Mycobiology

Research Article

Identification of Wild Yeast Strains and Analysis of Their β-Glucan and Glutathione Levels for Use in *Makgeolli* Brewing

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Abstract *Makgeolli*, also known as Takju, is a non-filtered traditional Korean alcoholic beverage that contains various floating matter, including yeast cells, which contributes to its high physiological functionality. In the present study, we assessed the levels of β-glucan and glutathione in various yeast strains isolated from traditional Korean *Nuruk* and selected a β-glucan- and glutathione-rich yeast strain to add value to *Makgeolli* by enhancing its physiological functionality through increased levels of these compounds. Yeast β-glucan levels ranged from 6.26% to 32.69% (dry basis) and were strongly species-dependent. Dried *Saccharomyces cerevisiae* isolated from *Nuruk* contained 25.53 μg/mg glutathione, 0.70 μg/mg oxidized glutathione, and 11.69 μg/g and 47.85 μg/g spermidine and L-ornithine monohydrochloride, respectively. To produce functional *Makgeolli*, a β-glucan- and glutathione-rich yeast strain was selected in a screening analysis. *Makgeolli* fermented with the selected yeast strain contained higher β-glucan and glutathione levels than commercial *Makgeolli*. Using the selected yeast strain to produce *Makgeolli* with high β-glucan and glutathione content may enable the production of functional *Makgeolli*.

Keywords β-Glucan, Glutathione, *Makgeolli*, Yeast

Glucan is a component of polysaccharides that consists of glucose molecules chemically bound by glycosidic bonds. They are classified as α-glucans and β-glucans according to the bonds connecting the monosaccharides (α- and β-glycosidic, respectively). β-Glucan is known to exert excellent immune-modulatory effects by activating the non-specific immune response of macrophages and promoting cell proliferation. β-Glucan also has various other functional effects, such as a cholesterol lowering effect [1], postprandial hypoglycemic effect [2], and anti-cancer and antioxidant effects [3-5]. Therefore, it has been used in cosmetics and as an anti-cancer drug and food additive. Recently it has gained focused as five functional polysaccharides with hyaluronic acid, chitosan and so on [6, 7]. β-Glucans are divided into β-1,3; β-1,4; and β-1,6 glucans based on the glycosidic bonds, and their functions have been reported to vary based on these structural differences. The most important are the β-1,3 glucans, which have immune regulation functions and anti-cancer effects. β-Glucans are synthesized by both prokaryotic and eukaryotic organisms. Some are secreted from cells, but most exist as cell wall components in microorganisms [8]. In the yeast *Saccharomyces cerevisiae*, the cell wall contains β-(1,3)-D-glucan, β-(1,6)-D-glucan, chitin, and mannoproteins. Yeast β-glucans have the basic structure of (1 → 3),(1 → 6)-β-D-glucan. Due to the structural and molecular weight differences between yeast β-glucan and the β-glucans from plants, yeast β-glucan is a well-known immunomodulator that has a strong positive effect on human and animal immune systems [8, 9].

Glutathione is a tripeptide of three amino acids: glutamic acid, cysteine, and glycine, and it is the most abundant non-protein thiol compound in almost all eukaryotic cells. It has important biological roles, including protection against oxidative stress, control of redox potential, detoxification of toxins, protein folding, and organic sulfur storage and transport [10], as well as immune reinforcement effects [11, 12]. Because of these biological effects, glutathione is

Mycobiology 2014 December, **42**(4): 361-367
http://dx.doi.org/10.5941/MYCO.2014.42.4.361
pISSN 1229-8093 • eISSN 2092-9323

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Received September 24, 2014
Revised October 8, 2014
Accepted October 8, 2014

used in the food industry as an additive to improve the nutritional value of products. Although glutathione is widely distributed in nature, many researchers have attempted to mass-produce it using yeast [13, 14]. Thus, glutathione from yeast and β-glucan are regarded as good functional foods for the prevention of diseases, although they are still relatively expensive compared to other functional materials.

Makgeolli is a traditional Korean alcoholic beverage made by simultaneous two-step fermentation using grains and yeast. After fermentation, it becomes white and cloudy in appearance due to the presence of unfiltered rice material, and it has evenly blended sweet, acidic, and bitter flavors. *Makgeolli* has higher health functionality than *Yakju*, a Korean traditional alcoholic beverage similar to Japanese Sake that is filtered after fermentation [15-17]. These suspended solids in *Makgeolli* include microorganisms, such as yeast and lactic acid bacteria, as well as starches, oligosaccharides, organic acids, peptides, and various other biologically useful substances [15]. In a study aimed at increasing *Makgeolli* functionality, Kang et al. reported that *Makgeolli* peptides showed angiotensin I-converting enzyme (ACE) inhibitory activity [18]. In the present study, we investigated the levels of β-glucan and glutathione in yeast strains isolated from *Nuruk*, a Korean traditional fermentation starter that is produced by the natural proliferation of fungi on crushed grain and is used in the fermentation of *Makgeolli*. We selected a β-glucan- and glutathione-rich yeast to add value to *Makgeolli* by increasing the levels of these physiologically functional compounds. The results of this research may be used to increase the functionality of yeast-containing products, such as bakery products or other foods containing whole yeast cells.

MATERIALS AND METHODS

Isolation and identification of wild yeast strains. Wild yeast strains were isolated from Korean traditional *Nuruk* collected from several provinces in South Korea. The microorganisms were sent to Macrogen, Inc. (Seoul, Korea) for identification via PCR using a PTC-225 Peltier Thermal Cycler (MJ Research, Reno, NV, USA) and primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The 18S rRNA genes were sequenced using these same primers and the ABI PRISM Big Dye Terminator Cycle Sequencing Kit in an ABI PRISM 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA). The sequences obtained were used in a BLAST search of GenBank (www.ncbi.mlm.nih.gov/BLAST), and then edited using BioEdit software [19].

Quantitative analysis of the β-glucan content in yeast cells. Yeast strains were cultured in potato dextrose agar (PDA) broth medium at 25°C for 48 hr. Cultured cells were harvested by centrifugation and lyophilized. β-Glucan content in dried yeast cells was quantified using a Megazyme kit (Megazyme Inc., Wicklow, Ireland).

Intracellular glutathione content analysis of yeast cells. Yeast strains were cultured in PDA medium at 25°C for 72 hr, and then freeze-dried. The dried yeast (20 mg) was transferred to a bottle containing beads, and then 1 mL of 50% methanol was added. The yeast cells were homogenized using an automated homogenizer (Precellys 24 lysis/homogenizer; Bertin Technologies, Montigny-le-Bretonneux, France) three times at 6,500 rpm for 10 sec each. After the cell pellet was extracted for 2 hr at room temperature, the supernatant of the final sample was obtained by centrifugation (10,000 ×g, 5 min, 4°C) and was analyzed by ultra-high performance liquid chromatograph (UHPLC).

Glutathione and its metabolites were analyzed with a UPLC system (Agilent 1290 Infinity; Agilent, Santa Clara, CA, USA) interfaced with an accurate-mass quadrupole time-of-flight instrument (Agilent 6520 with Jet Stream Technology). The QTOF-MS instrument was operated with an electrospray ion source (Agilent Jet Stream Technology) in positive mode. It was set at 350°C with drying gas at a flow rate of 12 L/mL, a nebulizer pressure of 45 psi, Vcap of 2,000 V, skimmer voltage of 65 V, and fragmentor voltage of 170 V. The mobile phase was in gradient mode with 0.1% formic acid in distilled water (A) and 0.1% formic acid in acetonitrile (B), with a flow rate of 0.9 mL/min. The analysis was performed with an ACE Excel C18 column (3 μm, 4.6 × 150 mm) at a column temperature of 30°C and an injection volume of 0.3 μL.

***Makgeolli* production using selected yeast.** The white rice (Dongsongnonghyup; Cheorwon O-Dae Rice, Cheorwon, Korea), Cozy (saccharification: 60 sp.; Joeungoksik, Gyeonggi-do, Korea), and purified enzyme (saccharification: 300 sp.; Hangukhyoso, Hwaseong, Korea) used for *Makgeolli* production were purchased and used as is. The yeast strains (*S. cerevisiae*) used in this study were isolated from preserved *Nuruk* collected from various regions of Korea [19]. *Lactobacillus* (*Lactobacillus plantarum JS1*) strains were isolated and purified from commercial *Makgeolli*. The first soak was fermented for 2 days at 25°C at a water ratio of 200% with Cozy, lactic acid bacteria, and yeast in a bottle. In the second soak, the same water ratio was used, and the Cozy and ground rice steeped in water were added and then incubated for 5 days at 25°C. Cozy and purified yeast were added at 35 sp./g of rice. Yeast and lactic acid bacteria were added at 0.06% and 0.012% of the *Makgeolli* mash, respectively.

Quantitative analysis of β-glucan content in *Makgeolli*. To obtain a pellet of the *Makgeolli*, after shaking to mix, a 150-mL aliquot was obtained and centrifuged at 3,000 rpm for 10 min. The β-glucan content of the collected pellets was analyzed by using the Megazyme β-glucan assay kit. The water content of the pellets was measured with an infrared moisture analyzer (MAC 50/NH; RADWAG, Radom, Poland). The final β-glucan content in *Makgeolli* is presented as a dry basis.

Statistical analysis. The raw data were analyzed to assess the significance of differences between samples using analysis of variance (ANOVA), and means were compared using Duncan's multiple range test (honestly significant differences). All statistical analyses were conducted with SAS 9.2 statistical software (SAS Institute Inc., Cary, NC, USA). *p*-values less than 0.05 were considered significant. Glutathione data are presented as box-and-whisker plots.

RESULTS AND DISCUSSION

Identification of wild yeast strains isolated from *Nuruk* and β-glucan content analysis. A list of the wild yeast strains isolated form Korean traditional *Nuruk* and their identification information are presented in Table 1. The isolated yeast strains identified by 18S rRNA sequencing included 30 strains of *Saccharomyces* sp., 11 strains of *Candida* sp., 21 strains of *Wickerhamomyces* sp., 1 strain of *Pichia* sp., and 1 strain of *Torulaspora* sp. Yeast β-glucans containing (1,3)-(1,6)-β bonds are typically insoluble, and they have been shown to possess a variety of pharmacological activities, including anti-cholesterolemic activity, hypoglycemic activity, acceleration of heavy metal excretion, and immune system stimulation [20-23]. In an attempt to identify strains with high glucan content, we assessed the β-glucan levels in the isolated wild yeast strains, and the results are shown in Table 1. The levels ranged from 6.26% to 32.68%. Among the 64 yeast strains tested, strain 230-9, identified as *Candida glabrata,* showed the highest β-glucan content (32.68%). The strain with the lowest β-glucan content (6.41%) was 197-12, which was identified as *Wickerhamomyces anomalus*. Among the identified *S. cerevisiae* strains, the highest β-glucan content was observed in strain 89-3-1 (25.95%), whereas the lowest content was found in strain 115-1 (7.68%). *S. cerevisiae* is the most common yeast, and it is widely used as a food and beverage starter. In addition,

Table 1. Identification and β-glucan contents analysis of wild yeast strains

No.	β-Glucan contents (% w/w, dry basis)	Strains	Similarity (%)
98-2	13.88 ± 0.14	*Saccharomyces cerevisiae*	100
94-2	17.21 ± 0.12	*S. cerevisiae*	98
91-2	15.35 ± 0.34	*S. cerevisiae*	99
90-2	19.40 ± 1.39	*S. cerevisiae*	99
89-3-1	25.95 ± 0.76	*S. cerevisiae*	99
89-1-3	22.16 ± 0.83	*S. cerevisiae*	99
64-3	13.38 ± 0.84	*S. cerevisiae*	99
300-6	6.66 ± 0.48	*Wickerhamomyces anomalus*	99
298-8	6.26 ± 1.15	*W. anomalus*	99
297-6	6.35 ± 1.24	*W. anomalus*	99
294-5	7.01 ± 1.03	*W. anomalus*	100
289-7	7.35 ± 0.88	*W. anomalus*	99
289-5	7.23 ± 1.66	*W. anomalus*	99
284-3	22.99 ± 1.15	*Candida glabrata*	100
284-16	23.94 ± 1.5	*C. glabrata*	99
284-14	24.69 ± 1.27	*C. glabrata*	98
284-11	23.97 ± 0.58	*C. glabrata*	99
282-6	18.01 ± 2.64	*S. cerevisiae*	98
282-1	16.92 ± 0.92	*S. cerevisiae*	99
272-7	17.62 ± 1.35	*S. cerevisiae*	98
271-4	18.61 ± 0.74	*S. cerevisiae*	100
271-2	20.18 ± 1.68	*S. cerevisiae*	100
270-5	15.99 ± 0.20	*S. cerevisiae*	99
270-10	22.71 ± 0.67	*S. cerevisiae*	99
268-1	22.29 ± 0.42	*S. cerevisiae*	98
263-4	9.32 ± 0.39	*Candida lusitaniae*	98
239-1	8.59 ± 1.63	*W. anomalus*	99
235-9	6.83 ± 1.05	*W. anomalus*	97
230-9	32.68 ± 2.81	*C. glabrata*	99
230-4	9.38 ± 0.48	*W. anomalus*	99
226-1	8.58 ± 0.94	*W. anomalus*	100
225-9	13.04 ± 1.87	*C. lusitaniae*	98
225-8	10.89 ± 0.29	*W. anomalus*	99
225-6	8.69 ± 0.67	*W. anomalus*	99
225-2	12.68 ± 2.19	*C. lusitaniae*	98
215-2	7.34 ± 0.83	*W. anomalus*	99
206-7	7.59 ± 2.38	*S. cerevisiae*	99

Table 1. Continued

No.	β-Glucan contents (% w/w, dry basis)	Strains	Similarity (%)
197-13	6.69 ± 1.73	*W. anomalus*	99
197-12	6.41 ± 1.59	*W. anomalus*	100
197-1	6.85 ± 1.08	*W. anomalus*	99
192-4	19.05 ± 1.23	*S. cerevisiae*	98
183-3	23.67 ± 1.19	*S. cerevisiae*	98
174-2	6.89 ± 1.07	*W. anomalus*	99
172-6	18.43 ± 0.56	*S. cerevisiae*	98
170-4	12.28 ± 0.95	*Pichia jadinii*	99
166-10	6.55 ± 1.40	*W. anomalus*	99
157-1	18.77 ± 0.31	*S. cerevisiae*	99
155-1	10.88 ± 0.96	*S. cerevisiae*	98
141-9	7.11 ± 3.29	*W. anomalus*	99
139-4	7.63 ± 0.44	*W. anomalus*	99
133-6	14.63 ± 2.09	*C. lusitaniae*	99
132-4	8.52 ± 2.91	*W. anomalus*	99
126-2	17.57 ± 1.62	*S. cerevisiae*	99
115-1	7.68 ± 0.63	*S. cerevisiae*	97
114-5	26.83 ± 1.22	*S. cerevisiae*	99
113-8	10.90 ± 0.31	*S. cerevisiae*	98
112-4	27.04 ± 3.23	*C. glabrata*	99
112-3	28.36 ± 3.34	*C. glabrata*	99
111-5	24.66 ± 0.90	*S. cerevisiae*	99
110-2	14.83 ± 0.44	*S. cerevisiae*	97
109-3	11.94 ± 0.24	*S. cerevisiae*	99
105-10	18.19 ± 2.86	*Torulaspora delbrueckii*	99
H4-2	16.91 ± 0.76	*S. cerevisiae*	99
H1-5	14.52 ± 1.03	*S. cerevisiae*	99

it is also sold as a source of β-glucan due to its nutraceutical and biological effects [20, 24, 25]. It is interesting to note that the yeast β-glucan levels were strongly dependent on the species. In the present study, the β-glucan content of the different yeast species varied, and *C. glabrata* > *S. cerevisiae* > *Candida lusitaniae* > *W. anomalus* (Fig. 1). This suggested that β-glucan content is influenced by the yeast species.

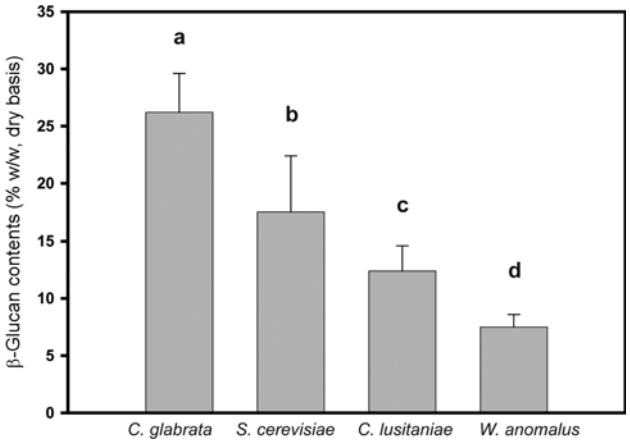

Fig. 1. β-Glucan contents of yeast strains isolated from *Nuruk*. *C. glabrata*, *Candida glabrata*; *S. cerevisiae*, *Saccharomyces cerevisiae*; *C. lusitaniae*, *Candida lusitaniae*; *W. anomalus*, *Wickerhamomyces anomalus*.

Glutathione contents of wild yeast strains. Glutathione (GSH) has various derivatives. GSH is oxidized by exposure to hydroperoxides, and redox cycling of GSH is central to the cellular response to oxidative stress. Polyamines (putrescine, spermidine, and spermine), which were analyzed in this study, are a family of molecules that are derived from ornithine through a decarboxylation process [26-32]. The amount of GSH and GSH derivatives in wild yeast cells has not yet been reported. In the present study, the glutathione and GSH derivative contents in yeast isolated from *Nuruk* were quantitatively analyzed with a UHPLC system, and their distribution was confirmed (Table 2, Fig. 2). The *S. cerevisiae* isolated from *Nuruk* contained 25.53 μg/mg GSH, 0.70 μg/mg oxidized glutathione, and 11.69 μg/g and 47.85 μg/g spermidine and L-ornithine monohydrochloride, respectively. In the present study, the glutathione content of *S. cerevisiae* ranged from 14.14 mg/g to 47.25 mg/g. For comparison, Zechner-Krpan *et al.* [23] reported GSH levels of 0.1~1% dry cell weight, Whistler *et al.* [28] reported 80~4,320 mg/L GSH, and Penninckx [30] reported high GSH concentrations of up to 10 mM in most living cells (from prokaryotes to eukaryotes). The levels of GSH and GSH derivatives in *S. cerevisiae* strains isolated from *Nuruk* are presented as box-and-whisker plots (Fig. 2). The range of GSH and GSH oxidized contents

Table 2. Glutathione and its derivatives contents in *Saccharomyces cerevisiae* isolated from *Nuruk*

Compounds	RT (min)	Exact mass [M + H]+	Actual mass [M + H]+	Mass error (ppm)	Contents
Glutathione	2.43	308.0911	308.0907	−1.298	25.53 ± 8.37[a]
Glutathione oxidized	1.83	613.1592	613.1585	−1.142	0.70 ± 0.37[a]
Spermidine	1.10	146.1652	146.1656	2.737	11.69 ± 10.31[b]
L-Ornithine monohydrochloride	1.31	169.0738	169.0741	1.774	47.85 ± 19.42[b]

Values are presented as number or mean ± SD.
[a]μg/mg, [b]μg/g in dried yeast.

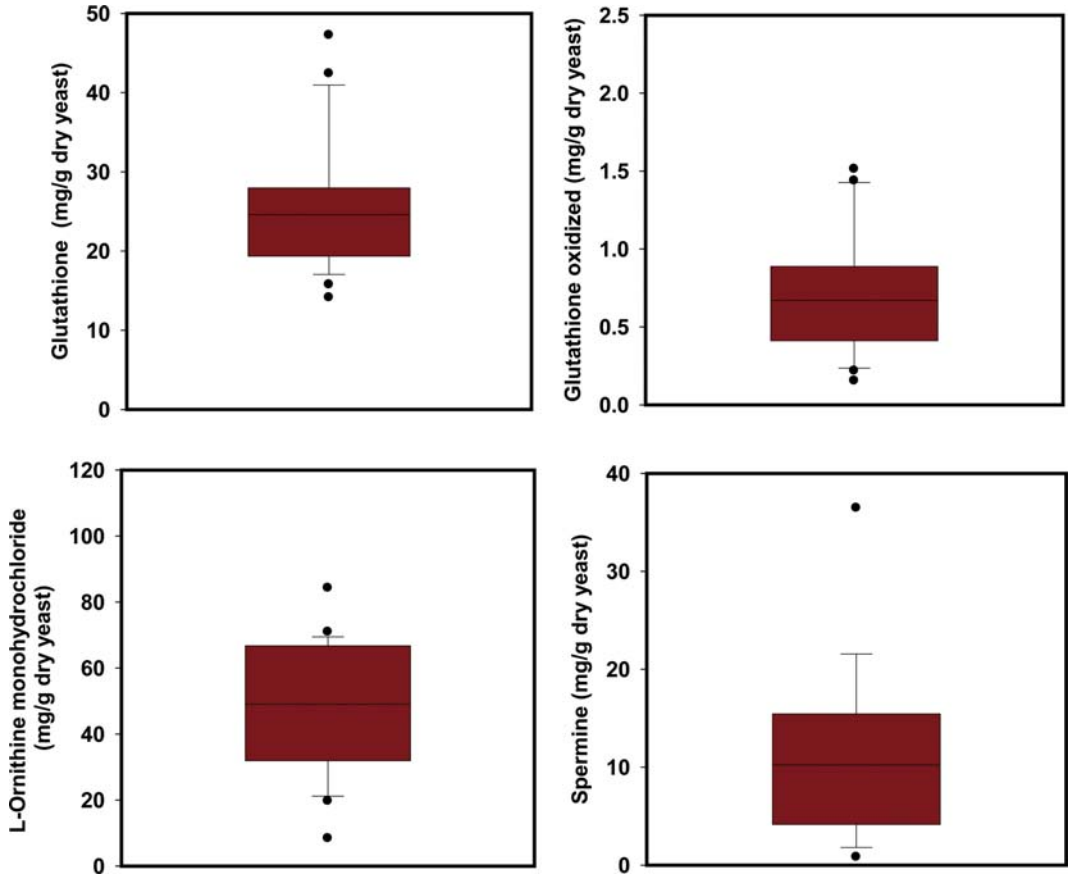

Fig. 2. Box-and-whisker plots of the levels of glutathione derivatives in *Saccharomyces cerevisiae* strains isolated from *Nuruk*.

was 30 and 1, respectively, and the interquartile ranges (IQR) were 9 and 0.4, respectively. However, the ranges for L-ornithine monohydrochloride and spermidine were approximately 70 and 35, respectively, and the IQR were 35 and 10, respectively. The distributions of GSH, oxidized GSH, and spermidine contents skewed to the right, whereas L-ornithine monohydrochloride content was symmetric.

β-Glucan- and glutathione-rich *Makgeolli* production using a selected wild yeast strain. In order to develop an improved functional *Makgeolli*, we produced *Makgeolli* (SY-1 *Makgeolli*) using the yeast strain 89-3-1, which contained high β-glucan and glutathione contents. Then, the β-glucan and glutathione contents of the SY-1 *Makgeolli* were compared to those of commercial *Makgeolli,* and the results are presented in Fig. 3. The levels of β-glucan and glutathione in the SY-1 *Makgeolli* were significantly higher than those of commercial sterilized and non-sterilized *Makgeolli*. In fact, the β-glucan level in SY-1 *Makgeolli* was six times higher than that in commercial *Makgeolli*, and the glutathione level was two times higher. It has been reported that commercial *Makgeolli* containing high levels of β-glucan showed high ACE inhibitory activity [1]. Therefore, SY-1 *Makgeolli* fermented with 89-3-1 yeast would be expected to have superior physiological functionality.

In sterilized *Makgeolli*, only β-glucan was detected, and glutathione was not. It was thought that the β-glucan was not completely destroyed in the sterilization process due to swelling and agglomeration of the glucan particles, whereas glutathione, a tripeptide, was modified or destroyed by

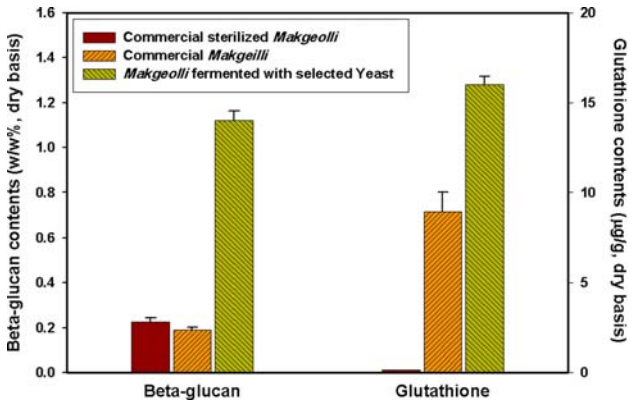

Fig. 3. β-Glucan and glutathione contents of commercial *Makgeolli* and *Makgeolli* produced with the selected high β-glucan and glutathione yeast strain.

sterilization [27]. Thus, from a functional standpoint, non-sterile *Makgeolli,* which contains higher amounts of functional substances, has higher functionality than sterilized *Makgeolli*. Based on the results presented here, production of *Makgeolli* with the selected yeast, which has high β-glucan and glutathione contents, can enhance its functionality; therefore, it may be possible to produce functional *Makgeolli*. Further studies are needed to evaluate the physiological effects of *Makgeolli* with high β-glucan and glutathione contents and to identify the underlying mechanisms of its health functions.

ACKNOWLEDGEMENT

This study was carried out with the support of "Cooperative Research Program for Agricultural Science & Technology Development (Project No. PH009070)".

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