	Physical property[2)]		Sensory property[3)]	
	Hardness (g/cm^2)	Volume expansion (mm^3)	Hardness	Volume expansion
Control[4)]	654±138.3^{NS}	25,016^c	9.7^a	8.3^c
Isolate 12	624±122.9	31,784^a	6.4^c	11.6^a
Isolate 37	721±162.4	27,729^b	7.3^{bc}	11.1^{ab}
Isolate B1	703±212.6	23,966^c	8.8^{ab}	9.7^{bc}

[1)]The same letter in the same column indicates no significant differences (p<0.05). NS, not significantly different among 4 groups (p<0.05).
[2)]n=20
[3)]Evaluated by 10 panelists trained over a period of 2 weeks using 15-unstructured line scales.
[4)]*Yugwa* made from waxy rice steeped for 15 days was used as a control.

In conclusion, *yugwa* made by using starters, especially isolate 12 isolated from the steeping medium, showed enhanced physical and sensory qualities than *yugwa* made by using the traditional lengthy steeping method. It was found that the addition of starter culture results in not only a desirable quality of the *yugwa*, such as low hardness and high volume expansion, but also in reduction in necessary steeping period. Among the 3 starters used for *yugwa* preparation, isolate 12, identified as *B. amyloliquefaciens* was the most promising, and these results suggest the possibility for the use of starter culture in commercial *yugwa* production.

Acknowledgments

This research was supported by a research grant from the Agriculture R&D Promotion Center, under the Ministry of Agriculture and Forestry, Korea. We also thank Kyue Heun Kim, President of ShinKung Tradition Food Co., for providing the *yugwa* production system.

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