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Feasibility of Brewing *Makgeolli* Using *Pichia anomala* Y197-13, a Non-*Saccharomyces cerevisiae*

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***Makgeolli* is a traditional rice wine favored by the general public in Korea. This study investigated the fermentation and sensory characteristics of using wild yeast strains for brewing *makgeolli*. A non-*Saccharomyces cerevisiae* strain was isolated from *nuruk* and termed Y197-13. It showed 98% similarity to *Pichia anomala* and had an optimal growth temperature of 25°C. *Makgeolli* was manufactured using *koji*, *jinju nuruk*, and improved *nuruk* as fermentation agents. Y197-13 *makgeolli* brewed with *koji* had alcohol and solids contents of 11.1% and 13.9%, respectively. Sweet sensory characteristics were attributed to residual sugars in *makgeolli* with 6% alcohol. The *makgeolli* had a fresh sour taste and carbonated taste. Volatile component analysis showed the isoamyl alcohol, phenylethyl alcohol, isoamyl acetate, and fatty acid, including ethyl oleate and ethyl linoleate, relative peak area was higher in Y197-13 *makgeolli* than in *makgeolli* with *Saccharomyces cerevisiae*. These results suggest the wild yeast, Y197-13, as a candidate for brewing *makgeolli*.**

Keywords: *Makgeolli*, wild yeast strain, *nuruk*, *Pichia anomala*

Makgeolli is produced through a process in which rice, such as non-glutinous rice, is steamed, mixed with a fermentation agent and water, and then fermented. Unlike *cheonghju* or *yakju,* which are clear liquors produced *via* filtration, *makgeolli* contains essential amino acids, proteins, sugars, and live yeasts because the fermented product is not filtered. Thus, it has unique nutritional characteristics [19]. Compared with wine, however, which is very popular because of its antioxidant components, such as resveratrol and polyphenols, few studies have been conducted on the characteristics of *makgeolli*. As a liquor with diverse nutritional and functional characteristics, *makgeolli* has drawn attention in the context of the increasing popularity of health products.

To brew *makgeolli*, *nuruk*, a naturally fermented carbohydrate containing various microorganisms, is used as a fermentation agent. *Aspergillus oryzae* and *Aspergillus flavus* have reportedly been isolated from *nuruk* [37]. Some fungi of the *Aspergillus* genus, including *A. flavus*, produce carcinogenic toxins, termed aflatoxins. The US Department of Agriculture (USDA) Agricultural Research Service (ARS) reported that nut trees and corn grew well, without aflatoxin contamination, when they are exposed to *Pichia anomala*. In a study conducted in California, the detection rate for *A. flavus* dropped by more than 97% in a pistachio tree sprayed with *P. anomala*, compared with an untreated pistachio tree. If pistachio, almond, and other nut trees are sprayed with *P. anomala*, the yeast competes with and inhibits the growth of the fungus *A. flavus*, which degrades nut nutrients and impairs the growth of the trees. *P. anomala* has also harmful effects on food production and storage [4]. As a food spoilage yeast, *P. anomala* may cause chemical changes in yogurt, bread, sugary cakes [17], and wine [28] by producing ethyl acetate [22]. However, *P. anomala* produces phenylethyl alcohol, which has a rose-like aroma in the alcoholic beverage [23], and is used for sensory quality of sugarcane wine (*cachaca*) [26]. In addition, the best balance of the ethyl acetate and higher alcohol by *P. anomala* are important for the aroma, in the grape must fermentation [22].

However, in food and beverage fermentations, the beneficial properties of *P. anomala* are related to its positive role in food preservation. *P. anomala* increases the pH of animal feeds, such as silage, by consuming lactic acid [10]. Although a few studies report that *P. anomala* causes pathogenic infection [3], it is classed at a biosafety level of 1 (BSL-1), indicating that it is safe for consumption by healthy individuals [5].

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The number of studies on *makgeolli* has gradually grown with its increase in consumption. These include studies on the functional characteristics of *makgeolli,* such as its anticancer effect [30], and the improvement of *makgeolli* quality through the addition of subsidiary materials [27]. Studies characterizing yeasts used for *makgeolli* brewing are still in their early stages, unlike studies characterizing yeasts for the Japanese alcoholic beverage sake. A few domestic brewers use their own yeasts for *makgeolli* brewing, whereas most companies use yeasts from foreign wines, bread yeasts, or yeasts produced by a few domestic companies, which produce *makgeolli* with similar sensory characteristics. This study was conducted to explore the characteristics of novel yeasts for brewing *makgeolli*.

Makgeolli is a low-alcohol beverage with an alcohol content of 6% to 8%. Thus, unlike previous studies that identified yeasts that yield a high alcohol content [11, 29], in this study, wild yeasts isolated from traditional Korean *nuruk* were characterized to identify a yeast strain that optimally produces the sensory characteristics of *makgeolli*, such as a unique carbonated taste and a sweet taste, regardless of its low alcohol production. Identifying novel yeast with new properties for *makgeolli* brewing is expected to improve *makgeolli* quality. This is highly desirable in the current *makgeolli* market, which depends on the use of additional ingredients to produce a sweeter taste, owing to the uniformity of domestic yeasts. To select yeasts for *makgeolli* brewing, approximately 1,000 yeasts isolated from approximately 300 *nuruks* stored at the Korea Food Research Institute were screened. The carbon dioxide gas, acid, and flavor production abilities of the yeasts during alcohol fermentation were analyzed and compared. Two hundred six yeasts that had an ability to produce a fruity flavor, sugar, alcohol, and acids were primarily selected. The yeasts that produced an unfavorable odor, such as a pungent odor, a soy sauce smell, and a medium smell, and film-forming yeasts during the fermentation were discarded. Among the final 10 selected wild yeast strains, 9 strains were *S. cerevisiae* and 1 strain turned out to be *P. anomala*. Among the 9 *S. cerevisiae* strains, *S. cerevisiae* Y98-5 was selected for its excellent sensory characteristics. In this study, *S. cerevisiae* Y98-5 and *P. anomala* Y197-13 were selected for *makgeolli* brewing, and the sensory and physicochemical characteristics of the *makgeolli* produced by these two strains were compared to assess the feasibility of using *P. anomala* Y197-13 for *makgeolli* brewing.

Materials and Methods

Strains and Materials

Yeasts isolated from *nuruk* were used in this study. Y98-5 was isolated from the Gongju area of Chungnam Province. Y197-13 was isolated from the Jeongpyung area of Chungbuk Province. *Koji* [saccharogenic power (sp; glucoamylase activity) 85] was purchased from Seoul Jangsoo, Inc. (Jincheon, Chungbuk, Korea). *Jinju nuruk* (JN, sp 300) was purchased from Jinjugokja industry Laboratory (Jinju, Gyeongnam, Korea), and an improved *nuruk* (IN, sp 1800) was purchased from Korea Enzyme Co., Ltd. (Hwaseong, Gyeonggi, Korea). For the non-glutinous rice, Odaechiak rice was purchased from Shinlimnonghyup (Wonju, Gangwon, Korea).

Strain Identification by PCR

Strains were cultured on potato dextrose agar (Difco, MD, USA) at 30°C for 48h. Total DNA was first extracted using the InstaGene Matrix (BIO-RAD) at 56°C for 15 min after vortex mixing and then extracted again at 99°C for 10 min. The solution was centrifuged at 12,000 rpm to separate total DNA, followed by PCR. PCR was performed using an MJ Research PTC-225 Peltier Thermal Cycler (Reno, NV, USA). The ITS1 forward primer (5'-TCCGTAGGTGAA CCTGCGG-3') and ITS4 reverse primer (5'-TCCTCCGCTTATTGA TATGC-3') were used to amplify the conserved ITS region of the yeast 18S rRNA gene [33, 35]. The PCR reaction mixture contained 3 µl of 10× reaction buffer, 1 µl of 10 mM dNTPs, 1 µl of 5× Band Doctor (Solgent, Daejeon, Korea), 1 µl of 10 pmol primer, 1 µl of template DNA (fungal genomic DNA, 25 ng/µl), 21.5 µl of ultra-pure water, and 0.2 µl of 2.5 U/ml EF-Taq polymerase (Solgent, Daejeon, Korea) in a total volume of 30 µl. The PCR consisted of 35 cycles of initial denaturation at 94°C for 5 min, denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. After the final extension at 72°C for 10 min, the PCR product was stored at 4°C. The amplified PCR products were separated by electrophoresis on a 1% agarose gel and then purified using a multiscreen filter plate (Millipore Corp., Billerica, MA, USA). After PCR, sequencing was conducted at Macrogen, Inc. (Seoul, Korea). The PCR product underwent a direct PCR sequencing reaction using the PRISM BigDye Terminator. Hi-Di formamide (Applied Biosystems, Foster City, CA, USA) was added to the amplified DNA sample and the samples were incubated at 95°C for 5 min and then placed on ice for 5 min. DNA was analyzed using the ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA, USA). The nucleotide sequence was aligned using the BioEdit sequencing program. Similarity searches for nucleotide sequences were performed using the Web-based BLAST algorithm of the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov).

Preparation of Koji Extract Medium

A *koji* extract medium was prepared by mixing *koji* (steamed rice on which *A. oryzae* had been cultivated) and distilled water at a ratio of 1:3 (w/v), followed by agitation at 62°C for 5 h. After centrifugation at 6,000 rpm for 20 min, the soluble solids were adjusted to a final concentration of 14% [12]. *Koji* extract medium was autoclaved.

Inoculation of Isolated Yeasts

The yeasts were precultured twice on PDA medium (Difco, Detroit, MI, USA) at 29°C for 24 h, followed by suspension in sterile 0.85% NaCl. The absorbance of the suspensions was measured using a turbidimeter (Biolog, Hayward, CA, USA) at 590 nm. The suspensions were adjusted to 2×10^7 CFU/ml and inoculated.

Alcohol and Flavor Production Capacity

To measure the yeasts' capacity for alcohol production, 10 ml of *koji* extract medium was placed in a test tube with a Durham tube, and carbon dioxide gas production rates were compared at 5°C, 15°C, 25°C, 35°C, and 39°C for 3 days. To measure flavor production capacity, eight experts on traditional liquors working at KFRI assessed the sensory characteristics after the 3-day fermentation.

***Makgeolli* Brewing**

The first brewing [yeast, 0.02% and *koji* (sp 85):distilled water :: 38:62] was performed to reach 36% of the total *makgeolli* volume, and was followed by fermentation at 25°C for 2 days. The second brewing (64% of the total *makgeolli* volume, steamed non-glutinous rice:water::32:68) was then performed, followed by fermentation at 25°C for 7 days. After compression, *makgeolli* was prepared by 120 mesh filtering. When JN (sp 300) and IN (sp 1800) were used instead of *koji* (sp 85), the amylolytic activity was adjusted. *Makgeolli* brewing was performed in triplicates.

Chemical Analysis

Alcohol content was analyzed by gas chromatography (GC; Agilent 6890N, Palo Alto, CA, USA) after filtering the samples with 0.45 μm syringe filters (Xpertek, Rivonia, Republic of South Africa). A DB-ALC2 column (30 m length × 0.53 mm diameter; 2 μm film thickness; J & W Scientific, Folsom, CA, USA) was used, with helium as the carrier gas and the following temperatures: oven, 70°C isothermal; inlet, 200°C; and detector FID, 250°C. A standard curve was generated, and alcohol content was calculated as the peak area [12]. The concentration of soluble solids was measured with a hand-held refractometer (ATAGO Pocket PAL-1; ATAGO Co. Ltd., Tokyo, Japan) and recorded in Brix units (% sucrose). The pH was measured with a model D-51 pH meter (HORIBA, Kyoto, Japan). Total acid was measured as citric acid concentration and was determined by adding two to three drops of phenolphthalein indicator to a 10 ml sample and titrating with 0.1 N NaOH until the solution turned light green. Phthalic acid (Sigma-Aldrich, St. Louis, MO, USA) was used as the standard [24]. Reducing sugar concentration was determined by measuring the absorbance at 550 nm using the dinitrosalicylic acid method [21], a glucose standard (Sigma-Aldrich, St. Louis, MO, USA), and the HP 8453 diode array UV/VIS spectrophotometer (Agilent, Palo Alto, CA, USA).

Organic Acid Analysis

A slurry of 5 100–200 mesh anion-exchange (Cl^- form) resin (Bio-Rex 5, Bio-rad, USA) was prepared in deionized water. The sample was applied to the column and the sugars were eluted with deionized water. Two milliliters of sulfuric acid [20% (v/v) in deionized water] and deionized water was added to collect 10 ml of eluent containing the acids [2], and filtered through a 0.2 μm syringe filter, and 3 μl was injected into a UHPLC (Agilent 1290 Infinity, CA, USA) equipped with a Zorbax SB-Aq (5 μm, 4.6 × 250 mm, Agilent, CA, USA) column. The column oven temperature was 35°C and the absorbance was 210 nm. The mobile phase was 20 mM phosphate buffer (pH 2.0) : ACN :: 99:1 (v/v); and the flow rate was 1 ml/min.

Volatile Compound Analysis

After equilibrating the sample (5 ml) for 20 min at 60°C, the sample was applied to a 50/30 μm DVB/carboxen/PDMS Stable Flex fiber (Supelco, Bellefonte, PA, USA) for 40 min and analyzed by GC for 1 min using solid phase microextraction (SPME) [7]. An Agilent 7890A GC (Agilent, Palo Alto, CA, USA) and a Stabilwax-DA column (30 m length × 0.25 mm diameter × 0.25 μm film thickness; Restek Corp., Bellefonte, PA, USA) were used for the analysis. The GC oven temperature was held at 60°C for 5 min and then set to rise to 200°C at a rate of 3°C/min. The injector temperature was 250°C; helium was used as the carrier gas and the flow rate was 2 ml/min. The direct capillary interface of the MSD (Agilent 5975C) was set at a temperature of 250°C; the ion source temperature was 230°C; ionization voltage was 70 eV; mass range was 40–550 amu; and scan rate was 2.2 scans/s. The identities of the volatile compounds were confirmed by comparing retention indices (RIs), mass library (NIST 05a), and aromatic properties [12].

Sensory Analysis

Eight panelists, most of them experienced in sensory attribute analysis, were selected on the basis of interest and availability from the KFRI. Nine-point hedonic scales (1, dislike extremely; 9, like extremely) for flavor, sweetness, sourness, bitterness, and overall acceptability were used to evaluate the *makgeolli*.

Statistical Analysis

The mean separation of the experimental parameters was determined by analysis of variance (ANOVA). The statistical analysis was performed using SAS for Windows ver. 7.2 (Statistical Analysis Systems Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

Microbial Identification

ITS of Y98-5 was sequenced by direct PCR. An 847 bp nucleotide sequence was obtained. A comparison between this sequence and type strain FN393997 sequence showed to have 99% similarities with the sequence in *S. cerevisiae*. The ITS of Y197-13 was sequenced by direct PCR. A 588 bp nucleotide sequence was obtained and compared with sequences, which showed that the sequence was 98%

Table 1. Identification of wild yeast strains Y98-5 and Y197-13.

Query				Subject					Identities		
Name	Length	Start	End	Gene	Accession no.	Length	Start	End	Match	Total	Similarity (%)
Y98-5	847	1	847	*Saccharomyces cerevisiae*	FN393997	848	1	844	844	847	99
Y197-13	530	53	582	*Pichia anomala*	AY046221	530	1	530	522	530	98

Table 2. Brewing characteristics of wild yeast strains for *makgeolli.*

Yeast	Fermentation time (h)		Flavor	Foam	Biofilm
	8	24			
Y98-5	-	++++	Sweet, fruity	+	-
Y197-13	++	++++	Sweet, fruity, sour	-	-

Cells were grown in *koji* extract medium at 25°C for 24 h. Growth is indicated by gas to fill the Durham tube as follows: ++, 50%; ++++, 100%. Foam and biofilm are indicated as follows: +, positive; -, negative.

similar with that of *P. anomala* (AY046221) (Table 1). The analyzed 588 bp nucleotide sequence of the Y197-13 strain was registered in GenBank, under the accession number JX566694. *Wickerhamomyces anomalus* and a *Pichia anomala* are a synonym [16].

Fermentation Characteristics of Isolated Yeasts

Ethanol is produced by yeast, which grows on sugars derived from starch by fungi in the *koji*. This is one of the factors that most strongly influences the quality of *makgeolli* and increases with the level of fermentation [13]. Ethanol production can be crudely estimated by measuring the amount of carbon dioxide gas produced during the fermentation process [14]. Y98-5 did not produce gas after 8 h, and to fill the whole Durham tube 24 h after the start of fermentation in *koji* extract medium. Y197-13 produced gas to fill half of the Durham tube after 8 h, and to fill the whole tube after 24 h. These results showed that fermentation by Y197-13 was faster than by Y98-5. In terms of flavor characteristics, Y98-5 produced a sweet and fruity flavor in *koji* extract medium, whereas Y197-13 produced a sweet, fruity, and sour flavor. Y98-5 in *koji* extract medium formed foam. Neither Y98-5 nor Y197-13 formed a biofilm in *koji* extract medium (Table 2).

To determine the optimal temperatures for fermentation by the yeasts, the fermentation rates at temperatures of 5°C, 15°C, 25°C, 35°C, and 39°C were compared (Fig. 1). At 5°C, neither yeast produced gas, indicating that the two yeasts could not ferment alcohol or grow at this temperature. At 15°C, Y98-5 produced gas to fill 25% of the Durham tube in 1 day after the start of fermentation, and gas to fill the whole tube after 2 days. Y197-13 did not produce gas the day after the start of fermentation, but produced gas to fill 50% of the Durham tube after 2 days, and to fill the whole tube after 3 days. At a fermentation temperature of 25°C, alcohol fermentation by both yeasts produced gas to fill the whole Durham tube in 1 day after the start of fermentation. At 35°C and 39°C, the alcohol fermentation by Y98-5 was the same as at 25°C, and Y197-13 produced gas to fill 75% of the Durham tube after 1 day, and gas to fill the whole tube after 2 days. Thus, the optimal fermentation temperature of Y197-13 was determined to be 25°C.

Suitability of Isolated Yeasts for Brewing

Non-glutinous rice, fermentation agent (*koji*, JN, or IN), yeast, and distilled water were mixed in a final volume of 1,500 ml. The brewing properties of yeast Y197-13 in the presence of each fermentation agent were analyzed (Fig. 2, Table 3).

In the case of Y98-5, the alcohol content of *makgeolli* was 14.6% for *koji*, 14.9% for JN, and 15.8% for IN. Thus, the alcohol content increased in proportion to the sp value.

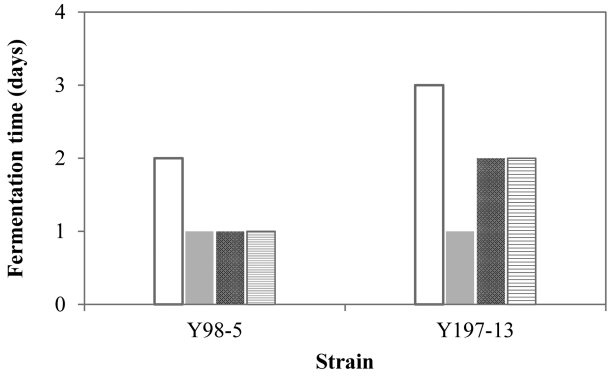

Fig. 1. Alcohol fermentation capacity of wild yeast strains at different fermentation temperatures.

Wild yeast strains Y98-5 and Y197-13 were inoculated into *koji* extract medium. Fermentation time was the time required for the Durham tube to become filled with carbon dioxide gas. □, 15°C; ■, 25°C; ■, 35°C; ≣, 39°C.

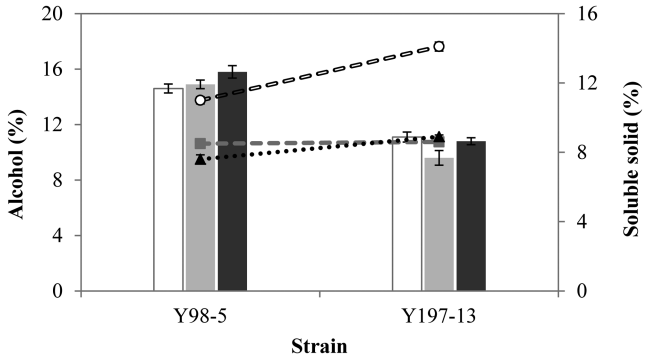

Fig. 2. Alcohol content (bars) and soluble solids (symbols) of *makgeolli* brewed using yeast strains Y98-5 and Y197-13 with different fermentation agents.

Alcohol content using *koji* (□), *jinju nuruk* (JN, ■), and improved *nuruk* (IN, ■). Soluble solids using *koji* (○), JN (■), and IN (▲). Values represent the mean ± SD (n = 3).

Table 3. Chemical characteristics of *makgeolli* using Y98-5 and Y197-13 brewed with different fermentation agents.

Yeast	FA[a]	Reducing sugar (mg/ml)	pH	Total acid (%)	Organic acid (mg/ml)					
					Oxalic	Malic	Lactic	Acetic	Citric	Succinic
Y98-5	*Koji*	12.12 ± 0.3	3.53 ± 0.02	0.65 ± 0.03	ND[b]	0.14 ± 0.02	0.28 ± 0.09	0.02 ± 0.01	3.49 ± 0.18	0.26 ± 0.05
	JN[c]	6.68 ± 0.15	4.15 ± 0.03	0.2 ± 0.03	ND	0.16 ± 0.04	1.37 ± 0.08	0.18 ± 0.03	0.13 ± 0.02	0.19 ± 0.04
	IN[d]	5.78 ± 0.15	3.79 ± 0.05	0.2 ± 0.05	0.57 ± 0.05	0.2 ± 0.05	0.78 ± 0.11	0.16 ± 0.03	0.12 ± 0.03	0.22 ± 0.04
Y197-13	*Koji*	39.67 ± 1.37	3.39 ± 0.04	0.73 ± 0.05	ND	0.35 ± 0.01	0.32 ± 0.01	0.18 ± 0.02	3.53 ± 0.15	0.32 ± 0.02
	JN	15.92 ± 0.12	3.28 ± 0.02	0.87 ± 0.04	ND	0.02 ± 0.02	3.51 ± 0.16	0.11 ± 0.03	ND	0.1 ± 0.03
	IN	16.48 ± 0.5	3.25 ± 0.03	0.64 ± 0.04	0.22 ± 0.03	0.69 ± 0.09	4.53 ± 0.17	0.38 ± 0.03	0.05 ± 0.02	0.22 ± 0.04

Results are presented as mean ± SD (n = 3).

[a] FA, fermentation agent.

[b] ND, not detected.

[c] JN, *jinju nuruk.*

[d] IN, improved *nuruk.*

In the case of Y197-13, the alcohol content of the *makgeolli* was 11.1% for *koji*, 10.8% for IN, and 9.6% for JN. Thus, in the case of Y197-13, even if the sp was low, *koji* (rice *nuruk*) was a more appropriate fermentation agent than JN (wheat *nuruk*) or IN, which is milled wheat coated with an amylolytic enzyme.

Solids content is reportedly inversely proportional to the alcohol content, as the starch in rice is degraded to sugar and used simultaneously as a carbon source and a fermentation substrate [14]. In this study, the *makgeolli* brewed using *koji*, and Y98-5 had the highest solids content and the lowest alcohol content. The solids content was lower in the *makgeolli* brewed using JN and IN, showing that more degraded sugar was converted into alcohol. The solids content of *makgeolli* prepared with *koji* and Y197-13 was the highest, and also had the highest alcohol content. The solids content of *makgeolli* prepared using JN and Y197-13 was similar to that prepared using Y98-5. These results suggest that the degraded sugar was converted into alcohol and simultaneously used to produce carbon dioxide gas (carbonated taste of Y197-13 *makgeolli*) and water (low alcohol content of Y197-13 *makgeolli*).

The pH can be used as a marker for organic acids, carbon dioxide, and other substances produced during fermentation [31]. The pH of Y98-5 *makgeolli* was lowest when brewed using *koji*, and highest when brewed with JN. These results are consistent with those of a study by Han *et al*. [8], who reported a pH of 3.5–4.2 for *makgeolli* brewed using *S. cerevisiae*. In Y197-13 *makgeolli*, the pH was highest when using *koji* and lowest when brewed using JN and IN. These results are similar to those of Lee *et al*. [20], who reported a pH of 3.1 for Pusan sansung *makgeolli*. Thus, in Pusan sansung *makgeolli*, which used *nuruk*, the presence of yeast such as *Pichia anomala* is expected. Pusan sansung *makgeolli* has a sour taste that is preferred by Pusan residents. The total acid content of Y98-5 *makgeolli* was the highest when brewed with JN. The reducing sugar content was highest in Y197-13 *makgeolli* brewed using *koji*, indicating that the strong sweet taste can be attributed to incomplete fermentation. Thus, *makgeolli* brewed using Y197-13 and *koji* as a fermentation agent is expected to have an appropriate flavor in contrast to the plain flavor of conventional *makgeolli*, which has a low alcohol content.

P. anomala varies in terms of its environmental habitat, growth pattern, metabolism, stress tolerance, and anti-microbial characteristics. It is isolated from plant seeds, fruit skins, human tissue, milk products, baked products, salted foods, waste water, tree secretions, and marine environments [34]. The level of sugar fermentation by *P. anomala* varies depending on the species, but is usually weak. *P. anomala* was recently granted "qualified presumption of safety (QPS)" status by the European Food Safety Authority (EFSA) and is considered to be beneficial to food biotechnology [32].

Organic Acid Content and Sensory Attributes of *Makgeolli*

Y98-5 *makgeolli* contained 3.49 mg/ml of freshly sour citric acid, 0.28 mg/ml of round, sour lactic acid, and 0.02 mg/ml of pungent, sour acetic acid when brewed using *koji*. When brewed using JN, the *makgeolli* contained 1.37 mg/ml of sour lactic acid, which is added primarily to prevent the growth of putrefactive pathogens early in the fermentation process, and 0.13 to 0.19 mg/ml of other organic acids. In the *makgeolli* brewed using IN, 0.57 mg/ml of bitter oxalic acid was detected; this was not detected in the *makgeolli* brewed using *koji* or JN (Table 3). Thus, the *makgeolli* brewed using IN had a bitter taste. Y197-13 *makgeolli* brewed using *koji* contained 3.53 mg/ml citric acid and 3.51 mg/ml lactic acid; citric acid was not detected in the *makgeolli* brewed with JN. The *makgeolli* brewed with IN had an organic acid composition of 4.53 mg/ml lactic acid; 0.69 mg/ml malic acid; 0.22–

Table 4. Preference test[a] for *makgeolli* brewed using different yeasts and fermentation agents (N = 8).

Yeast	FA[b]	Flavor	Sweet taste	Sour taste	Bitter taste	Overall
Y98-5	*Koji*	6.25 ± 1.28[a]	4.50 ± 0.93[b]	5.00 ± 0.76[ab]	4.88 ± 0.99[ab]	5.88 ± 1.13[a]
	JN	5.00 ± 1.07[c]	3.88 ± 0.83[bc]	4.13 ± 0.99[bc]	4.38 ± 0.74[abc]	4.75 ± 1.28[ab]
	IN	5.13 ± 0.83[bc]	3.25 ± 1.04[c]	4.50 ± 0.76[abc]	3.63 ± 1.06[c]	4.25 ± 0.89[b]
Y197-13	*Koji*	6.13 ± 1.13[ab]	5.50 ± 1.20[a]	5.25 ± 1.28[a]	5.00 ± 1.07[a]	6.00 ± 1.41[a]
	JN	4.63 ± 1.06[c]	4.13 ± 1.25[bc]	3.88 ± 1.25[c]	4.25 ± 1.28[abc]	4.50 ± 1.60[b]
	IN	4.25 ± 1.04[c]	4.38 ± 0.92[b]	3.63 ± 1.41[c]	3.75 ± 1.49[abc]	3.75 ± 1.04[b]

[a] 9, like extremely; 1, dislike extremely.

[b] FA, fermentation agent.

JN and IN are the same as described in Table 3.

Means with superscript letters (a–c) indicate significant differences at the 5% level, as determined by Duncan's multiple range test.

0.38 mg/ml oxalic, acetic, and succinic acid; and 0.05 mg/ml citric acid. Thus, IN was inappropriate as a fermentation agent in Y97-13 *makgeolli* because its acid content is too high. Because lactic acid accounted for the majority of the organic acids, and the fresh, sour citric acid taste was rarely detected in the *makgeolli* using JN, *koji* was determined to be the most suitable *makgeolli* fermentation agent. In addition, the total acid content was higher in Y197-13 *makgeolli* than in the Y98-5 *makgeolli*. Thus, if *koji* is used as the fermentation agent with Y197-13, the *makgeolli* will have a fresh and sour taste.

The alcohol content of the *makgeolli* brewed with Y98-5 and Y197-13 and different fermentation agents was diluted to 6% to yield the alcohol content of conventional *makgeolli*, and its sensory attributes were evaluated (Table 4). The overall preference for Y197-13 *makgeolli* using *koji* was significantly higher than for the other *makgeolli*, and was followed by Y98-5 *makgeolli* using *koji*, and then the other *makgeolli* batches, with scores ranging from 3.75 to 4.75 points ($p < 0.05$). The flavor preference score was 6 points for all the samples that used non-glutinous rice as a source of *nuruk*, which suggests that the use of non-glutinous rice as a source improves the aroma. The flavor preference score was significantly lower for JN and IN *makgeolli*, which both used wheat ($p < 0.05$). For both yeasts, the *makgeolli* produced with *koji* had a fruity flavor. In particular, the Y197-13 *makgeolli* had both a fresh flavor and a carbonated taste. Thus, it may be possible to produce *makgeolli* brews with a sour taste without the need for the addition of acids to the conventional *makgeolli*. The sweet taste was the strongest for Y197-13 *makgeolli* using *koji*, which had a high solids level. The *makgeolli* obtained using JN tasted like *nuruk* (earthy). The *makgeolli* obtained using IN had a bitter taste ($p < 0.05$). The panel members expressed the greatest preference for Y197-13 *makgeolli* using *koji*. The *makgeolli* that was brewed using *koji* had a carbonated taste and a fruity flavor and freshness. Thus, *koji* was shown to be appropriate for the production of Y197-13 *makgeolli*.

Volatile Components of *Makgeolli*

The volatile component of the *makgeolli* with the best sensory characteristics (6% alcohol content), which was brewed using *koji* as a fermentation agent and Y98-5 or Y197-13, was extracted using SPME and analyzed using GC/MS. A total of 35 volatile compounds were identified (Table 5). The functional groups of volatile compounds comprised six alcohols, which included ethyl alcohol; 25 esters, which included ethyl acetate; one acid; one aldehyde; and two ketones. The *makgeolli* brewed using both yeasts had the highest ethyl hexadecanoate, a weak waxy odor, a sweet but creamy flavor with no aftertaste, and a mild texture [1]. Thus, the ethyl hexadecanoate was responsible for a full-bodied creamy flavor, and was present at a higher level in Y197-13 *makgeolli*. Isoamyl alcohol, which is produced from the amino acid leucine during *makgeolli* brewing, has a banana flavor, so it is considered an important alcohol composite that significantly affects the flavor and taste of *makgeolli* [18]. In this study, the isoamyl alcohol of Y197-13 *makgeolli* was 10-fold higher than that brewed withY98-5. Phenylethyl alcohol, which is found in natural oils, such as rose and orange flower oils, has rose and honey flavors [6]. In this study, the phenylethyl alcohol of the different preparations of *makgeolli* were similar. Acids, which are produced *via* the Embdem Meyerhof Parnas pathway (EMP) and hexose monophosphate pathway (HMP), bind to the alcohols produced during fermentation, and thereby produce various types of esters. Thus, a low acid content is essential for good flavor and the taste of *makgeolli* [18]. In this study, the acid of Y197-13 *makgeolli* was approximately 2-fold higher than that made with Y98-5.

Ester compounds are more important than alcohol for the flavor and taste of liquors. As a type of fatty acid ethyl ester (FAEE), they are considered important flavor composites of beer and *cheonghju* [25, 36]. Among ester compounds, isoamyl acetate has a light, fruity flavor; is an important ester that produces pear, banana, and apple flavors; and is added to carbonated water or syrup [9]. The isoamyl acetate was slightly higher in Y197-13 *makgeolli* than in

Table 5. Volatile compounds of *makgeolli* brewed using different yeasts with *koji.*

No.	RT[a]	RI[b]	Compound	Y98-5	Y197-13
1	2.531	<1,000	Ethyl acetate	ND[c]	0.298
2	2.876	<1,000	Ethyl alcohol	15.863	13.467
3	4.381	1,047	Propyl alcohol	0.093	0.053
4	5.548	1,109	2-Methyl butanoate	0.039	0.069
5	5.877	1,122	Isoamyl acetate	0.127	0.182
6	8.447	1,222	Ethyl caproate	2.146	0.636
7	8.624	1,228	Isoamyl alcohol	0.203	2.176
8	10.875	1,304	3-Hydroxy-2-butanone	ND	0.097
9	13.485	1,388	3-Ethoxy-1-propanol	0.226	ND
10	14.849	1,431	Ethyl caprylate	2.568	1.102
11	15.521	1,453	Isoamyl caproate	0.275	ND
12	15.736	1,460	Acetic acid	0.047	0.102
13	17.457	1,516	Propyl octanoate	0.022	ND
14	17.993	1,534	Ethyl nonylate	0.052	ND
15	18.381	1,546	2,3-Butanediol	0.185	0.427
16	21.153	1,640	Ethyl caprate	4.605	3.524
17	21.455	1,650	Isoamyl caprylate	4.245	0.035
18	23.542	1,723	Propyl decanoate	0.093	0.057
19	24.425	1,755	Isobutyl decanoate	0.144	ND
20	26.331	1,825	Phenethyl acetate	1.225	1.415
21	27.118	1,855	Ethyl laurate	7.145	3.021
22	27.486	1,869	Isopentyl decanoate	0.582	0.03
23	28.83	1,867	Phenylethyl alcohol	3.264	3.488
24	29.177	1,921	Propyl laurate	0.086	ND
25	29.944	1,934	Isobutyl laurate	0.048	ND
26	31.483	2,027	2-Pentadecanone	0.038	0.032
27	32.36	2,063	Ethyl myristate	10.048	11.285
28	32.623	2,074	Isoamyl laurate	0.283	0.116
29	34.224	2,142	Octadecanal	0.438	0.105
30	34.611	2,158	Ethyl pentadecanoate	0.252	0.384
31	34.876	2,170	*n*-Butyl myristate	0.16	0.167
32	37.281	2,276	Ethyl hexadecanoate	25.675	33.082
33	37.486	2,285	Ethyl 9-hexadecenoate	1.108	1.398
34	42.928	2,489	Ethyl oleate	8.363	10.967
35	44.269	>2,500	Ethyl linoleate	10.352	12.285
			Total	100	100

Data are presented as % values of the total peak area.

[a] RT, Retention time.

[b] RI, Retention indices were determined using C10~C25 as external reference.

[c] ND, not detected.

that of Y98-5 *makgeolli*. Ethyl caproate, which is a flavor component of *cheonghju* [15], has a strong and diffusive fruity-winey odor. The ethyl caproate was more than 3-fold higher in Y98-5 *makgeolli* than in that prepared with Y197-13. Fatty acids that are mild, sweet, and oily with no odor [1], such as ethyl oleate and ethyl linoleate, were also the main composites of *makgeolli*. The ethyl oleate plus ethyl linoleate of Y197-13 *makgeolli* was higher than that of Y98-5 *makgeolli*. These results are consistent with the higher solids content of the Y197-13 *makgeolli*, which was sweeter and had more body.

In conclusion, the *Pichia anomala* Y197-13, non-*S. cerevisiae* isolated in this study produced enough alcohol, fruity flavor, mild body, and sweet taste for *makgeolli*

brewing. Thus, it is deemed appropriate for the production of high-quality *makgeolli* brews.

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