A Dissertation for the Degree of Doctor of Philosophy

Development Yeast Strains to Manufacture a *Makgeolli* and High Shearing Homogenization Process for *Makgeolli* Manufacturing

전통주(막걸리) 누룩의 특성 및 이를 활용한 막걸리 제조와 고압 균질 처리의 영향에 관한 연구

By

Sang Hoon Song

Department of Food Science and Biotechnology

Seoul National University

February, 2014

Development Yeast Strains to Manufacture a *Makgeolli* and High Shearing Homogenization Process for *Makgeolli* Manufacturing

By

Sang Hoon Song

Dissertation
Submitted as partial fulfillment of the requirements
for the degree of Doctor of Philosophy

under the supervision of professor Hyong Joo Lee

at the
School of Agricultural Biotechnology
Seoul National University

February, 2014

Abstract

A variety of *nuruk* was collected from all around of provinces in Korea, and their microflora profiles were analyzed at the species level. Total 42 *nuruk* were collected and when viable cell numbers in *nuruk* were enumerated, average cell numbers of bacteria, fungi, yeast, and lactic acid bacteria from all *nuruk* were 7.21, 7.91, 3.49, and 4.88 log CFU/10 g, respectively. There were no significant differences in viable cell numbers of bacteria or fungi according to regions collected. *Bacillus amyloliquefaciens* and *B. subtilis* were predominant bacterial strains in most *nuruk.* A significant portion of *nuruk*, 13 out of 42 *nuruk*, contained food-borne pathogens such as *B. cereus* or *Cronobacter sakazakii.* There were various species of lactic acid bacteria such as *Enterococcus faecium* and *Pediococcus pentosaceus* in *nuruk*. It was unexpectedly found that only 13 *nuruk* among 42 *nuruk* contained *Aspergillus oryzae,* the representative saccharifying fungi in *makgeolli,* whereas a fungi *Lichtheimia corymbifera* was widely distributed in *nuruk*. It was also found that *Pichia jadinii* was predominant yeast strain in most *nuruk*, but the representative alcohol fermentation strain, *Saccharomyces cerevisiae,* was isolated from only 18 out of 42 *nuruk*. These results suggested that a variety of species of

fungi and yeast were distributed in *nuruk* and involved in the fermentation of *makgeolli.* In this study, total 64 bacterial species, 39 fugal species, and 15 yeast species were identified from *nuruk*. Among these strains, 37 bacterial species, 20 fungal species, and 8 yeast species were distributed less than 0.1%.

The applicability of *Saccharomycopsis fibuligera* CJN1020, *Pichia jadinii* CJN1287 and *Saccharomyces cerevisiae* CJN1514 isolated from Korean *nuruk* to manufacture *makgeolli* was investigated. These yeasts were all suitable for the manufacture of both white rice *makgeolli* by a steam method and white rice-brown rice-wheat flour (WBW) *makgeolli* by a complex method. Upon physicochemical evaluation, there was no difference of final ethanol concentration between white rice *makgeolli* and WBW *makgeolli.* But it had difference of final ethanol yields according to yeasts species. Final ethanol yields by *Saccharomycopsis fibuligera* and *Pichia jadinii* were 16% each and by *Saccharomyces cerevisiae* was 18%. Upon sensory evaluation, WBW *makgeolli* samples received higher scores than white rice *makgeolli* in overall preference. GC/MS analysis revealed that the aromatic compounds of WBW *makgeolli* were richer than those of white rice *makgeolli*. These results suggest that the complex method is

suitable for manufacturing *makgeolli* using brown rice and wheat flour.

High shearing homogenization (HSH), as an alternative of Holder pasteurization, was applied to inactivate the yeast levels in the fermented rice wine (FRW). The storage stability of FRW is very important because the fermentation of rice wine during distribution is still progressed. Holder pasteurization traditionally used might loss nutrients and quality of FRW. In HSH, the applied pressure at 25000 psi with 5 circulations of treatment reduced over 4 log cycles of yeast. In addition, the effects of HSH and thermal treatment on the inactivation of yeast and volatile components in the FRW were compared. The D-values of yeast in FRW were 28 min at 50 °C and 4.18 min at 65 °C. The HSH at 25000 psi with 5 circulations were equivalent to the thermal treatment at 80 °C for 3 min. Total 34 volatile components were identified, including 8 alcohols, 18 esters, 2 acids, 1 carbonyls, 3 hydrocarbons, 2 miscellaneous. Volatile components having fruit aroma were found more in the samples with HSH, while components having fatty and oily characters were more detected in the samples with thermal treatments.

We studied the effect of high shearing process (HSP) on the flow behavior of a colloidal dispersion obtained from the coarse filtered

rice fermentation. The translucency, a key major characteristic of the dispersion, is kinetically changed due to the settling of particles. To delay the phase separation of particles, HSP was applied to the colloidal dispersion. The applied pressure of HSP was varied from 7000 psi to 25000 psi, and for 7000 psi and 25000 psi the process was repeated. The mean particle sizes were dropped from 129.69 μm to about 5.24 μm when HSP was applied. In addition to the particle size, viscosity, volume fraction, settling velocity, turbidity and ζ-potential were measured to understand the flow behavior of the dispersion. The viscosity and the volume fraction were inversely proportional to the particle size. The settling velocities were reduced by applying HSP from ~4 x 10^{-2} m/s to ~3 x 10^{-7} m/s. The phase separation from solid particles in the dispersion was significantly delayed by applying HSP.

Keywords: *makgeolli*; *nuruk*; microflora; *Saccharomycopsis fibuligera*; *Pichia jadinii*; high shearing homogenization; yeast inactivation; microfludization; settling velocity

Students ID: 2008-30343

Contents

Chapter 1.

Analysis of Microflora Profile in Korean Traditional *Nuruk*

Abstract

A variety of *nuruk* was collected from all around of provinces in Korea, and their microflora profiles were analyzed at the species level. Total 42 *nuruk* were collected and when viable cell numbers in *nuruk* were enumerated, average cell numbers of bacteria, fungi, yeast, and lactic acid bacteria from all *nuruk* were 7.21, 7.91, 3.49, and 4.88 log CFU/10 g, respectively. There were no significant differences in viable cell numbers of bacteria or fungi according to regions collected. *Bacillus amyloliquefaciens* and *B. subtilis* were predominant bacterial strains in most *nuruk.* A significant portion of *nuruk*, 13 out of 42 *nuruk*, contained food-borne pathogens such as *B. cereus* or *Cronobacter sakazakii.* There were various species of lactic acid bacteria such as *Enterococcus faecium* and *Pediococcus pentosaceus* in *nuruk*. It was unexpectedly found that only 13 *nuruk* among 42 *nuruk* contained *Aspergillus oryzae,* the representative saccharifying fungi in *makgeolli,* whereas a fungi *Lichtheimia corymbifera* was widely distributed in *nuruk*. It was also found that *Pichia jadinii* was predominant yeast strain in most *nuruk*, but the representative alcohol fermentation strain, *Saccharomyces cerevisiae,* was isolated from only 18 out of 42 *nuruk*. These results suggested that a variety of species of

fungi and yeast were distributed in *nuruk* and involved in the fermentation of *makgeolli.* In this study, total 64 bacterial species, 39 fugal species, and 15 yeast species were identified from *nuruk*. Among these strains, 37 bacterial species, 20 fungal species, and 8 yeast species were distributed less than 0.1%.

Key words: *makgeolli*, Korean rice wine, *nuruk*, microflora profile, identification

1.1. Introduction

Makgeolli is a traditional turbid rice wine in Korea, and of which consumption is continuously increasing due to the recent globalization campaign of Korean ethnic foods led by the Korean government (16). It is usually brewed using rice as a main ingredient and *nuruk* as a fermentation agent (13,22). *Nuruk* is a starter culture made with wheat flour and fermented spontaneously by various microorganisms such as fungi, yeasts, and bacteria as well as lactic acid bacteria inoculated from nature (4). For *makgeolli* production, diverse types of microorganisms are involved in the saccharification, fermentation, and ripening processes. Fungi and bacteria such as *Bacillus subtilis* in *nuruk* saccharify the rice starch and thereby produce glucose, and subsequently yeast cells conduct alcoholic fermentation using glucose to produce ethanol and carbon dioxide (14).

In *nuruk*, the fungi such as *Aspergillus* sp., *Rhizopus* sp., *Mucor* sp., yeasts such as *Saccharomyces cerevisiae*, *B. subtilis*, and various lactic acid bacteria has been reported by many research groups (3). *Aspergillus oryzae* isolated from *nuruk* was characterized (15,21) and the useful fungus and *S. cerevisiae* isolated from traditional *nuruk* was also characterized and used to ferment rice wine or *yakju* (6,11).

The microbiota in *nuruk* and their biochemical roles in the fermentation process of *makgeolli* have been documented (2,7,8), but these studies were restricted to the microbiota in one or several *nuruk* produced in specific area in Korea. *Nuruk* is widely produced in almost all provinces in Korea and it is presumed that microbiota in *nuruk* produced in different province is different from the other one. Because the quality and organoleptic properties of *makgeolli* is clearly dependent on the *nuruk* used for fermentation, the microbiota in *nuruk* is very important in the quality and organoleptic properties of *makgeolli*. Although the importance of the microbiota of the *nuruk*, there are poor studies on the distribution and difference of microbiota in *nuruk* produced in diverse areas.

In this study, a variety of *nuruk* was collected from all around of provinces in Korea, and their microflora profiles were analyzed.

1.2. Materials and Methods

1.2.1. Collection of *nuruk*

Nuruk were purchased from local markets all around provinces in Korea and grouped according to their regions collected.

1.2.2. Enumeration of microorganisms in *nuruk*

The viable cell number of total bacteria, fungi, yeasts, and lactic acid bacteria in *nuruk* were enumerated. Ten grams of *nuruk* were homogenized with 100 mL of sterile saline solution for 2 h and serially diluted. For the enumeration of total bacteria, serially diluted sample solution was spread onto nutrient agar and counted the colonies formed after 2 days of incubation at 30℃. For fungi and yeasts, potato dextrose agar and yeast mold agar containing 20 μg/mL of chloramphenicol was used, respectively, and plates were incubated at 25℃ for 4 days. For the enumeration of lactic acid bacteria, MRS agar was used to grow colonies and anaerobic system gas pak (BBL, Becton Dickinson, Franklin Lakes, NJ, USA) was used to make anaerobic environment and incubated at 37℃ for 3 days.

1.2.3. Identification of the isolates

To identify the isolates from the *nuruk* at the species level, colonies grown on agar plate were resuspended with 50 mM EDTA solution containing 50 mg/mL of lysozyme and incubated at 37℃ for 16 h. Genomic DNAs of total bacteria, fungi, yeasts, and lactic acid bacteria were extracted and purified according to the manufacturer's manual using Wizard genomic DNA purification kit (Promega, Madison, WI, USA). For each individual genomic DNA, polymerase chain reaction (PCR) was performed to clone 16S rRNA or 18S rRNA genes. Reactions contained 20-80 ng of genomic DNA, 1× PCR reaction buffer, 2.5 mM dNTP mixture, 20 pmol of forward primer (27F for total bacteria and lactic acid bacteria; 5'-AGAGTTTGATCATGGCTCAG-3', NS1 for yeasts; : 5'-GTAGTCATATGCTTGTCTC-3', and ITS 1 for fungi; 5'-TCCGTAGGTGAACCTGCGG-3'), 20 pmol of reverse primer (1492R for total bacteria and lactic acid bacteria; 5'-GGATACCTTGTTACGACTT-3', NS8 for yeasts; 5'-TCCGCAGGTTCACCTACGGA-3', ITS4 for fungi; 5'-TCCTCCGCTTATTGATATGC-3'), and 2 units of Ex-Taq polymerase (TaKaRa, Tokyo, Japan) in a final volume of 50 μL. The temperature

profile consisted of 5 min initial denaturation at 95℃ followed by 40 cycles of 95℃ for 30 s, 60℃ for 1 min, and 72℃ for 45 s followed by a final extension at 72℃ for 7 min and the reaction was performed using MyCycler Thermal Cycler System (Bio-Rad, Hercules, CA, USA). The amplified PCR products were electrophoresed using 0.8%(w/v) agarose gel and the DNA band which showed estimated size was eluted from the gel and purified using Wizard SV Gel and PCR clean-up system (Promega). The nucleotide sequences of purified 16S rRNA or 18S rRNA gene were determined using ABI PRISM BigDye ™ Terminator Cycle Sequencing Kits(Applied Biosystems Co., Carlsbad, CA, USA) with ABI PRISM 3730XL Analyzer (Applied Biosystems Co.). Sequences were compared to those available 16S rRNA sequences in GenBank databases using BLAST program (http:/www.ncbi.nlm.nih.gov./blast/). The nearly identical sequences were aligned using the CLUSTAL X program (12) and the phylogenetic trees were constructed using MEGA4 program by neighbor-joining method (18).

1.3. Results & Discussion

1.3.1. Quantitative analysis of microorganisms in *nuruk*

Total 42 *nuruk* were collected from all around of provinces in Korea; 11 *nuruk* were collected from Chungcheong-do (CC, including Dangjin, Hapdeok, Buyeo, and Hongseong), 3 were from Gangwon-do (GW, including Sokcho and Yangyang), 5 were from Gyeonggi-do (GG, including Seongnam, Pocheon, and Gapyeong), 9 were from Gyeongsang-do (GS, including Andong, Munkeong, Yeochon, Kyeongju, Changwon, Haman, jinju, Tongyoung, and Geoje), 4 were from Jeju-do (JJ, including Jeju-si, Seongeup, and Pyoseon), and 10 were from Jeolla-do (JL, including Jeonju, Imsil, Namwon, Sunchang, Gunsan, Gwangju, Mokpo, Boseong, Haenam, and Muan). When viable cell numbers in *nuruk* were enumerated, average cell numbers of bacteria, fungi, yeast, and lactic acid bacteria from all *nuruk* were 7.21, 7.91, 3.49, and 4.88 log CFU/10 g, respectively (Table 1). These viable cell numbers of bacteria, fungi, yeast, and lactic acid bacteria were ranged from 4.73 (JL NR4) to 11.76 log CFU/10 g (GS NR3), from 1.79 (JL NR6) to 12.46 log CFU/10 g (GS NR4), from none to 7.77 log CFU/10 g (GS NR8), and from none to 11.42 log CFU/10 g (GW NR1), respectively. In this result, viable cell number of fungi was the highest

and that of bacteria was second highest. Some *nuruk* did not contain yeast or lactic acid bacteria; 13 out of 42 *nuruk* and 6 out of 42 *nuruk* did not have yeast and lactic acid bacteria, respectively.

Nuruk were grouped according to their provinces from which each *nuruk* was collected and the average viable cell numbers of bacteria, fungi, yeast, and lactic acid bacteria were calculated according to province of which *nuruk* was collected. As shown in Table 2, there were no statistically significant differences ($p > 0.05$) in average viable cell numbers of bacteria or fungi according to the province from which they were isolated. In contrast, there was highest average viable cell number of yeast in *nuruk* collected from Jeju-do (5.71 log CFU/10 g) and the average viable cell number of *nuruk* collected from Gyeonggi-do (1.61 log CFU/10 g) was the lowest. Similarly average viable cell number of lactic acid bacteria was highest in *nuruk* collected from Jeju-do (8.03 log CFU/10 g) and lowest in *nuruk* collected from Jeolla-do (3.62 log CFU/10 g). The reason why the average viable cell number of yeast or lactobacilli was different according to provinces they collected is not clear, though there are some patterns that viable cell numbers of yeast and lactic acid bacteria were highest in *nuruk* collected from Jeju-do and statistically lowest in *nuruk* collected from Gyeonggi-do.

Source	Total bacteria	Fungi	Yeast	Lactic acid bacteria
CC[1)] NR1	5.62±0.11[2)]	8.23±0.17	ND[3)]	3.16±0.04
CC NR2	6.74±0.14	9.11±0.18	ND	3.99±0.04
CC NR3	7.35±0.12	9.10±0.33	7.38±0.31	4.10±0.44
CC NR4	6.24±0.22	8.54±0.44	4.58±0.11	ND
CC NR5	5.54±0.27	7.11±0.14	2.67±0.45	3.49±0.44
CC NR6	7.65±0.23	9.24±0.30	4.54±0.33	3.00±0.22
CC NR7	6.67±0.24	8.69±0.25	3.48±0.36	5.26±0.27
CC NR8	8.28±0.30	6.61±0.52	3.55±0.44	3.45±0.35
CC NR9	8.20±0.32	8.99±0.15	3.27±0.31	3.55±0.42
CC NR10	7.75±0.22	8.29±0.23	4.82±0.17	5.46±0.45
CC NR11	8.23±0.31	8.48±0.26	ND	7.31±0.21
GW NR1	5.47±0.14	8.33±0.17	4.05±0.09	11.42±0.39
GW NR2	7.41±0.17	9.24±0.20	3.77±0.10	5.36±0.07
GW NR3	7.04±0.14	8.75±0.18	5.62±0.15	7.16±0.17
GG NR1	4.97±0.08	6.47±0.19	3.47±0.06	5.93±0.08
GG NR2	8.11±0.16	8.17±0.16	ND	6.24±0.08
GG NR3	8.32±0.13	8.08±0.12	ND	ND
GG NR4	7.56±0.11	6.06±0.12	4.58±0.11	5.63±0.07
GG NR5	6.90±0.12	3.80±0.11	ND	3.63±0.05
GS NR1	5.68±0.09	6.57±0.18	ND[2)]	ND
GS NR2	5.70±0.11	8.28±0.17	3.29±0.19	4.90±0.06
GS NR3	11.76±0.17	8.43±0.18	ND	4.24±0.08
GS NR4	6.53±0.13	12.46±0.25	5.76±0.14	4.45±0.06
GS NR5	6.73±0.07	7.56±1.02	3.97±0.05	5.38±0.14
GS NR6	9.32±0.19	8.28±0.17	5.88±0.15	5.42±0.07
GS NR7	6.54±0.14	8.73±0.20	5.54±0.17	7.52±0.30
GS NR8	8.93±0.30	9.63±0.19	7.77±0.18	7.17±0.15
GS NR9	7.69±0.20	7.25±0.19	7.36±0.14	9.54±0.29
JJ NR1	7.15±0.19	8.64±0.17	6.28±0.18	8.39±0.26
JJ NR2	9.04±0.18	6.24±0.32	6.00±0.15	8.28±0.16
JJ NR3	9.04±0.18	8.57±0.26	5.36±0.14	8.20±0.24
JJ NR4	7.62±0.13	4.82±0.26	5.19±0.35	7.23±0.33
JL NR1	5.65±0.10[1)]	10.33±0.19	4.15±0.03	5.29±0.16
JL NR2	7.62±0.20	8.20±0.21	4.18±0.06	5.24±0.16
JL NR3	6.55±0.13	6.22±0.13	3.79±0.09	5.76±0.07
JL NR4	4.73±0.09	6.17±0.12	ND	ND
JL NR5	6.16±0.09	8.38±0.13	ND	ND
JL NR6	5.36±0.11	1.79±0.04	ND	ND

JL NR7	8.87±0.18	7.81±0.16	ND	6.30±0.08
JL NR8	6.30±0.20	8.81±0.18	5.48±0.14	3.22±0.04
JL NR9	8.71±0.18	9.11±0.18	ND	4.25±0.27
JL NR10	7.04±0.14	8.65±0.17	7.04±0.18	6.10±0.23
Mean±SD	7.21±1.41	7.91±1/76	3.40±2.57	4.88±2.70

(Unit: log CFU/10 g)

1) CC: Chungcheong-do, GW: Gangwon-do, GG: Gyeonggi-do, GS: Gyeongsang-do, JJ: Jeju-do, JL: Jeolla-do

2) Means±SD of triplicate experiments

3) ND: not detected

Table 1. Viable cell number of total bacteria, fungi, yeast, and lactic acid bacteria in *nuruk* collected from various provinces in Korea.

Province	Bacteria	Fungi	Yeast	Lactobacilli
Chungcheong-do	7.12±1.02[1)a2)]	8.40±0.84[a]	3.12±2.34[ab]	3.89±1.82[b]
Gangwon-do	6.64±1.03[a]	8.77±0.46[a]	4.48±1.00[ab]	7.98±3.11[a]
Gyeonggi-do	7.12±1.35[a]	6.52±1.79[a]	1.61±2.24[b]	4.29±2.60[b]
Gyeongsang-do	7.65±2.01[a]	8.58±1.70[a]	4.40±2.86[ab]	5.40±2.66[ab]
Jeju-do	8.21±0.97[a]	7.07±1.87[a]	5.71±0.52[a]	8.03±0.54[a]
Jeolla-do	6.70±1.37[a]	7.58±2.38[a]	2.46±2.75[ab]	3.62±2.65[b]

(Unit: log CFU/10 g)

1) Means±SD of triplicate experiments

2) The same superscripts in a column are not significantly different each other at $p<0.05$.

Table 2. Average viable cell number of total bacteria, fungi, yeast, and lactic acid bacteria in *nuruk* collected from various provinces in Korea.

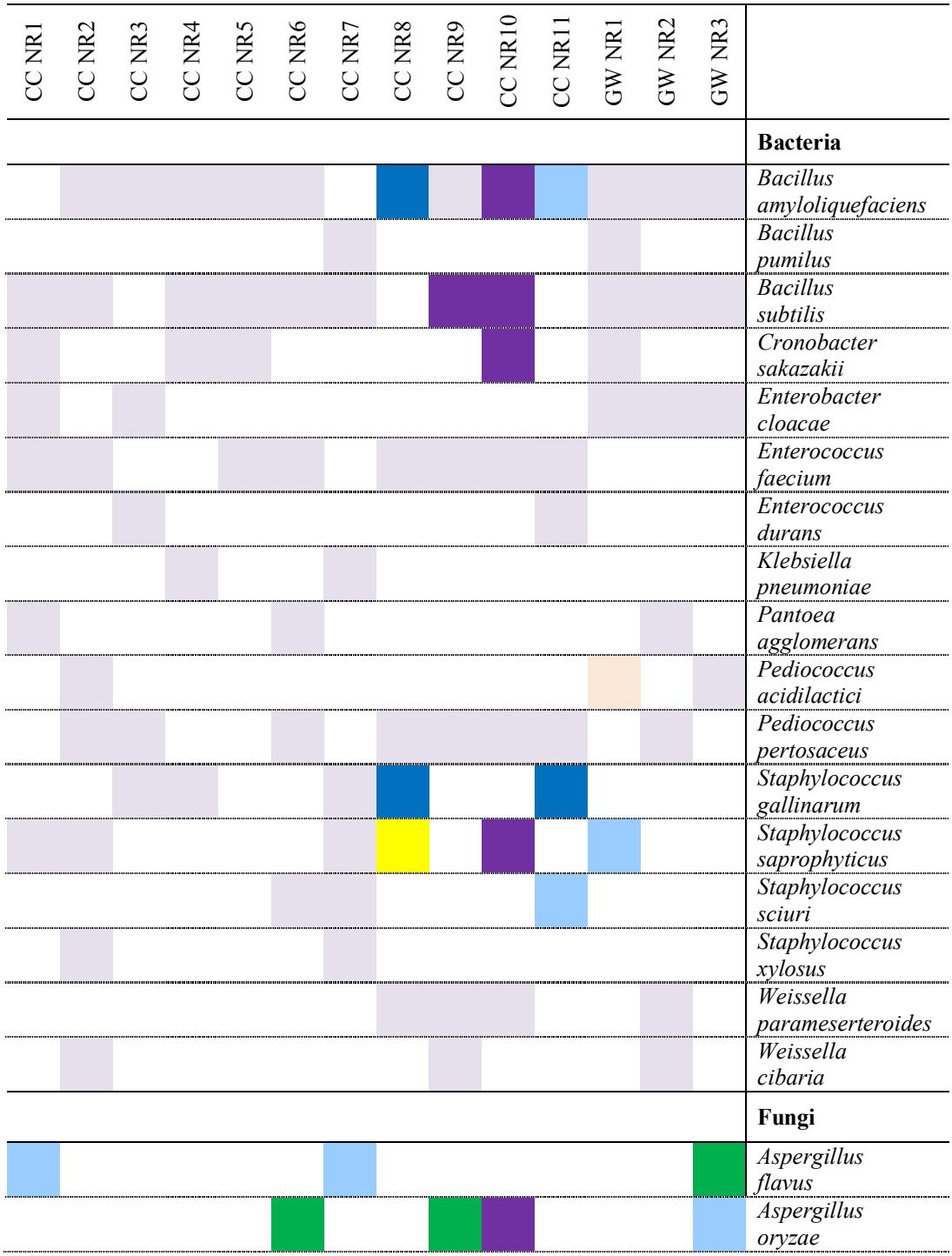
1.3.2. Identification of bacteria

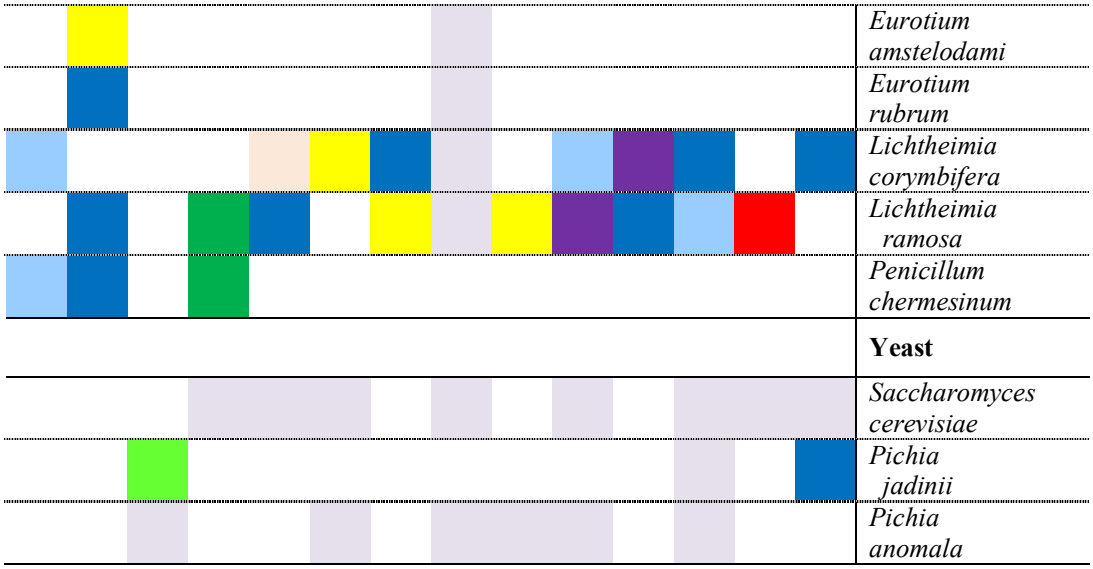
All microorganisms isolated from each *nuruk* were identified at the species level using nucleotide sequence analysis of 16S rRNA gene followed by phylogenetic tree analysis and the result was shown in Fig. 1. *Bacillus amyloliquefaciens* existed in most *nuruk* collected from Chungcheong-do and Gangwon-do and was the most predominant bacteria in *nuruk* JL NR7 of which proportion was over 80%. *Bacillus subtilis* was detected in 27 out of 42 *nuruk* and 20-29% of microorganisms were *B. subtilis* in several *nuruk* such as GG NR2, GG NR5, and GS NR6. A significant portion of *nuruk*, 13 out of 42 *nuruk*, contained food-borne pathogens such as *B. cereus* or *Cronobacter sakazakii,* and in some *nuruk* such as GG NR5 and JJ NR4, over 40 and over 30% of microorganisms were *C. sakazakii,* respectively. This result indicated that there are serious problems in sanitation during the manufacturing process of some *nuruk*.

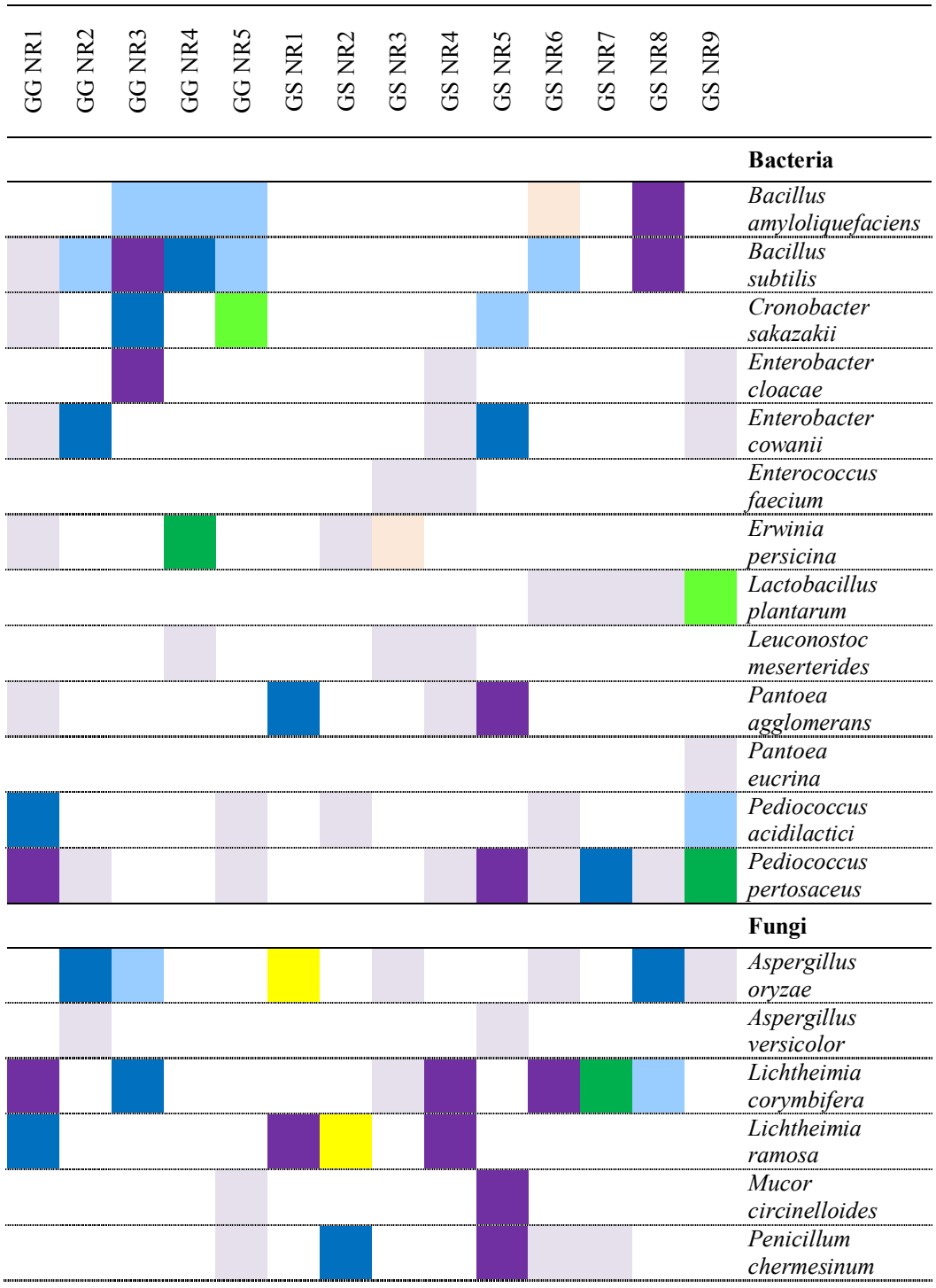
When the lactic acid bacteria isolated from *nuruk* was identified, various species of lactic acid bacteria such as *Enterococcus faecium, Lactobacillus plantarum, Leuconostoc mesenteroides, Pediococcus acidilactici, P. pentosaceus, Weissella paramesenteroides,* and *W. cibaria* were detected. *E. faecium* and *P. pentosaceus* were

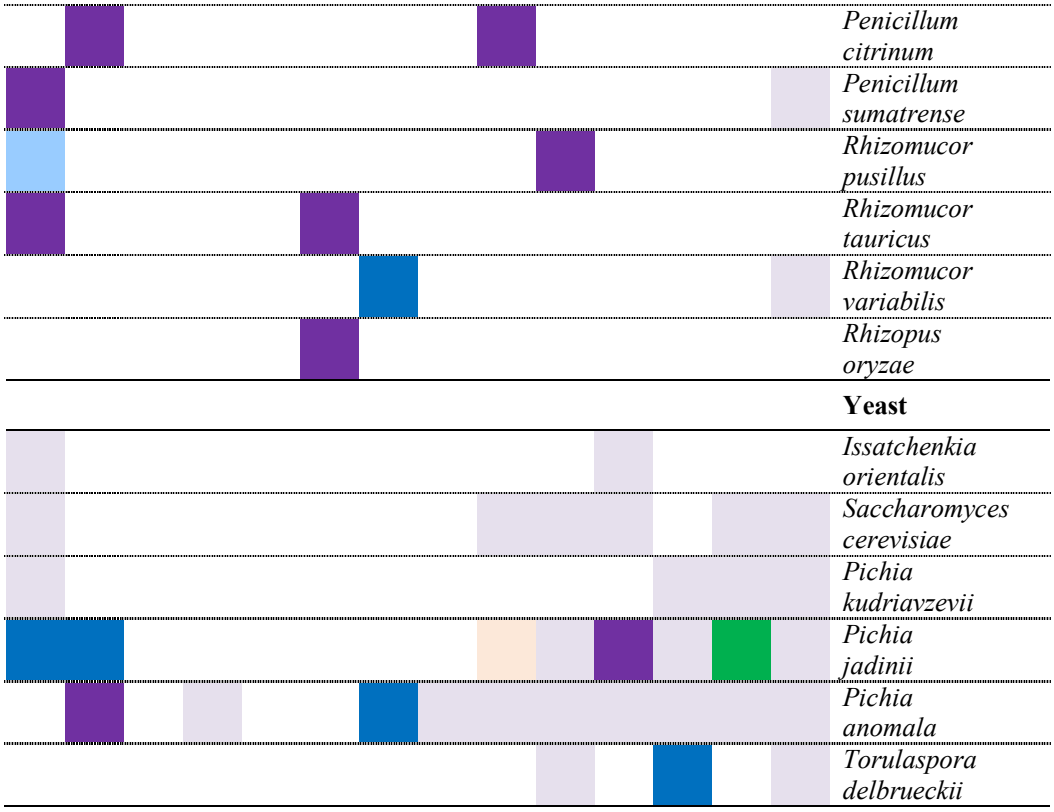
widely distributed in 18 and 26 *nuruk,* respectively, and in GS NR9, over 30% of microorganisms were *P. pentosaceus.*

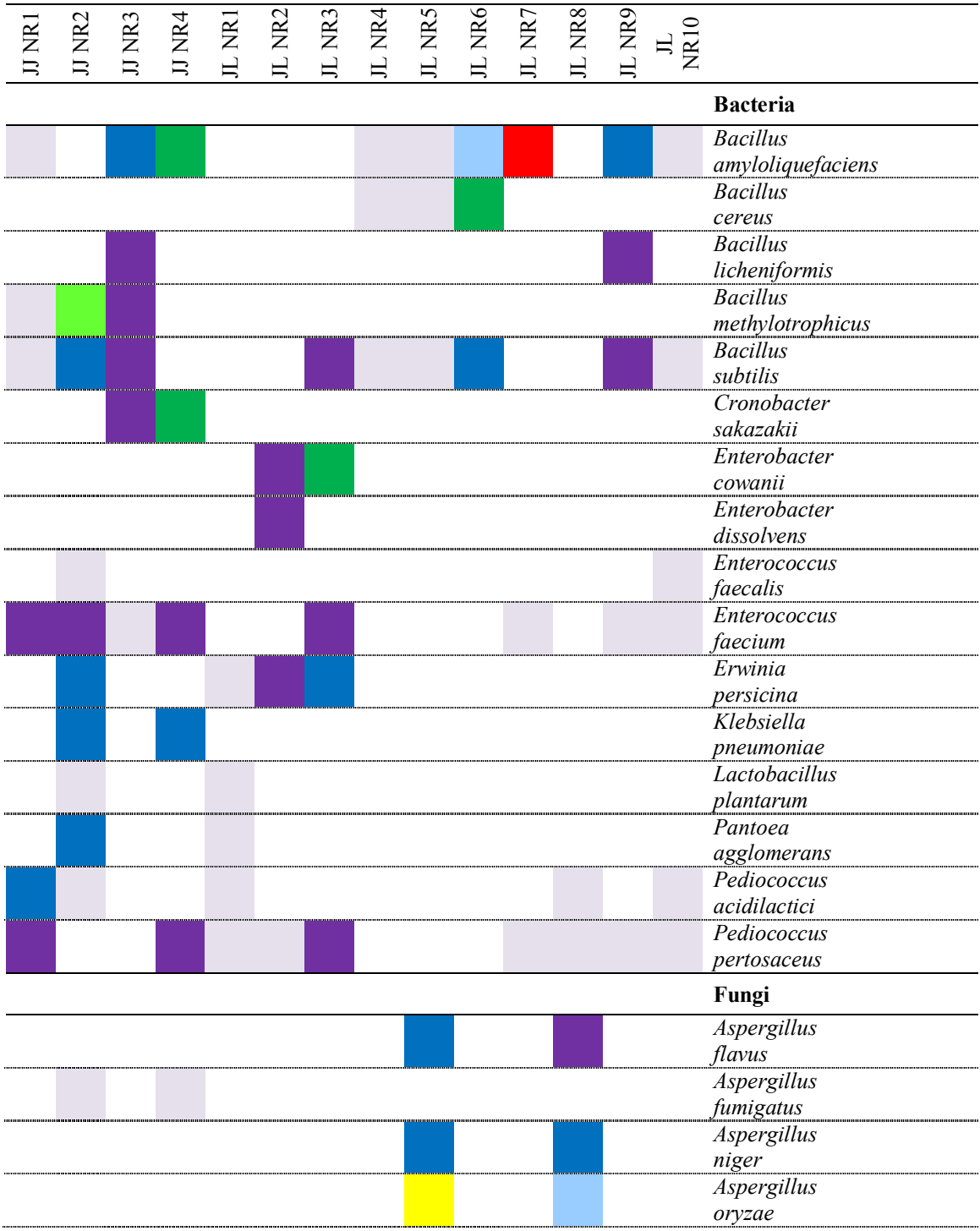
Lee and Yu (13) isolated three lactic acid bacteria from *nuruk*, and identified as *Lactococcus lactis* subsp. *lactis* NR C-1, *Leuconostoc mesenteroides* subsp. *mesenteroides* NR K-3, and *Pediococcus pentosaceus* NR T-1, respectively, by morphological, physiological and biochemical characterization. When their results were compared with this study, the predominant species of lactic acid bacteria in *nuruk* was different in both study, and furthermore much more species of lactic acid bacteria were detected in this study. Yu *et al.* (22) reviewed the research papers on the microorganisms of *nuruk* and summarized that bacteria in *nuruk* were probably not considered as important microorganisms in traditional Korean liquor fermentation, even though *Bacillus* and lactic acid bacteria were continually isolated from *nuruk*.


CC NR1
CC NR2
CC NR3
CC NR4
CC NR5
CC NR6
CC NR7
CC NR8
CC NR9
CC NR10
CC NR11
GW NR1
GW NR2
GW NR3
Bacteria
Bacillus amyloliquefaciens
Bacillus pumilus
Bacillus subtilis
Cronobacter sakazakii
Enterobacter cloacae
Enterococcus faecium
Enterococcus durans
Klebsiella pneumoniae
Pantoea agglomerans
Pediococcus acidilactici
Pediococcus pertosaceus
Staphylococcus gallinarum
Staphylococcus saprophyticus
Staphylococcus sciuri
Staphylococcus xylosus
Weissella parameserteroides
Weissella cibaria
Fungi
Aspergillus flavus
Aspergillus oryzae


Eurotium amstelodami
Eurotium rubrum
Lichtheimia corymbifera
Lichtheimia ramosa
Penicillum chermesinum
Yeast
Saccharomyces cerevisiae
Pichia jadinii
Pichia anomala


GG NR1
GG NR2
GG NR3
GG NR4
GG NR5
GS NR1
GS NR2
GS NR3
GS NR4
GS NR5
GS NR6
GS NR7
GS NR8
GS NR9
Bacteria
Bacillus amyloliquefaciens
Bacillus subtilis
Cronobacter sakazakii
Enterobacter cloacae
Enterobacter cowanii
Enterococcus faecium
Erwinia persicina
Lactobacillus plantarum
Leuconostoc meserterides
Pantoea agglomerans
Pantoea eucrina
Pediococcus acidilactici
Pediococcus pertosaceus
Fungi
Aspergillus oryzae
Aspergillus versicolor
Lichtheimia corymbifera
Lichtheimia ramosa
Mucor circinelloides
Penicillum chermesinum


Penicillum citrinum
Penicillum sumatrense
Rhizomucor pusillus
Rhizomucor tauricus
Rhizomucor variabilis
Rhizopus oryzae
Yeast
Issatchenkia orientalis
Saccharomyces cerevisiae
Pichia kudriavzevii
Pichia jadinii
Pichia anomala
Torulaspora delbrueckii


JJ NR1
JJ NR2
JJ NR3
JJ NR4
JL NR1
JL NR2
JL NR3
JL NR4
JL NR5
JL NR6
JL NR7
JL NR8
JL NR9
JL NR10
Bacteria
Bacillus amyloliquefaciens
Bacillus cereus
Bacillus licheniformis
Bacillus methylotrophicus
Bacillus subtilis
Cronobacter sakazakii
Enterobacter cowanii
Enterobacter dissolvens
Enterococcus faecalis
Enterococcus faecium
Erwinia persicina
Klebsiella pneumoniae
Lactobacillus plantarum
Pantoea agglomerans
Pediococcus acidilactici
Pediococcus pertosaceus
Fungi
Aspergillus flavus
Aspergillus fumigatus
Aspergillus niger
Aspergillus oryzae

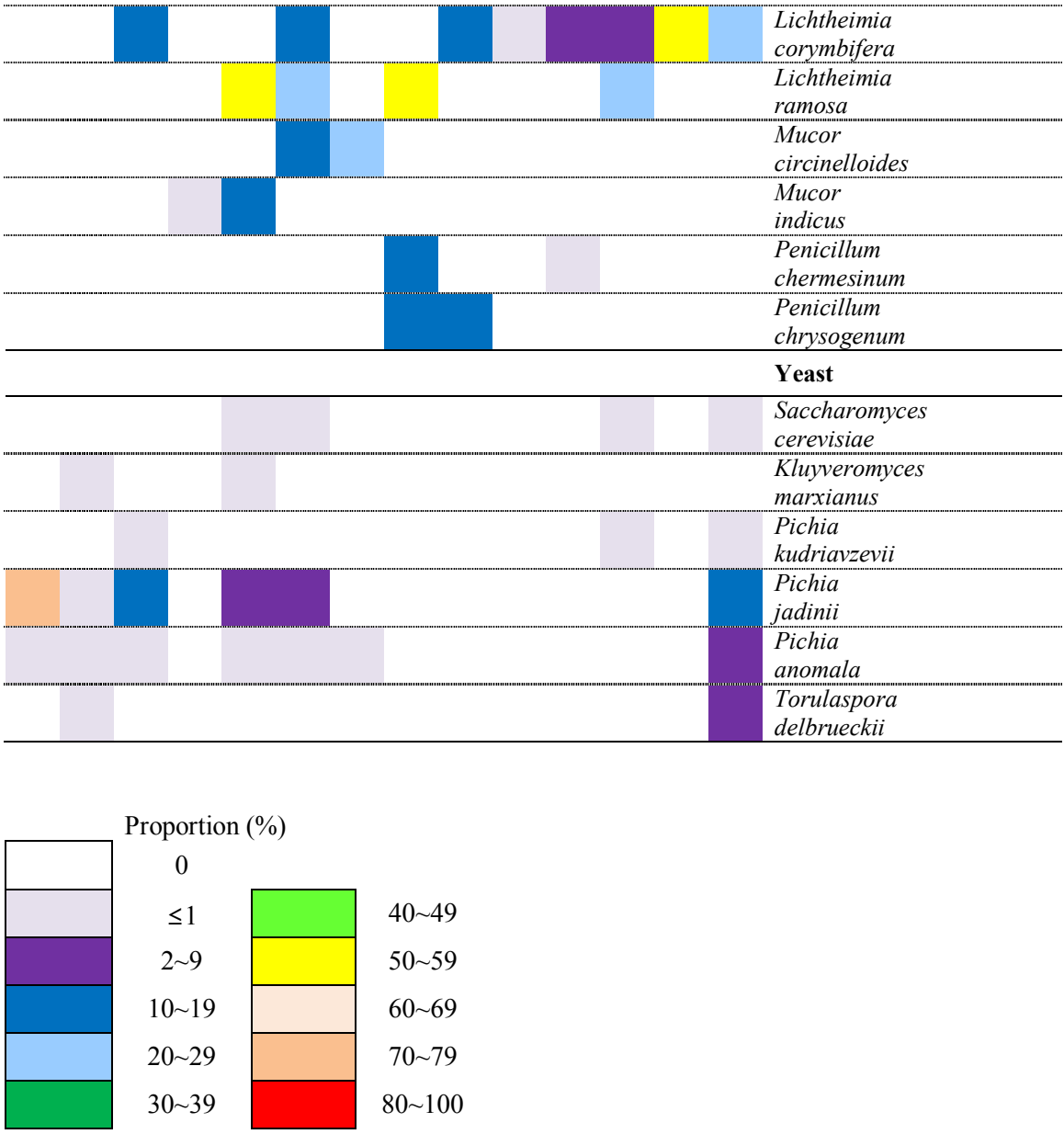


Figure 1. Microflora profile of *nuruk*.

Microorganisms for which the proportion was over 0.1% are presented.

1.3.3. Identification of fungi

Among 42 *nuruk*, only 13 *nuruk* contained *Aspergillus oryzae*, and this result was unexpected because it has been known that *A. oryzae* is the representative saccharifying fungi in the fermentation of *makgeolli*, though the other fungi such as *Mucor* spp. or *Rhizopus* spp. have also been used to ferment *makgeolli* (5,9). A variety species of fungi were isolated from *nuruk* including *Aspergillus flavus, A. fumigatus, A. niger, A. versicolor, Eurotium amstelodami, E. rubrum, Lichtheimia corymbifera, L. ramose, Mucor circinelloides, M. indicus, Penicillium chermesinum, P. chrysogenum, P. sumatrense, Rhizomucor pusillus, R. tauricus, R. variabilis, Rhizopus oryzae.* Proportion of *A. oryzae* was over 50% in GS NR1 and JL NR5, and was below 50% in other *nuruk*. *Lichtheimia corymbifera* and *L. ramose* were widely distributed in *nuruk*, 24 and 18 *nuruk,* respectively. In addition, the proportion of these fungi in *nuruk* was very high, and the proportion of *L. corymbifera* was over 50% in CC NR5, CC NR6, and JL NR9. Also the proportion of *L. ramose* was over 50% in CC NR7, CC NR9, GW NR2, GS NR2, JL NR1, and JL NR4.

The predominant fungi in *nuruk* collected from Chungcheong-do were *A. flavus* (CC NR1), *L. corymbifera* (CC NR1, CC NR5, CC

NR6), *P. chermesinum* (CC NR1), *Eurotium amstelodami* (CC NR2), *L. corymbifera* (CC NR4, CC NR10), and *L. ramose* (CC NR4, CC NR7, CC NR9, CC NR11). *L. ramose* (GW NR1, GW NR2) and *L. corymbifera* (GW NR3) were predominant in *nuruk* collected from Gangwon-do, and *Rhizomucor pusillus* (GG NR1) and *A. oryzae* (GG NR 2, GG NR3) were predominant in *nuruk* collected from Gyeonggi-do. Also, *A. oryzae* (GS NR1), *L. ramose* (GS NR2), and *L. corymbifera* (GS NR7, GS NR9) were predominant fungi in *nuruk* collected from Gyeongsang-do, and *L. corymbifera* (JJ NR3) was predominant in *nuruk* collected from Jeju-do. In addition, *L. ramose* (JL NR1, JL NR2, JL NR4, JL NR8), *Mucor circinelloides* (JL NR3) *A. oryzae* (JL NR5, JL NR8), *L. corymbifera* (JJ NR9, JL NR10) were predominant fungi in *nuruk* collected from Jeolla-do. Park *et al.* (17) isolated and identified 159 strains of fungi from *nuruk* collected from several regions in Korea at the genus level, and there were many differences in numbers and distributions of fungi from each *nuruk* according to their collected region of which results were similar with this study. In their study, *Absidia* spp. were the most frequently isolated from every *nuruk* sample, but *Penicillium* sp. or *Mucor* sp. were not detected of which result was different from that of this study.

Kim *et al.* (10) isolated 10 strains of fungi and examined their saccharogenic enzyme activity, and reported that *Aspergillus* sp. and *Rhizopus* sp. were the predominant strains which showed high liquefying activity in *nuruk*.

1.3.4. Identification of yeast

For yeast, *Pichia jadinii* was predominant strain in most *nuruk*, that is, the proportion of *P. jadinii* in CC NR3, GS NR4, GS NR8, and JJ NR1 were over 40, 60, 30, and 70%, respectively. *P. jadinii* was detected in 17 out of 42 *nuruk*, and unexpectedly, *Saccharomyces cerevisiae,* the representative fermentation strain, was isolated from only 18 out of 42 *nuruk*.

Besides the strains mentioned above, minor strains of which distribution was less than 0.1% was found in *nuruk*, and these minor bacterial strains were identified as *Bacillus circulans, B. flexus, B. infantis, B. licheniformis, B. megaterium, B. sonorensis, B. vallismortis, B. velezensis, B. vietnamensis, Citrobacter braakii, Cronobacter muytjensii, Cupriavidus gilardii, Enterobacter asburiae, E. cloacae, E. cancerogenus, E. hormaechei, E. ludwigii, E. pulveris, E. turicensis, Enterococcus durans, Erwinia soli, Erwinia tasmaniensis, Escherichia coli, Escherichia hermannii, Klebsiella variicola, Lactobacillus brevis, L. casei, L. coryniformis, L. rhamnosus, Leuconostoc citreum, Leuconostoc fallax, Pantoea calida, P. gaviniae, P. stewartii, Pseudomonas aeruginosa, Staphylococcus kloosii,* and *S. xylosus.* The minor fungi strains included *Aspergillus clavatus, A. tritici,*

Cladosporium cladosporioides, C. sphaerospermum, C. uredinicola, Eurotium chevalieri, E. intermedium, Irpex lacteus, Mucor racemosus, Penicillium cinnamopurpureum, P. commune, P. crustosum, P. fellutanum. P. funiculosum, P. phoeniceum, P. sumatrense, P. waksmanii, Rhizopus microsporus, Saccharomycopsis fibuligera, and *S. racemosum.* Also the minor yeast strains isolated from *nuruk* were *Candida glabrata, C. tropicalis, Clavispora lusitaniae, Issatchenkia orientalis, Kluyveromyces lactis, Pichia fabianii, P. farinose,* and *P. guilliermondii.*

In this study, total 64 bacterial species, 39 fugal species, and 15 yeast species were isolated and identified from *nuruk*. Among these strains, 37 bacterial species, 20 fungal species, and 8 yeast species were distributed less than 0.1%. When You *et al.* (23) reviewed the research papers on the microorganisms from Korean *traditional nuruk*, they summarized that total number of fungal species identified is 38 species among 12 different genus and total number of yeast species is up to 18 species from different 8 genus. The total number of fungal and yeast species in their review was very similar with this study, but the species showed in their review were somewhat different from this study. Furthermore the number of total bacterial species was 19 which was

smaller than the total number in this study.

Some studies on the microorganisms isolated from *nuruk* have been undertaken, but these studies were mainly focused on the availability on the fermentation of *makgeolli* and dealt with saccharification activity of fungi or ability of alcohol fermentation of yeasts (1). Also studies of the changes of microflora of *makgeolli* or diversity of microorganisms in *makgeolli* have been done by several research groups, these microfloral changes or microflora profiles were examined mainly during the fermentation process of *makgeolli* (19,20). Although a variety of researches have been done for identification of microorganisms in *makgeolli*, no one has been reported on the microflora profiles in *nuruk*. In this study, the microorganisms isolated from *nuruk* were identified at the species level based on the nucleotide sequence of 16S rRNA gene and the microflora profiles in *nuruk* collected from all around of Korea were analyzed for the first time.

1.4. References

1. Baek SY, Yun HJ, Choi HS, Hong SB, Koo BS, Yeo SH. Screening and characteristics of useful fungi for brewing from commercial *nuruk* in Chungcheong Provinces. Kor J Microbiol Biotechnol. 2010;38:373–378.

2. Chung HK. Studies on *kokja* of high quality. (Part 1) Preparation of new type *kokja* and its activity. Food Sci Biotechnol. 1970;2:88-92.

3. Hong Y, Kim YB, Park SO, Choi EH. Microflora and physicochemical characteristics of *nuruk* and main mashes during fermentation of a traditional Andong *soju*. Food Sci Biotechnol. 1997;6:297-297.

4. Jang JH. History of Korean traditional rice wine. Kor J Dietary Cult**.** 1989;4:271-274.

5. Jo GY, Lee CW. Isolation and identification of the fungi from *nuruk*. J Korean Soc Food Nutr. 1997;26:759-766.

6. Jung HK, Park CD, Park HH, Lee GD, Lee IS, Hong JH. Manufacturing and characteristics of Korean traditional liquor *Hahyangju* prepared by *Saccharomyces cerevisiae* HA3 isolated from traditional *nuruk*. Korean J Food Sci Technol. 2006;38:659-667.

7. Kang TY, Oh GH, Kim K. Isolation and identification of yeast

strains producing high concentration of ethanol with high viability. Kor J Appl Microbiol Biotechnol. 2000;28:309-315.

8. Kim CJ, Oh MJ, Kim SY. Studies on the induction of available mutants of *takju* yeast by UV light irradiation. (Part 1) on the selection and identification of the mutants. J Korean Agric Chem Soc. 1975;18:10-15.

9. Kim HS, Hyun JS, Kim J, Ha HP, Yu TS. Characteristics of useful fungi isolated from traditional Korean *nuruk*. J Korean Soc Food Nutr. 1997;26:767-774.

10. Kim HS, Hyun JS, Kim J, Ha HP, Yu TS. Enzymological characteristics and identification of useful fungi isolated from traditional Korean *nuruk*. Kor J Microbiol Biotechnol. 1998;26:456-464.

11. Kim IH, Park WS. Comparison of fermentation characteristics of Korean traditional alcoholic beverage with different input step and treatment of rice and *nuruk* Korean-style bran *koji*. Korean J Diet Cul. 1996;11:339-348.

12. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. Clustal W and Clustal X

version 2.0. Bioinformatics 2007;23:2947-2948.

13. Lee JH, Yu TS. Identification and characteristics of lactic acid bacteria isolated from *nuruk*. Korean J Biotechnol Bioeng. 2000;15:359-365.

14. Lee JS, Heo GY, Lee JW, Oh YJ, Park JA, Park YH, Pyun YR, Ahn JS. Analysis of *kimchi* microflora using denaturing gradient gel electrophoresis. Int J Food Microbiol. 2005;102:143-150.

15. Lee SS, Park DH, Seong SK, Yoo JY. Studies on the fungal isolates (*Aspergillus* species) inhabiting at the cereals in Korea. Korean J Mycol. 1997;25:35-45.

16. No WS. Zymurgy. Backsan Publishing, Seoul, Korea. 2007;319-320.

17. Park JW, Lee KH, Lee CY. Identification of filamentous molds isolated from Korean traditional *nuruk* and their amylolytic activies. Kor J Appl Microbiol Biotechnol. 1995;23:737-746.

18. Rzhetsky A, Nel M. A simple method for estimating and testing minimum evolution trees. Mol Biol Evol. 1992;9:945-967.

19. Seo MJ, Ryu SR. Screening and characteristics of ethanol tolerant strain *Saccharomyces cerevisiae* SE211. Kor J Microbiol Biotechnol. 2002;30:216-222.

20. Seo MY, Lee JK, Ahn BH, Cha SK. The changes of microflora during the fermentation of *takju* and *yakju*. Kor J Food Sci Technol. 37:61-66.

21. Yu KW, Seong SK, Lee SS, Yoo JY. Studies on the fungal isolates of mucorales collected from Korean home made *mejus* and *nuluks*. Korean J Mycol. 1996;24:280-292.

22. Yu TS, Kim J, Kim HS, Hyun JS, Ha HP, Park MG. Bibliographical study on microorganisms of traditional Korean (until 1945). J Korean Soc Food Nutr. 1996;25:170-179.

23. Yu TS, Kim J, Kim HS, Hyun JS, Ha HP, Park MG. Bibliographical study on microorganisms of traditional Korean *nuruk* (since 1945). J Korean Soc Food Nutr. 1998;27:789-799.

Chapter 2.

Physicochemical Properties of Korean Rice Wine (*Makgeolli*) Fermented by Yeasts Isolated from Korean Traditional *Nuruk*

Abstract

The applicability of *Saccharomycopsis fibuligera* CJN1020, *Pichia jadinii* CJN1287 and *Saccharomyces cerevisiae* CJN1514 isolated from Korean *nuruk* to manufacture *makgeolli* was investigated. These yeasts were all suitable for the manufacture of both white rice *makgeolli* by a steam method and white rice-brown rice-wheat flour (WBW) *makgeolli* by a complex method. Upon physicochemical evaluation, there was no difference of final ethanol concentration between white rice *makgeolli* and WBW *makgeolli*. But it had difference of final ethanol yields according to yeasts species. Final ethanol yields by *Saccharomycopsis fibuligera* and *Pichia jadinii* were 16% each and by *Saccharomyces cerevisiae* was 18%. Upon sensory evaluation, WBW *makgeolli* samples received higher scores than white rice *makgeolli* in overall preference. GC/MS analysis revealed that the aromatic compounds of WBW *makgeolli* were richer than those of white rice *makgeolli*. These results suggest that the complex method is suitable for manufacturing *makgeolli* using brown rice and wheat flour.

Key words: alcoholic beverages, brewing, fermentation, rice, yeast

2.1. Introduction

Rice has long been the staple food of Korea and grain wines that use rice and *gokja* as the main ingredients were developed early in Korea's history. Although its origin is not clear, *makgeolli*, generally called *takju*, is a Korean traditional rice wine that has long been manufactured and consumed in Korea (13). Originally rice or sticky rice was used as the primary ingredient and *gokja* was used as the fermentation agent during manufacture of *makgeolli*, however, rice was replaced by wheat flour in 1963 due to the food policy of Korean government. In addition, the use of yeasts originating from Japan has resulted in loss of the legitimacy of *makgeolli*. Moreover, modification of processes used to manufacture *makgeolli* to reduce the production cost have resulted in loss of its traditional flavor (4, 13).

Allowance of the use of rice in the manufacturing of *makgeolli* in late 1980s resulted in improved quality of *makgeolli* and many studies of *makgeolli* have been conducted since then (13). Manufacturing of *makgeolli* using various carbohydrate sources such as grains and root and tuber crops has been attempted; however, raw materials for the fermentation of *makgeolli* have not been diversified due to the increases in manufacturing costs and the limitations of flavor

improvement (11,12,14).

Brown rice is replaceable ingredient of *makgeolli*, with superior nutritional characteristics including high levels ash, fiber, calcium, phosphorus, ferrous, magnesium, vitamin B1, B2, B6, and K, nicotinic acid (niacin), pantothenic acid, folic acid, inositol, choline, and phytic acid as well as antioxidant materials such as vitamin E or gamma oryzanol which can remove the reactive oxygen species. These antioxidant materials are important nutrients that absorb toxic materials and wastes in the human body (20). However the use of brown rice as the main ingredient in the manufacture of *makgeolli* has been hampered by the rapid growth of yeasts and lactic acid bacteria due to the rich nutrients in brown rice, which results in an imbalance of liquefaction of starch and alcohol production processes followed by a negative effect on the flavor, taste, and color of *makgeolli* (1,2,18).

Therefore, this study was conducted to investigate the organoleptic and flavour properties as well as biochemical characteristics of *makgeolli* produced with *Saccharomycopsis fibuligera, Pichia jadinii,* or *Saccharomyces cerevisiae* isolated from Korean traditional *nuruk* and to establish the manufacturing process of *makgeolli* made with brown rice.

2.2. Materials and Methods

2.2.1. Materials

Purchased rice and sticky rice were cultivated at Cheolwon, Gangwon-do, Korea in 2012 and wheat flour was acquired from CJ Cheiljedang, Co. Ltd. (Seoul, South Korea). *Ipguk*, a fermentation agent, was purchased from Choeun Goksik Co. (*Aspergillus* sp.; saccharification power = 80). *Nuruk* was acquired from Songhak Gokja (Gwangju, South Korea) and the enzymes used were Decozyme-Rhizoforte (92% glucoamylase and 8% α-amylase; saccharificaion power = 30,000) from Proma Co. (Seoul, South Korea). *Saccharomyces cereivsiae* (CJN1514), *Saccharomycopsis fibuligera* (CJN1020), and *Pichia jadinii* (CJN1287) were isolated from Korean traditional *nuruk* and *Saccharomyces cerevisiae* (CJN51286) was used as a control.

2.2.2. Preparation of yeast culture

Yeast cultures for the fermentation of *makgeolli* were prepared by inoculating one colony of yeast into 5 mL of YM broth (yeast extract 3 g, malt extract 3 g, peptone 5 g, dextrose 10 g, distilled water 1 L) followed by incubation at 26℃ for 2-3 days.

2.2.3. Evaluation of fermentation characteristics according to raw materials and manufacturing process

Makgeolli was fermented according to the type of raw material and manufacturing process using *Saccharomyces cereivsiae* (CJN1514), *Saccharomycopsis fibuligera* (CJN1020), and *Pichia jadinii* (CJN1287). In the steam method, fermentation was conducted using either white rice alone or a mixture of white rice and brown rice. In the non-stream method, fermentation was accomplished using either white rice alone, a mixture of white rice and brown rice, or a mixture of white rice, brown rice, and wheat flour. In the steam and non-steam complex method, fermentation was conducted using a mixture of white rice and brown rice or a mixture of white rice, brown rice, and wheat flour. The complex method in this study means that both steamed white rice and wheat flour and wheat flour and non-steamed brown rice were used for the fermentation.

Fermentation suitability according to the type of raw material and manufacturing process for each yeast strain was considered good when the alcohol yield of the fermented mother brew was over 15%, the acidity of *makgeolli* was below 8.3, and no bad taste or odor were organoleptically detected. Fermentation suitability was considered bad

when alcohol yield of fermented mother brew was over 15% the acidity of *makgeolli* was over 8.3, and bad taste or odor are organoleptically detected. Yeast tested were considered to be unsuitable for *makgeolli* fermentation when the alcohol yield of fermented mother brew was lower than 15%.

2.2.4. *Damgeum* and fermentation by steam method

Mother brew was manufactured by adding distilled water and culture broth of yeast cells into *ipkuk* followed by fermentation at 26℃ for 72 h. First-stage *damgeum* was processed by adding *ipkuk* and distilled water into fermented mother brew and agitation followed by fermentation at 26℃ for 72 h. Second-stage *damgeum* was processed by mixing first-stage mother brew, hard-boiled rice, and water followed by fermentation at 26℃ for 90 h. When white rice and brown rice were used for the *damgeum* process, they were first hard-boiled by soaking the non-glutinous rice or brown rice for 2h, removing water at room temperature for 1 h and then steaming at 121℃ for 55 min after which they were allowed to cool.

2.2.5. *Damgeum* and fermentation by non-steam method

Mother brew was manufactured by adding distilled water and culture broth of yeast cells into *ipkuk* followed by fermentation at 26℃ for 72 h. First-stage *damgeum* was processed by adding *ipkuk* and distilled water into fermented mother brew and agitation followed by fermentation at 26℃ for 72 h. Second-stage *damgeum* was processed by mixing first-stage mother brew, rice flour, water, and purified enzyme followed by fermentation at 26℃ for 96 h.

When white rice and brown rice were used for *damgeum* process, they were first converted to flour. Similarly, when white rice brown rice, and wheat flour were used for the second-stage *damgeum* process, the rice was converted to flour. Both types of rice flour were prepared by soaking the non-glutinous rice or brown rice for 2 h, removing water at room temperature for 1 h and then grinding.

2.2.6. *Damgeum* and fermentation by steam and non-steam complex method

First-stage *damgeum* using white rice and brown rice was processed by adding white hard-boiled rice, purified enzyme, *Songhak*-

gokja, and distilled water into fermented mother brew and agitation followed by fermentation at 27℃ for 72 h. When white rice, brown rice, and wheat flour were used, first-stage *damgeum* was processed by adding white hard-boiled rice, steamed wheat flour, purified enzyme, *Songhak-gokja*, and distilled. Second-stage *damgeum* was processed by mixing first-stage mother brew, brown rice flour, water, and purified enzyme, and fermenting the mixture at 27℃ for 96 h. Hard-boiled rice was prepared by soaking the non-glutinous rice for 1 h, removing water at room temperature for 1 h and then steaming at 121℃ for 55 min followed by cooling. Wheat flour was pre-kneaded using a small amount of distilled water and steamed under the same condition as white rice. Brown rice flour was prepared by soaking brown rice for 2 h, removing water at room temperature for 1 h and then grinding.

2.2.7. Fermentation of *makgeolli*

Fermented raw liquor was homogenized by fine-grinding using a grinder and then screened through a 100-meshed sieve. Distilled water subsequently added to raw liquor to give an alcohol content 6%, after which aspartame was added to a final concentration of 110 ppm to

give sweetness. Prepared *makgeolli* was then post-fermented at 25℃ for 16 h to give a carbonated flavor and then stored at 10℃.

2.2.8. Sensory evaluation

Sensory evaluation of *makgeolli* was conducted at 24 h after the *damgeum* process was completed. Twenty five professional sensory evaluators among researchers at CJ Foods R&D, CJ Cheiljedang (Seoul, South Korea) were selected as panels for sensory evaluation, and overall acceptance was evaluated using the 5-point scale method.

2.2.9. Statistical analysis

Statistical analysis was performed using the SPSS software package (Ver. 18.0, SPSS Inc., Chicago, IL, USA). The values were then analyzed by Duncan's multiple range test (DMRT) and all results were expressed as the mean±SD in each examined group and $p < 0.05$ indicating significance.

2.2.10. Analysis of general component and flavors in *makgeolli*

Alcohol content in *makgeolli*: A total of 30 mL of distilled water were

added to 100 mL of *makgeolli* in 300-500-mL flask and distilled until the volume of residual liquid reached 70 mL after which distilled water was added to give a final volume 100 mL. The alcohol content of the sample was then measured at 15℃ using an automatic alcohol analyzer (Density Meter DMA4500, Anton Paar Gmbh, Österreich, Austria).

Titratable acidity : *Makgeolli* samples were filtered through Whatman NO. 2 filter paper and 2 drops of BTB-NR mixed indicator (neutral red 0.1 g, bromothymol blue 0.2 g, 95% ethyl alcohol 300 mL) were then applied to 10mL of filtrate. Next, the sample was titrated with 0.1 N NaOH solution and acidity was calculated based on the volume of NaOH solution used to neutralize the sample solution

Reducing sugar content : To determine the reducing sugar content, a modification of Somogyi's method was used. 2 mL of *makgeolli* and 10 mL of solution A (potassium sodium tartarate tetrahydrate, 90 g, sodium phosphate tribasic dodecahydrate 225 g, cupric sulfate pentahydrate 30 g, potassium iodoate 3.5 g) were mixed in a 1,000 mL flask and the final volume was adjusted to 30 mL with distilled water. In addition, a blank composed of 20 mL of distilled water and 10 mL of

solution A was prepared for comparison. Samples was boiled for 3 min and then rapidly cooled. Next, 10 mL of solution B (potassium oxalate 90 g, potassium iodide 40 g) and 10 mL of solution C (2 N H_2SO_4) were added and mixed well to dissolve the precipitate. The dissolved solution was then titrated with solution D (sodium thiosulfate pentahydrate 13 g, sodium carbonate 0.1 g) until the color changed from brown to green or bluish green, and after which a few drops of starch solution were added and the sample was titrated again until the purple color changed to a pale sky color. The reducing sugar content was then calculated based on the volume of solution D used for titration.

Analysis of volatile components in *makgeolli* : Each 30 mL of *makgeolli* sample was mixed using a magnetic stirrer at 300 ppm for 60 min after 60 mL of redistilled methylene chloride and 100 μL of an internal standard compound, 2-ethyl-1-hexnol [1,000 μg/mL (w/v) in methylene chloride], were added. After that, the sample was washed twice with 50 mL distilled water and then the solvent layer was separated by centrifugation for 10 min at 3,000 rpm. Finally, the supernatant was dehydrated with sodium sulfate and a fraction containing only volatile components was obtained using solvent-

assisted flavor extraction (SAFE) method. Dehydrated sample was dispersed by dropping into a round flask at 40°C and mixing at 300 rpm below 2×10^{-5} torr, after which it was collected into two traps surrounded by liquid nitrogen and concentrated to a final volume of 0.1 mL using a gentle stream of nitrogen gas.

The chromatographic analysis was carried out using an HP 7890A gas chromatograph/5975C mass selective detector (GC/MSD) (Hewlett-Packard Co., Palo Alto, CA, USA). A DB-5 (30 m length ×0.25 mm i.d. × 0.25 mm film thickness, J&W Scientific, Folsom, CA, USA) fused silica capillary column was employed to separate volatile compounds. Helium, as a carrier gas, was run with a constant flow rate of 0.8 mL/min. One microliter of the sample extract was injected at a split ration of 1:10. The oven temperature program was held at 40℃ for 5 min, raised to 120℃ at 4℃/min until 200℃ and then to 220℃ at 16℃/min until 220℃ and finally maintained at 220℃ for 10 min. The injector and detector temperatures were 230℃ and 250℃, respectively. The other GC/MS conditions were follows; electron impact ionization, 70 eV; mass scanning range, 35 -550 amu. The GC-MS condition of each sample was described in Table 1.

Each volatile component was tentatively identified by comparing their mass spectral data with those of on-computer library (Wiley 7n mass spectral database, Hewlett-Packard Co.) and manual interpretation. The retention indices (RI) of volatile components were calculated with *n*-paraffins from C_7 to C_{22} as external standards. The relative peak areas of volatile components identified were determined by comparing their peak areas with that of 2-ethyl-1-hexnol, an internal standard compound, on total ion chromatograms of GC/MS. Mean values of two replicates were presented.

Column	DB-5 (30 m length, 0.25 mm i.d. × 0.25 ㎛ film thickness)
Column	Helium 0.8 mL/min, constant flow
Inlet temp.	230℃
Transfer line temp.	250℃
Oven temp.	40℃, 5 min → 4℃/min → 120℃ → 16℃/min, → 220℃, 10 min
Injection volume	1 μL
Split ratio	100:1
Mass scan range	35 ~ 550 m/z
Scanning rate	2.86 scan/sec
Ionization energy	70 Ev

Table 1. Operating conditions for GC/MS.

2.3. Results & Discussion

2.3.1. Evaluation of fermentation suitability according to raw materials and manufacturing process

Fermentation characteristics of raw materials and the manufacturing process used for production of *makgeolli* are shown in Table 2. The fermentation characteristics of *P. jadinii* (CJN1287) and *S. fibuligera* (CJN1020) prepared by the steam method using white rice were good. Those characteristics were also found to be good when the steam and non-steam complex method was applied, regardless of wheat flour usage. In contrast, when the non-steam method was applied, the sour taste was too strong, and *makgeolli* was not fermented normally when wheat flour was applied. This might have been due to minimization of the effect of other microorganisms such as lactic acid bacteria on fermentation according to the steam of starch materials in steam method or steam and non-steam complex method at first-stage *damgeum* process (9).

Fermentation characteristics of *S. cerevisiae* (CJN1514) were good when the steam method, non-steam method, or steam and non-steam complex method were used in the *damgeum* process, regardless of brown rice usage, accordingly it is estimated that the fermentation

suitability and stability of strain CJN 1514 were better than those of CJN1020 or CJN1287. *S. cerevisiae* (CJN1514) showed vigorous alcohol fermentation ability in the early stage of fermentation, and this ability led to the dominancy of strain CJN1514 in fermentation and stable fermentation when the non-steam method was used (9).

The steam method in the *damgeum* process using white rice was suitable for all strains (CJN1514, CJN1020, and CJN1287). However, in the case of the *damgeum* process using brown rice or wheat flour to enhance flavor, the steam and non-steam complex method were applicable to the successful fermentation.

Treatment	Ingredients			CJN 1020	CJN 1287	CJN 1514
	Steamed	Raw	Roasting			
Steam	WR[2)]	-	-	O[a]	O	O
	WR, BR	-	-	N.P[1)]	N.P	O
Non-steam	-	WR	-	∆	∆	O
	-	WR,BR	-	∆	∆	O
	-	WR,BR,WF[2)]	-	X	X	N.P
Steam and Non-steam	WR	BR	-	O	O	O
	WR, WF	BR	-	O	O	O

[1)] N.P.: Not performed

[2)] WR: white rice, BR: brown rice, WF: wheat flour

[a] Symbols : O, suitable; ∆, unsuitable; X, faulty

Table 2. Fermentation suitability of yeasts to manufacture *Makgeolli* according to treatment of ingredients.

2.3.2. Sensory evaluation of *makgeolli* prepared by the steam method or steam and non-steam complex method

The results of sensory evaluation of white *makgeolli* using the steam method and white rice, brown rice, and wheat flour *makgeolli* using the steam and non-steam complex methods are shown in Table 3. Overall acceptability increased when the stem and non-steam complex method was used for strain CJN1020, CJN1287 or CJN1514. It is likely that the raw brown rice used in the steam and non-steam complex method in the second-stage *damgeum* process enhanced the flavor of the *makgeolli* (18). Strain CJN1287 showed the highest overall acceptance score. In the steam and non-steam complex method using white rice, brown rice, or wheat flour, non-steamed brown rice is added in the second-stage *damgeum* process, which minimizes the nutritional destruction of brown rice by heating and suppresses the heat flavor; accordingly, this process may increase the organoleptic properties of *makgeolli* (9,18).

Physicochemical properties	*S. fibuligera* (CJN1020)		*P. jadinii* (CJN1287)		*S. cerevisiae* (CJN1514)	
	Rice	Rice brown rice wheat flour	Rice	Rice brown rice wheat flour	Rice	Rice brown rice wheat flour
Overall preference	3.30 ± 1.02^{b}	3.53 ± 0.74^{ab}	3.20 ± 0.62^{b}	3.96 ± 0.87^{a}	2.60 ± 0.57^{c}	3.20 ± 0.92^{b}

Table 3. Sensory evaluation of rice *Makgeolli* and rice-brown rice-wheat flour *Makgeolli.*

2.3.3. Comparison of general components of *makgeolli* prepared by the steam method or complex method

General components of white rice *makgeolli* prepared by the steam method or of white rice, brown rice, and wheat flour *makgeolli* prepared by the complex method are shown in Fig. 1. Changes in alcohol content were analyzed for ten days after the first- stage *damgeum* process.

The alcohol content of strains CJN1020, CJN1287, and CJN1514 was similar in response to both the steam method and complex method during the early stage of fermentation, but was higher for the complex method than the steam method during the middle stage of fermentation. This was likely a result of the difference in the dilution rate of mother brew due to differences in the amount of raw materials and water in the second-stage *damgeum* according to the manufacturing methods.

In the case of strains CJN1020 and CJN1287, the alcohol content obtained when the steam method was applied was lower than that obtained when the complex method was applied during the middle stage of fermentation, but was similar during the late stage of fermentation In the case of strain CJN1514, the alcohol content was

lower when the complex method was applied than when the steam method was applied during the middle stage of fermentation, possibly due to yeast requiring a greater adaptation time to utilize the non-steamed brown rice added during the second-stage *damgeum* (18).

The final alcohol content of strains CJN1020, CJN1287, and CJN1514 showed similar levels, regardless of manufacturing methods. The reason for the decreased alcohol content after 3 days of fermentation was that it was diluted due to the two-stage *damgeum* process, but there was no significant difference in alcohol fermentation among manufacturing methods. However, the ability of strain CJN1514 to ferment alcohol was better than that of strains CJN1020 and CJN1287.

To reduce the sugar content, strain CJN1514 was lowest among the strains tested, whereas strain CJN1020 was highest, regardless of the manufacturing methods. This was likely because the ability of strain CJN1514 to reduce sugars to alcohol was quite good, which resulted in a low residual sugar level (8). It is expected that changes in alcohol content by post-fermentation using strain CNJ1514 were minimal due to its low level residual sugar content (8,10).

When strain CJN1020 was used to produce *makgeolli,* the

sugar content of the fermented product was high. As a result, the texture and sweetness of the product immediately after fermentation would be good; however, changes in alcohol content and titratable acidity are expected during storage due to the residual sugar, and it is necessary for the stability of *makgeolli* during the storage should be verified (4, 10, 15).

The sugar content in the mother brew was not affected by the manufacturing methods, but rather by the microbial strains. Differences in the sugar content of the final mother brew according to strains were estimated to be due to the fermentation characteristics of each strain as well as amylolytic enzymes such as α-amylase or glucoamylase produced by microorganisms (8).

In the case of the titratable acidity of mother brew, the complex method showed lower acidity than the steam method, indicating that the complex method is more advantageous to alcohol fermentation by yeasts than lactic acid fermentation by lactic acid bacteria (9). Kim *et al.* (9) reported that the viable cell number of lactic acid bacteria in non-steamed *takju* was lower than that in steamed *takju* (9).

Acids in liquor prevent other microorganisms from contamination and maintain the balance of taste. The results of the

present study indicate that the acidity was maintained properly at the time of finishing alcohol fermentation, and that it did not increase significantly during fermentation when the steam method or complex method were applied, suggesting that alcohol fermentation occurred stably (16).

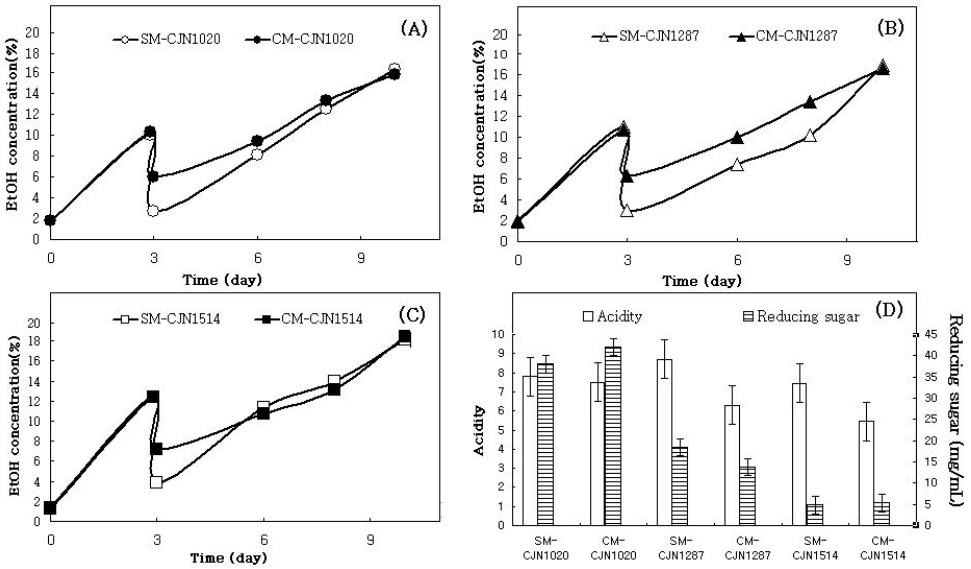


Figure 1. Physicochemical properties of non-diluted *Makgeollies* manufactured by SM and CM. A, CJN1020; B, CJN1287; C, CJN1514; D, acidity and reducing sugar; SM, steam method; CM, complex method.

2.3.4. Comparison of volatile components in *makgeolli* according to raw materials and manufacturing process

Volatile components of *makgeolli* found in this study are listed in Table 4 and 5. A total of 40 volatile components, including 11 alcohols, 20 esters, 2 carbonyls, 4 hydrocarbons, and 3 miscellaneous compounds, were tentatively identified from *makgeolli.* The occurrence of some compounds such as 2-furanmethanol, 1-hexanol, 2-propanol, 4-vinylphenol, ethylacetate, butyl lactate, ethyl nonanoate, ethyl 2-hydroxy-3-phenylpropanoate, limonene, and 4-methylbenzaldehyde were varied both qualitatively and quantitatively depending on the samples. Also, there were differences between *makgeolli* samples produced from white rice by the steam method and those produced from white rice, brown rice, and wheat flour *makgeolli* by the complex mixed method.

Among alcohols, 3-methyl-1-butanol and benzene ethanol were detected at high levels in all *makgeolli* samples. 3-Methyl-1-butanol, which is produced during fermentation and has malt-like and alcoholic-like odor note, can be related to the qualities of alcoholic beverages, and also benzeneethanol, which provides floral, sweet, and greenish odor note, was reported to contribute to the characteristic sensory

properties of alcoholic products even at low concentration (5,7). Such higher alcohols, which are produced during alcohol fermentation and known as fusel oils, can be also responsible for qualities of some liquors such as wine, beer and whisky in terms of both sensory properties and regulation aspects. These compounds can act as positive factors at concentrations lower than 300 mg/mL, but as negative factors at concentrations higher than 400 mg/mL. On the other hand, 1-hexanol and 2-propanol, the fusel oils identified in this study, could be detected only when the steam and non-steam mixed method were applied using strain CJN1020.

2,3-Butanediol, which contributes to the unique bitter taste as well as buttery odor note (3), can be formed by the fermentation of carbohydrates in *makgeolli*. On the other hand, 3- (methylthio)-1-propanol, which is a sulfur-containing compound with cooked vegetable-like and boiled potato-like odor note and also known as an off-odorant in wine and beer (7,17), can be produced via methionine metabolism in yeast cells during the fermentation process. In this study, relatively high contents of 2,3-butanediol and 3-(methylthio)-1-propanol were found when the steam and non-steam mixed method was applied using strain CJN1020 (Fig. 2A).

Ethyl esters, which carry fruity, floral, and perfume-like odor notes, are compounds produced by esterification of ethanol and organic acids during fermentation, and are generally found in alcoholic beverages such as wine, beer, and sake (19). Some ethyl esters, such as ethyl acetate, ethyl butyrate, and isopentyl acetate (isoamyl acetate), which have distinctive odor notes such as fruity and banana-like and relatively low aroma threshold values, can be related to fruity and floral flavor characteristics of *makgeolli,* and their content can be affected by fermentation conditions such as sugar content, type of yeast strains, and fermentation time (6). In our study, the relative contents of ethyl esters could be varied depending on both ingredients and yeast strains used. For the formation of ethyl-4-hydroxybutanoate, high content was observed in all samples made from only white rice regardless of yeasts inoculated. On the other hand, some odorants such as 4-mmethylbenzaldehyde with cherry-like odor note and limonene with sweet and citrus odor note could be found only in WBW *makgeolli* samples.

No.	RI[1)]	Compounds	Relative Peak Area(%)[2)]		
			Rice *makgeolli*		
			CJN1020	CJN1287	CJN1514
		Alcohols			
1	<800	3-methyl-1-butanol	19.583	18.64	18.763
2	804	2,3-butanediol	1.336	2.021	0.924
3	841	3-ethoxy-1-propanol	0.549	0.233	0.319
4	855	2-furanmethanol	0.046	0.032	0.037
5	874	1-hexanol	-	-	-
6	943	2-propanol	-	-	-
7	981	3-(methylthio)-1-propanol	0.718	0.835	0.541
8	1128	Benzeneethanol	21.825	17.378	18.676
9	1219	4-vinylphenol	-	-	-
10	1311	2-methoxy-4-vinylphenol	0.289	0.206	0.048
11	1504	2,4-bis(1,1-dimethylethyl)-phenol	0.059	0.046	0.04
		Esters			
12	<800	isobutyl acetate	0.078	0.074	-
13	804	ethyl butyrate	0.076	0.032	0.291
14	815	ethyl lactate	0.181	0.177	0.274
15	878	Isopentyl acetate (isoamyl acetate)	0.871	0.667	0.992
16	935	ethyl 3-hydroxybutyrate	0.093	0.083	0.198
17	963	ethyl acetate	-	-	-
18	968	butyl lactate	-	-	-
19	999	ethyl caproate	0.096	0.061	0.199
20	1071	ethyl-4-hydroxybutanoate	3.121	2.189	4.364
21	1179	diethyl succinate	0.083	0.096	0.16
22	1194	ethyl octanoate	0.238	0.153	0.648
23	1258	phenethyl acetate	0.413	0.316	0.461
24	1295	ethyl nonanoate	-	-	-

25	1394	ethyl decanoate	0.467	0.185	0.742
26	1443	ethyl 2-hydroxy-3-phenylpropanoate	0.035	-	-
27	1591	ethyl dodecanoate	0.194	0.128	0.191
28	1789	ethyl tetradecanoate	0.316	0.396	0.133
29	1986	ethyl hexadecanoate	0.546	0.472	0.204
30	2151	ethyl linoleate	0.087	0.098	0.033
31	2157	ethyl oleate	0.093	0.091	0.045
		Carbonyls			
32	909	dihydro-2(3H)-furanone	0.064	0.035	0.145
33	1027	limonene	-	-	-
34	1081	4-methylbenzaldehyde	-	-	-
		Hydrocarbons			
35	960	4-methylnonane	0.039	-	0.039
36	1198	dodecane	-	-	0.02
37	1277	pentadecane	-	-	-
38	1464	heptadecane	0.038	0.027	0.04
		Miscellaneous			
39	890	xylene	-	-	-
40	1250	1,3-bis(1,1-dimethylethyl)benzene	0.27	0.228	0.356

[1)] Retention indices were determined using *n-paraffins* C_7-C_{22} as external standards

[2)] Relative peak area (peak area of component/peak area of internal standard)

[3)] Flavor-Base 2010 Professional, Leffingwell & Associates ; Aroma threshold value is odor threshold in water

Table 4. List of volatile components of rice *makgeolli* manufactured by yeasts (CJN1020, CJN1287, CJN1514).

No.	RI[1)]	Compounds	Relative Peak Area(%)[2)]		
			WBW *makgeolli*		
			CJN1020	CJN1287	CJN1514
		Alcohols			
1	<800	3-methyl-1-butanol	15.267	18.022	17.772
2	804	2,3-butanediol	13.228	1.863	1.167
3	841	3-ethoxy-1-propanol	0.188	0.493	0.266
4	855	2-furanmethanol	-	0.049	0.34
5	874	1-hexanol	0.118	-	-
6	943	2-propanol	0.274	-	-
7	981	3-(methylthio)-1-propanol	2.699	0.85	0.513
8	1128	benzeneethanol	15.502	19.403	19.283
9	1219	4-vinylphenol	-	0.045	-
10	1311	2-methoxy-4-vinylphenol	0.386	0.291	-
11	1504	2,4-bis(1,1-dimethylethyl)-phenol	-	0.046	0.038
		Esters			
12	<800	isobutyl acetate	0.034	0.068	-
13	804	ethyl butyrate	0.039	-	0.157
14	815	ethyl lactate	-	0.163	0.264
15	878	Isopentyl acetate (isoamyl acetate)	0.435	0.673	0.92
16	935	ethyl 3-hydroxybutyrate	-	0.087	0.193
17	963	ethyl acetate	0.655	-	-
18	968	butyl lactate	0.075	-	-
19	999	ethyl caproate	0.104	0.055	0.178
20	1071	ethyl-4-hydroxybutanoate	0.208	2.438	-
21	1179	diethyl succinate	0.066	0.087	0.141
22	1194	ethyl octanoate	0.248	0.159	0.567
23	1258	phenethyl acetate	0.133	0.34	0.381

24	1295	ethyl nonanoate	0.038	-	-
25	1394	ethyl decanoate	0.256	0.246	0.638
26	1443	ethyl 2-hydroxy-3-phenylpropanoate	0.627	-	-
27	1591	ethyl dodecanoate	0.098	0.123	0.16
28	1789	ethyl tetradecanoate	0.333	0.332	0.126
29	1986	ethyl hexadecanoate	0.586	0.485	0.153
30	2151	ethyl linoleate	0.332	0.092	0.03
31	2157	ethyl oleate	0.352	0.085	0.042
		Carbonyls			
32	909	dihydro-2(3H)-furanone	-	0.04	0.142
33	1027	limonene	0.105	-	0.081
34	1081	4-methylbenzaldehyde	-	0.035	1.291
		Hydrocarbons			
35	960	4-methylnonane	-	-	0.039
36	1198	dodecane	0.025	-	0.028
37	1277	pentadecane	0.077	-	-
38	1464	heptadecane	-	-	0.081
		Miscellaneous			
39	890	xylene	0.034	-	-
40	1250	1,3-bis(1,1-dimethylethyl)benzene	0.259	0.239	0.329

[1)]Refer to Table 4

Table 5. List of volatile components of WBW *makgeolli* manufactured by yeasts (CJN1020,CJN1287,CJN1514).

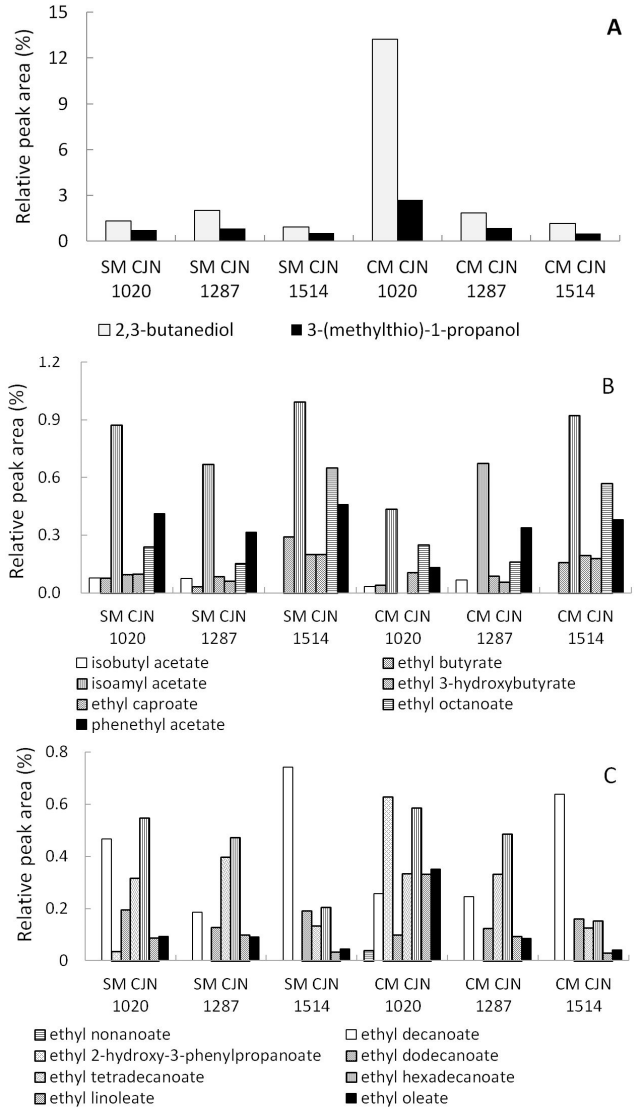


Figure 2. Volatile components distribution of *Makgeollies* manufactured by steam method (SM) and complex method (CM) using CJN1020, CJN1287, CJN1514. A, fruity and floral flavor alcohols; B, fruity and floral flavor esters; C, fatty and oily flavor esters.

2.4. References

1. Bae JH. Korean Patent 1000179660000 (Nov. 19, 1984).

2. Bae SM. The superiority of Korean traditional wines and their industrial application methods. Food Industry and Nutrition. 1999;4:9-12.

3. Bartowky EJ, Henschke PA. The 'buttery' attribute of wine-diacetyl - desirability, spoilage and beyond. International Journal of Food Microbiology. 2004;96:235-252.

4. Cho HK, Seo WT, Lee JY, Che KM. Quality characteristics of cereal *Makgeolli* rice *nuruk* prepared *Rhizopus oryzae* OCS01. J Korean Soc Food Sci Nutr. 2012;41:1002-1008.

5. Dragon G, Mussatoo SI, Oliveira JM, Teixeira JA. Characterization of volatile compounds in an alcoholic beverage produced by whey fermentation. Food Chemistry. 2009;112:929-935.

6. Duarte WF, Dias DR, Oliveira JM, Vilanova M, Teixeira JA. Esiva J.B.A., Schwan R.F., Raspberry(Rubus idaeus L.) wine: Yeast selection, sensory evaluation and instrumental analysis of volatile and other compounds. Food Research International. 2010;43:2303-2314.

7. Falqu E, Ferndez E, Dubourdieu D. Differentiation of white wines by their aromatic index. Talanta. 2001;54:271-281.

8. Ji YJ, Chung HJ. Changes in quality characteristics of *Makgeolli* during storage time. Korean J Food Culture. 2012;27:383-390.

9. Kim MJ, Kim BH, Han JK, Lee SY, Kim KS. Analysis of quality properties and fermentative microbial profiles of *Takju* and *Yakju* brewed with or without steam process. J Fd Hyg Satety. 2011;26:64-69.

10. Kim SM, Han A. Storage properties and biogenic amines production of *Makgeolli* brewed with different proportions of rice and wheat flour. Korean J Food Sci Technol. 2012;44:583-591.

11. Lee SB, Chang WG, Im Bjg, Kim DC. Studies on chemical components of fermented mash in the brewing of *Maggerley* (Korean wine). Korean J Microbiol 1969;7:153-158.

12. Lee TS, Choi, JY. Volatile flavor components in *Takju* fermented with mashed glutinous rice and barley rice. Korean J Food Sci Technol. 1998;30: 638-643.

13. Lee YS, Shin JS, Song YH, Moon SH, Rhee SY. Res. Agric. Hist. 2010;9:99-111.

14. Oh SH, O PS. Screening and identification of a potent fungus for producing raw corn meal saccharifying enzyme. Korean J Appl Microbiol Biotech. 1990;18:547-552.

15. Park CW, Jang SY, Park EJ, Yeo SH, Jeong YJ. Quality

characteristics of rice *Makgeolli* prepared by mashing types. Korean J Food Sci Technol. 2012;44:207-215.

16. Park JH, Bae SM, Yook C, Kim JS. Fermentation using uncooked rice for *soju* production. MS thesis. Yonsei Univ. 2004;12-38.

17. Seow YX, Ong PK, Liu SQ. Production of flavor-active methionol from methionine metabolism by yeasts in coconut cream. International Journal of Food Microbiology. 2010;143:235-240.

18. Song CJ, Park HJ. *Takju* brewing using the uncooked germed brown rice at second stage mash. J Korean Soc Food Sci Nutr. 2003;32:847-854.

19. Vanderhaegen B, Neven H, Coghe S, Verstrepen KJ, Verachtert H, Derdelinckx G. Evolution of chemical and sensory properties during aging of top-fermented beer. Journal of Agricultural and Food Chemistry. 2003;51:6782-6790.

20. Woo SM, Shin JS, Seong JH, Yeo SH, Choi JH, Kim TY, Jeong YJ. Quality characteristics of brown rice *Takju* by different *Nuruks*. J Korean Soc Food Sci Nutr. 2010;39:301-307.

Chapter 3.

Effects of High Shearing Homogenization on Viability of Yeast Cells and Flavor Components in the Non-Pasteurized Fermented Rice Wine *(Makgeolli)*

Abstract

High shearing homogenization (HSH), as an alternative of holder pasteurization, was applied to inactivate the yeast levels in the fermented rice wine (FRW). The storage stability of FRW is very important because the fermentation of rice wine during distribution is still progressed. Holder pasteurization traditionally used might loss nutrients and quality of FRW. In HSH, the applied pressure at 25000 psi with 5 circulations of treatment reduced over 4 log cycles of yeast. In addition, the effects of HSH and thermal treatment on the inactivation of yeast and volatile components in the FRW were compared. The D-values of yeast in FRW were 28 min at 50 °C and 4.18 min at 65 °C. The HSH at 25000 psi with 5 circulations were equivalent to the thermal treatment at 80 °C for 3 min. Total 34 volatile components were identified, including 8 alcohols, 18 esters, 2 acids, 1 carbonyls, 3 hydrocarbons, 2 miscellaneous. Volatile components having fruit aroma were found more in the samples with HSH, while components having fatty and oily characters were more detected in the samples with thermal treatments.

Key words: Fermented rice wine, High shearing homogenization, yeast inactivation, non-thermal treatment, D-value

3.1. Introduction

Fermented rice wine (FRW) is a worldwide product. Varied products are available in many countries. Among them, *Takju* (or *Makgeolli*) is the Korean traditional rice wine with 6–7% alcohol, and it is popularly produced and consumed in most of Asian countries with pasteurized or non-pasteurized form (25). Unlike other alcoholic beverages, *Takju* (TJ) is highly nutritious and functional because it contains proteins, sugars, vitamins, bioactive components, and various organic acids. *Takju* making process involves the use of a fungi starter known as *nuruk*, which saccharifies the rice starch during fermentation. *Nuruk* is a starter culture, made from wheat or grits moistened to make cake-shaped products through pressure and natural fermentation, allowing the growth of various natural types of microorganisms such as fungi, yeast and lactic acid bacteria (23). Since TJ is made through a rough filtration of fermented mash and contains turbid ingredients with many more microbes than wine or beer, its quality can be easily affected by storage conditions (30,36).

The most important microbial groups involved in the fermentation process of rice wine (RW) are mainly lactic acid bacteria and yeasts. When the growth of lactic acid bacteria overcomes the

growth of yeasts, lactic acid fermentation is favored and a final food product with lower pH value is obtained. However, if yeasts become the dominant microorganisms, the final products will have higher pH values. Such microbiological influences are easily found in many processed foods. In case of the olive fermentation, the lactic acid bacteria is dominated in the Spanish style, but the yeast is dominated in the Greek style olives (11,1,14). The main roles of yeasts in the processing of FRW are associated with the production of alcohols, ethyl acetate, acetaldehyde and organic acids components that are relevant for the development of taste and characteristics (24,27). Nevertheless, yeasts may have a negative role as they are responsible for the production of CO_2, clouding of brines, biofilm production and, probably, production of off flavors as well. Especially in the non-pasteurized rice wine (NPFRW), the yeast is able to grow continuously after packing and affects the product quality during storage. Since the yeast in the NPFRW continuously produces CO_2, often the bottle cap is not completely sealed to discharge the gases generated after bottling. It might have more chance to get contamination during storage (16,17). Thus, it is necessary to control the concentration of yeast once the role of yeast is completed during fermentation.

Thermal processing has long been recognized as an effective method to eliminate pathogenic vegetative cells in fluid foods such as juices and extend product shelf life by destroying spoilage microorganisms (28). However, traditional thermal processing methods can cause thermal damages on the components and loss of nutrients. Often it leads to reduce fresh-flavors in many beverages. Therefore low-temperature alternatives are being sought: particular attention has been given to the use of pressure treatment in food preservation. Especially, designing suitable heat treatment process for FRW is very complicated, because the solid particles mainly composed of carbohydrates became gelatinized during heating. The flow properties after heat treatment are fully changed. In addition, the thermal treatment may change the flavors in FRW as well.

High shearing homogenization (HSH), or commercially called microfludization (MF), is an emerging non-thermal technology that has demonstrated capability to inactivate various types of bacteria without significant loss of product quality (2,3,31). Disruption cells in HSH, or MF, is achieved by passing a cell suspension under high pressure through an adjustable, restricted orifice discharge valve. The major parameters determining efficiency are operating pressure and number

of circulations through the valve (6), suspension temperature (15), and homogenizer valve design (19). Disruption of cells results from non-specific tearing apart of the cell wall. Impingement is an important mechanism of disruption, particularly for yeasts (7,8).

Applying the HSH to FRW is worth to be investigated, since HSH could be a non-thermal processing which may avoid thermal degradation of FRW while reducing the yeast level. HSH has not been applied in the FRW. In this study, HSH was used to reduce the concentration of yeast cell in the FRW and the effects of HSH on the microbiological and flavor characteristics were compared to those from thermal treatments. In addition the equivalent effect of non-thermal treatment from HSH to the thermal treatment was estimated.

3.2. Materials and Methods

3.2.1. Rice wine (RW)

The RW was prepared by CJ Cheiljedang (Seoul, Korea). Making process of the rice wine was based on Seo et al. (29). The alcohol content in the samples was adjusted to 6% by dilution with distilled water. Samples were stored at 4 °C during the experiment.

3.2.2. High shearing homogenization (HSH)

HSH was conducted for RW with high shear fluid processor (Picomax-MN400, Micronox, Gyeonggi, Korea). The schematic diagram of the HSH system was described in Fig. 1. The HSH system provides applicable pressure from 3000 psi to 40000 psi. The interaction chamber contains a tube (diameter 35 mm, length 90 mm) and the fluid passes through the tube while maintaining the same pressure drop. The HSH process can be repeatedly performed by continuous circulation of the fluid in the high shear fluid processor. In this study, the number of circulation (N) and the applied pressure (P) were varied to investigate the effect of HSH on the microbiological characterization. P was varied at 7000, 10000, 15000, 20000, and 25000 psi. The number of circulation (N) was varied 1, 2, 3, 4 and 5 at each applied pressure.

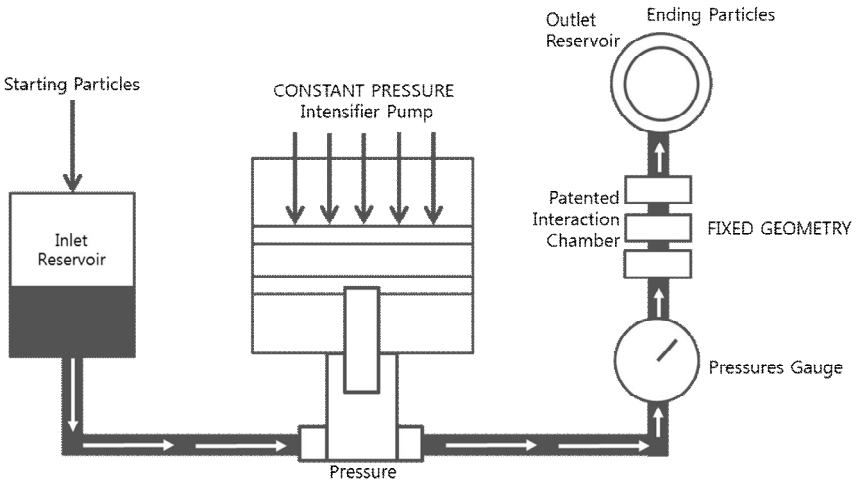


Figure 1. Schematic diagram of the experimental system used for high shearing homogenization.

3.2.3. Pasteurization in the cab-tube

Pasteurization of FRW was undertaken, using the Holder pasteurization (LTLT pasteurization) technique, following the procedure of the Human Milk Banking Association of North America (HMBANA) (32). The 10 ml samples of FRW from each participant were placed in 13 ml polypropylene tubes, which were then submerged (until the water reached a level of approximately 2 cm over the level of FRW) in a shaking water bath that was preheated to 50, 55, 60, and 65 °C (Table 1). The water did not touch the caps, to avoid microbiological contamination of FRW. All tubes in the batch contained the same amount of FRW. A control bottle, containing the same volume of FRW, was fitted with a calibrated thermometer to register FRW temperature during heat processing. This bottle was placed in the water bath with all the other tubes and positioned in the center of them. It underwent the same process as the rest of the batch at all times. The thermometer was located at a fixed position so that approximately 25% of the FRW volume was below the measuring point of the thermometer (to measure the temperature in the "cold point"). Once the temperature of the monitored control bottle had reached at the target pasteurization temperature, the heat treatment

continued for 20 to 40 min up on the target pasteurization temperature. The FRW temperature was monitored and recorded. After the heat processing, the FRW was rapidly cooled in an ice bath.

Pasteurization temperature (°C)	Pasteurization time (min)
50	0, 10, 20, 30, 40
55	0, 7, 15, 22, 30
60	0, 5, 10, 15, 20
65	0, 5, 10, 15, 20

Table 1. Pasteurization temperature and pasteurization time of fermented rice wine in a cap-tube test.

3.2.4. Total viable count of yeast in FRW

Yeast were cultured at 3M™ Petrifilm™ yeast and mold count plates (3M, MN, USA) at 20 °C incubator under aerobic conditions for 68 h. Yeast colonies were blue–green and formed small defined colonies by 3M directions. The viable cell counts of yeast were determined by colony forming unit (CFU)/ml in independent triplicate experiments. To assess sub-lethal injury caused by each treatment, the reduction factor was introduced. The logarithm of the reduction factor (log RF) was calculated as:

$$\text{Log RF} = \log (\text{CFU before treatment} / \text{CFU after treatment}) \quad (1)$$

3.2.5. Polarizing Microscope

To compare the degree of disruption of yeast caused by a treatment, morphological characteristics of yeast were observed using a High Resolution Polarizing Microscope (Axio Skop II, Carl Zeiss Vision KK Co., Germany). The diluted FRW were dyed with methylene blue solution and then prepared for examination by polarizing microscopy.

3.2.6. Analysis of volatile components

Volatile flavor components of FRW were studied by solvent assisted flavor evaporation (SAFE) and gas chromatography-tadem mass spectrometry (GC-MS/MS). The samples (30mL) were extracted with 60 ml of methylene chloride which was re-distilled before use. After 0.1 ml of 100 ppm dodecanoic acid methyl ester (v/v, in dichloromethane) was added as an internal standard, the sample which was suspended in methylene chloride was mixed with a magnetical stirrer at 3000 rpm for 10 min and then filtered (paper No. 41, Whatman, Maidstone, UK) under a vacuum. Volatile components were then separated from the non-volatiles using high-vacuum sublimation (HVS) at an operating vacuum that was typically below 10^{-5} Torr (4). The extract was dehydrated over anhydrous sodium sulfate, evaporated on a Vigreux column in a water bath at 40 °C, and then concentrated under a slow stream of nitrogen gas to obtain a final volume of 0.1 ml. GC-MS conditions are presented in Table 2. GC–MS analysis was performed using an Agilent 7890A gas chromatography-5975 mass selective detector (GC–MSD) (Agilent Technologies Inc., Palo Alto, CA). Volatile components were positively identified by comparing their mass spectra and RIs with those of the authentic components. When

standards were not available, components were tentatively identified with the aid of the Wiley 275 Imass spectral database (Hewlett-Packard, 1995). All key odorants were positively identified by comparing their mass spectra, linear retention indices (RIs), and aroma properties perceived at the sniffing port with those of authentic standards. The RI of each component was calculated using n-paraffins C_7–C_{22} as external references (34). The semi-quantitative analysis of volatile components was performed by comparing their peak areas to that of the internal standard component (0.1 ml of 1000 ppm 2-ethyl-1-hexanol, w/v) on the GC–MS total ion chromatogram.

Column	DB-5 (30 m length, 0.25 mm i.d. × 0.25 μm film thickness)
Carrier gas	Helium 0.8 mL/min, constant flow
Inlet temp.	230 °C
Transfer line temp.	250 °C
Oven temp.	40°C,5 min → 4°C/min →120°C → 16°C/min, → 220°C, 10min
Injection volume	1 μL
Split ratio	10:1
Mass scan range	35-550 m/z
Scanning rate	2.86 scan/sec
Ionization energy	70 eV

Table 2. Operating condition of GC and GC-MS for analyses of volatile components.

3.3. Results & Discussion

3.3.1. Effect of HSH on viability of yeast

To assess the influence of operating pressure and number of circulation (N) for loss of viability on high shearing homogenization, the changes of yeast viable cell counts by each treatment were shown in Figure 2 and 3.

As regards to the effect of pressure on the yeast cells in FRW, the reduction of yeast cells appeared insignificant for homogenizing pressure up to 20000 psi, because a reduction less than 1 log value of CFU is not relevant microbiologically or in terms of processes. The maximum reduction at 25000 psi was ca. 1 log value of CFU (Fig. 2).

The effects of the number of circulation (N) at different pressure on the reduction of yeast cells were shown in Fig. 3. The reduction rate of yeast cells by the number of circulation showed an applied pressure dependence. When the pressure is lower than 10000 psi, the reduction was not significant, but at > 15000 psi the reduction rate dramatically increased. At 25000 psi, each circulation clearly showed a log scale reduction of yeast cells. At N = 5 at 25000 psi, the reduction of yeast cells was over ca. 4 log cycle. It clearly demonstrated that the effect of pressure was not significant to reduce

the yeast cells in FRW, but as the number of circulation increased, the reduction dramatically increased. The microscope images of the intact yeast and post-homogenization disrupted yeast were illustrated in Fig. 4. The polarizing microscope experiments indicated that a large number of yeast was considerably disrupted after 3 circulations of homogenization at 25000 psi.

To assess loss of viability caused by a treatment, the reduction factor (eqn. 1) was introduced. The reduction factor (log RF) can quantify the effect of pressure and the number of circulation on the reduction of yeast cells in FRW. The slopes of each treatment indicated the magnitude of reduction of yeast cells using the circulation of HSH at different pressure. In Fig. 5, the log RF clearly increased as the N increased at each pressure. In addition, the higher pressure showed the higher slope reflecting the rate of reduction of yeast. Our study demonstrated that increasing operating pressure was thus concluded to significantly enhance the efficacy of high shearing homogenization in disrupting the yeast cells. It has been observed that several successive circulations of high shearing homogenization have an additive effect on viability reduction. Recently a similar result was reported that the microalgal cells to produce biofuel was significantly disrupted by

successive circulations of fluid through the HSH (13). As a similar study, the HSH was applied to control the growth of foodborne moulds in tomato juice (5). Indeed, under the conditions used for tomato juice, i.e. homogenization pressure up to 150 MP, inactivation of the moulds by a single homogenization treatment remained under 1 log unit, which is far insufficient for applications such as food pasteurization. The level of inactivation increased to almost 3 log units after 3 circulations of homogenization, and can probably be further increased by applying additional treatments. In fact, a multi-step processing seemed to influence the effectiveness of HSH in many food application (26,33,35). The reduction of cells using HSH might be non-specific so that disruption characteristics such as pressure or number of circulation vary according to microbial species (3,10,18,33,35). Disruption resulted from non-specific tearing apart of the cell wall. Impingement is an important mechanism of disruption, particularly for yeasts (7,8). The cell disruption is achieved through high-pressure impingement of accelerated cellular jet on the stationary valve surface as well as through pressured-drop-induced shear stress that the cell suspension experiences as it circulations from the valve to the chamber (13). Because of their larger size and different cell wall structure, disruption

of yeasts is generally easier than bacteria. These results were successfully confirmed by Bevilacqua et al. (3). It clearly demonstrated that the HSH for FRW is very effective to reduce the cell viability by disrupting cell walls.

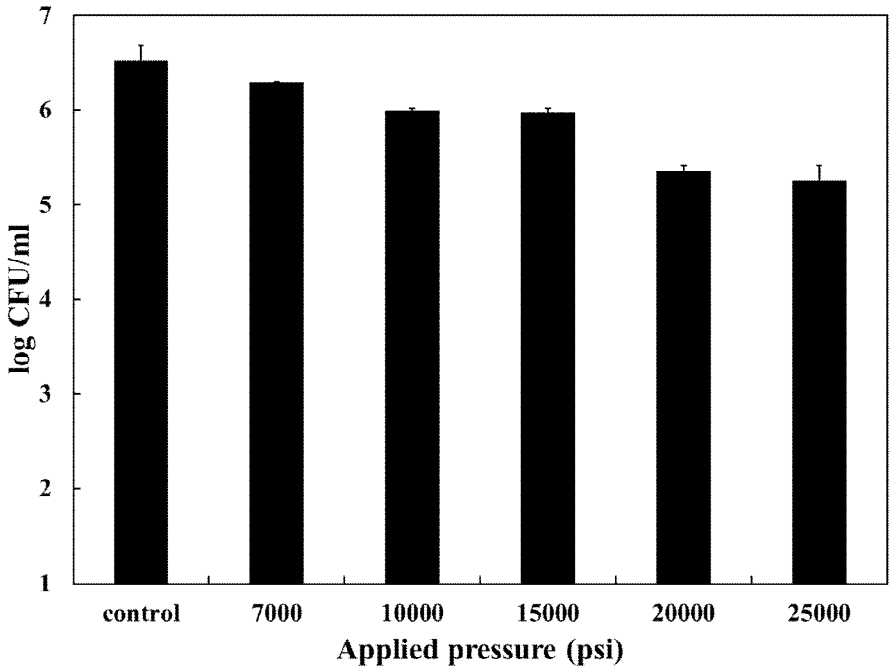


Figure 2. Changes of yeast viable cell counts by pressure treatment.

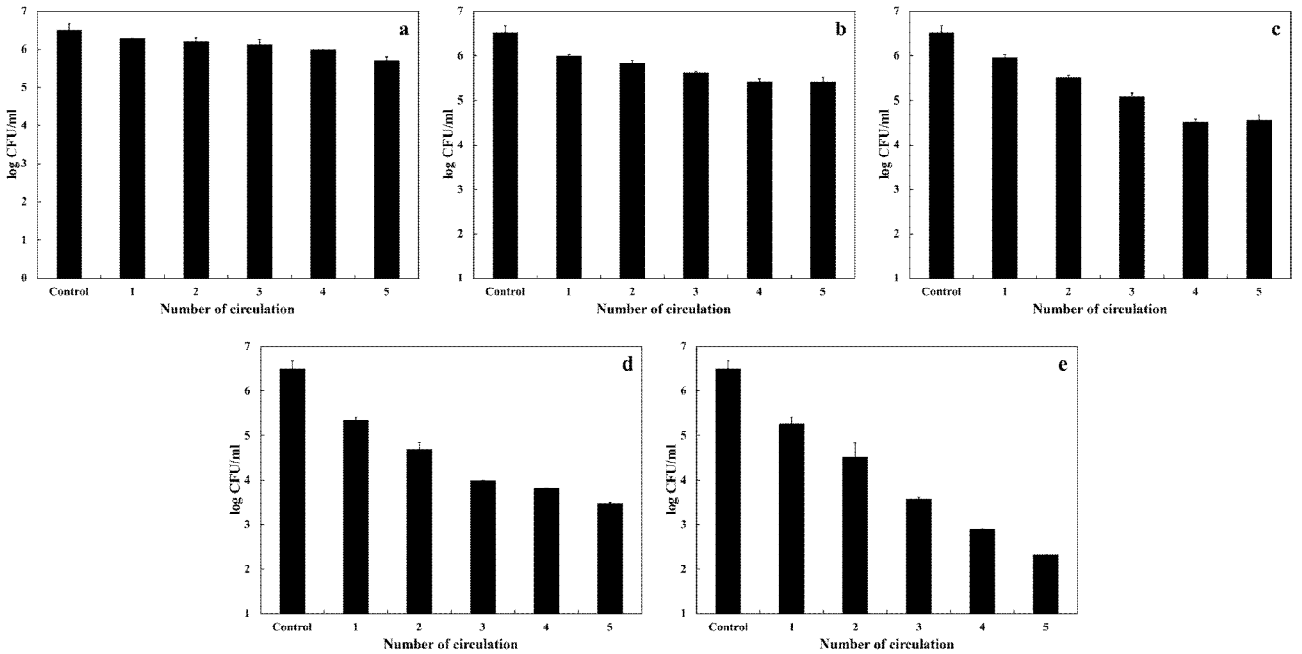


Figure 3. Changes of yeast viable cell counts by number of circulation.

(a) 7000psi, (b) 10000psi, (c) 15000psi, (d) 20000psi, (e) 25000psi.

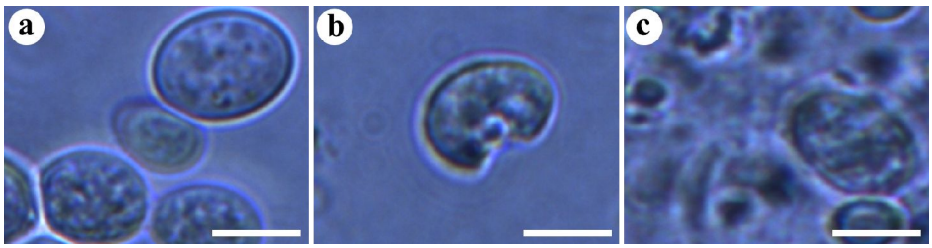


Figure 4. Microscopic images of the yeast: intact cells (a) and disrupted cells after three(b) and five(c) circulations through the high shearing homogenizer. Bar: 25 μm.

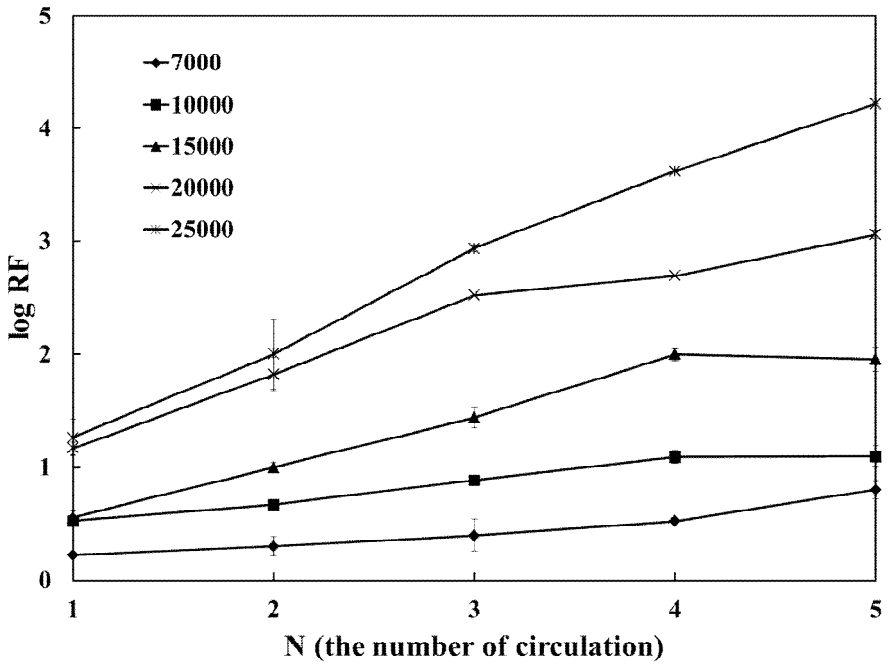


Figure 5. Effect of number of circulation (N) on the log RF at different pressure.

3.3.2. Effect of thermal treatment on the inactivation of yeast

To investigate the effect of thermal treatment on the inactivation of yeast in the FRW, the cab-tube containing the FRW were thermally treated in the high temperature region (50 to 65 °C). The prototypical thermal inactivation curves showing linear slopes between the log scale of yeast numbers and the time for the thermal treatment were obtained and the results were summarized in Table 3. The slope of each curve indicates the D-value of yeast at different temperature. The D-value of yeast is 28 min at 50 °C and it decreased to 4.18 min at 65 °C. The Z-value of yeast used in this study was estimated to be 19.1 °C. As shown in Table 3. The level of yeast in the FRW was decreased from 10^6 to 10^1 or 10^{-2} after thermal treatment. The inactivation rate significantly increased above 55 °C. The more thermal inactivation was found at the higher temperature. Furthermore, the longer thermal treatment time showed the higher inactivation. Our result showed somewhat different results compared to the traditional thermal inactivation process of yeast (21). According to Lee et al. (21), the inactivation of yeast observed during coming-up time (CUT), but no more inactivation was consequently observed. Our result showed the

inactivation continuously increased.

The part of FRW samples was placed in the cap-tube to evaluate the thermal properties of yeast in the FRW used for this study. The D-values of yeast in the high temperature region (70 to 100 °C) were estimated. D-value at 80 and 100 °C were evaluated to be 0.75 min (45 s) and 0.067 min (4 s), respectively. The D-value of yeast at 65.6 °C was reported to be 0.5-1.0 min (21). Since the yeast in FRW was not in the pure water but mixed with acids, alcohols, and other components, it is difficult to compare the D-values directly from the value found in other studies. The D-value estimated from this study was about two times higher than that from Lee et al. (21), but the Z-value from this study was close to the value in Lee et al. (21).

Temp. (°C)	D-value
70	2.509
75	1.373
80	0.751
85	0.411
90	0.225
95	0.123
100	0.067

Table 3. The estimated D-value (in min) of yeast in FRW at different temperatures in cap-tube heating.

3.3.3. Comparison of HSH and pasteurization

The significance of HSH on the inactivation of yeast in FRW was compared with the pasteurization, i.e., a thermal treatment. The D-value and Z-value from HSH were introduced. The time and the temperature in pasteurization were qualitatively equivalent to the number of circulation (N) and the applied pressure in HSH, respectively. The results of comparison were shown in Fig. 6. The data in Fig. 6 demonstrated that the physical meaning of D and Z were suitably applicable for HSH.

According to our result, the number of yeast (10^2 CFU) after 5 circulations at 25000 psi was equivalent to those for 16 min at 65 °C. In addition, it can be estimated to be equivalent to pasteurize for 3 min at 80 °C. In our study, the chemical analysis to compare the thermal treatment and HSH were conducted using samples treated 5 circulations for 25000 psi and 3 min for 80 °C. The results of GC analysis for volatile components in FRW were shown in Table 4. In Table 4, GC1, GC2, and GC3 indicated the samples from the control, i.e. FRW without applying HSH or pasteurization, HSH, and pasteurization, respectively.

Thirty-four volatile components were identified by GC–MS in

FRW (Table 4) and were grouped as 8 alcohols, 18 esters, 2 acids, 1 carbonyl, 3 hydrocarbons, and 2 miscellaneous components. Qualitative differences were observed in isobutyl acetate, ethyl 1-hydroxy-3-phenylpropanoate, and decanoic acid. Quantitative differences were found in 2-methyl-1-butanol, benzeneethanol, ethyl butyrate, ethyl octanoate, ethyl decanoate, ethyl tetradecanoate, ethyl linoleate, and ethyl oleate. High contents of 3-methyl-1-butanol and benzeneethanol were detected in all samples. 3-Methyl-1-butanol (banana-like aroma) is the main constituent of fusel oil. They form in the course of alcoholic fermentation as by-products of yeast metabolism, and serve as flavor and aroma carriers in beer, wine and spirits (22).

Benzeneethanol occurs widely in nature, being found in a variety of essential oils. 3-methyl-1-butanol were detected a similar quantity in GC2 and GC3, but benzeneethanol with floral, sweet, green flavor characteristics was found relatively lower levels in GC2. Relatively low amount of 2,3-butanediol(DL), an aliphatic dihydric alcohol usually found in the fermented beverages, such as wine, beer, and rice wine, were detected in GC3.

A group of esters were identified as wells. Ethyl decanoate,

isoamyl acetate and ethyl caproate which show floral and fruity characters were found in all samples but particularly higher amount were found in GC2 than in GC3. These components contribute positively to the overall FRW quality, and most of them have a mature flavor and fruity aroma that contribute to the fruity and floral sensory properties of FRW. Esters, such as ethly caproate and isoamyl acetate, are widely found in a variety of food products (9). In fermented beverages such as wine and beer, they are frequently in trace amounts, such that individually they are often below aroma threshold concentrations. As such, esters are extremely important for the flavor profile of fermented beverages (12).

The highest amount of ethyl lactate generally having cookie-like and rum-buttery characters was detected in the GC3 followed by GC1 and GC2. GC2 showed the lowest level of ethyl tetradecanoate, ethyl linoleate, and ethyl oleate which generally show oily and fatty characters. Relatively higher amount of these components were detected more in GC3 compared to GC2 and GC1. According to our analysis, GC3 showed relatively high amount of volatile components having oily, fatty and waxy characters. In addition, ethyl oleate and ethyl linoleate which found in GC3 are generally associated with the

odor descriptor “rancid” and it may cause a negative impact on the quality of FRW during storage and distribution.

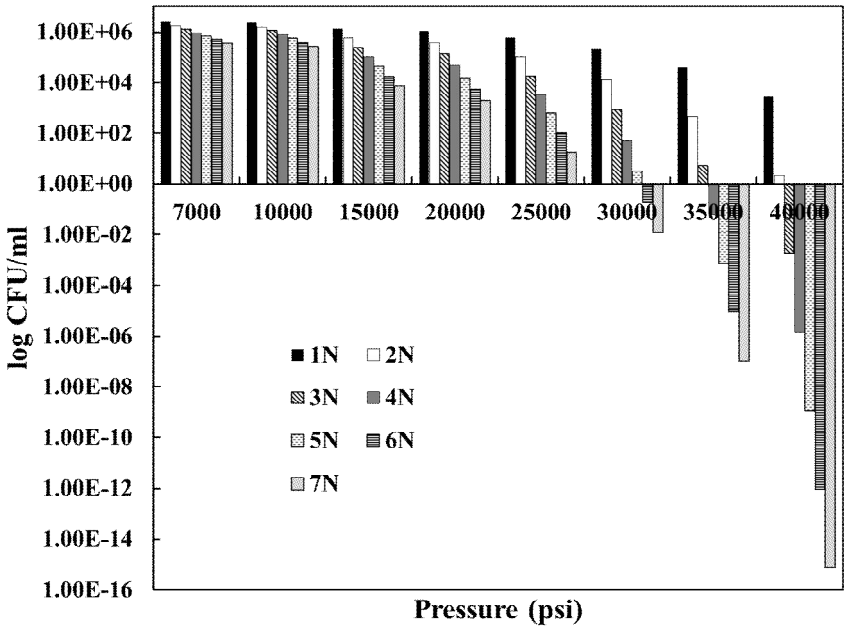


Figure 6. Comparison of yeast reduction at different pressure with different number of circulation.

No.	RI[1)]	Components	Relative Peak Area(%)[2)]		
			GC1	GC2	GC3
Alcohols					
1	<800	3-methyl-1-butanol	8.169±0.749	7.930±0.712	7.847±0.203
2	<800	2-methyl-1-butanol	0.838±0.144	0.398±0.240	1.024±0.057
3	<800	1,3-butanediol	0.152±0.007	0.145±0.032	0.115±0.017
4	<800	2,3-butanediol	0.018±0.006	0.019±0.004	0.010±0.009
5	837	3-ethoxy-1-propanol	0.544±0.059	0.575±0.082	0.569±0.136
6	978	3-(methylthio)-1-propanol	0.163±0.014	0.167±0.022	0.158±0.012
7	1118	benzeneethanol	7.123±0.599	6.749±0.144	7.694±0.020
8	1505	2,4-bis(1,1-dimethylethyl)-phenol	0.011±0.002	-	0.022±0.007
Esters					
9	<800	isobutyl acetate	-	0.025±0.001	0.025±0.001
10	802	ethyl butyrate	0.023±0.007	0.016±0.000	0.014±0.001
11	811	ethyl lactate	0.102±0.008	0.123±0.012	0.114±0.016
12	876	isoamyl acetate	0.194±0.023	0.212±0.006	0.185±0.010
13	933	ethyl 3-hydroxybutyrate	0.038±0.003	0.041±0.004	0.038±0.003
14	998	ethyl caproate	0.028±0.001	0.036±0.006	0.027±0.008
15	1057	ethyl-4-hydroxybutanoate	1.244±0.050	1.192±0.114	1.325±0.175
16	1177	diethyl succinate	0.020±0.005	0.017±0.001	0.021±0.002
17	1193	ethyl octanoate	0.036±0.001	0.041±0.001	0.017±0.002
18	1257	phenethyl acetate	0.092±0.007	0.094±0.002	0.094±0.001
19	1393	ethyl decanoate	0.061±0.016	0.117±0.012	0.071±0.009
20	1441	ethyl 1-hydroxy-3-phenylpropanoate	0.021±0.018	-	0.039±0.006
21	1590	ethyl dodecanoate	0.051±0.028	0.054±0.008	0.060±0.010
22	1753	2-oxohexanedioic acid	0.223±0.021	-	0.021±0.009
23	1795	ethyl tetradecanoate	0.110±0.104	0.056±.009	0.127±0.024
24	1991	ethyl hexadecanoate	0.360±0.236	0.123±0.018	0.346±0.044

25	2150	ethyl linoleate	0.021±0.007	0.015±0.002	0.048±0.010
26	>2200	ethyl oleate	0.031±0.006	0.020±0.002	0.072±0.004
Acids					
27	1171	octanoic acid	0.062±0.007	0.011±0.003	0.031±0.008
28	1367	decanoic acid	0.030±0.002	-	-
Carbonyls					
29	907	dihydro-2(3H)-furanone	0.031±0.002	0.035±0.003	0.037±0.005
Hydrocarbons					
30	1197	dodecane	0.018±0.007	0.012±0.001	0.012±0.005
31	1398	tetradecane	0.009±0.000	0.006±0.001	0.009±0.001
32	1473	heptadecane	0.027±0.004	0.025±0.003	0.040±0.023
Miscellaneous					
33	866	xylene	-	0.011±0.001	0.012±0.003
34	1250	1,3-bis(1,1-dimethylethyl)benzene	0.131±0.008	0.185±.010	0.189±0.107

1) Retention indices were determined using *n-paraffins* C_7-C_{22} as external standards
2) Relative peak area (peak are of component/peak area of internal standard)
3) Flavor-Base 2010 Professional, leffingwell & Associates ; Aroma threshold value is odor threshold in water

Table 4. Volatile flavor components of FRW by GC and GC-MS.

3.4. Conclusion

To reduce the yeast level in FRW, HSH was applied as a non-thermal treatment. The yeast levels were significantly reduced by adjusting applied pressure and the number of circulation of treatment. At over 20000psi of applied pressure, about 1 log cycle of reduction was observed and there was no significant reduction by increasing the applied pressure. But, as the number of circulation of treatment increased the reduction of yeast level became significant. Especially at higher applied pressure, the reduction dramatically increased and showed higher dependence of the number of circulation. D-value and Z-value of the yeast in FRW were determined from the thermal treatment. The D-values in the high temperature region (70 to 100 °C) were estimated. D-value at 80 and 100 °C were 0.75 min (45 s) and 0.067 min (4 s), respectively. The non-thermal treatment at 25000 psi with 5 circulations were equivalent to the thermal treatment at 80 °C for 3 min. Total 34 volatile components from the control, HSH (non-thermal treatment), and thermal treatment were found. The volatile components from HSH (GC2) showed higher amount of esters, such as ethyl caproate, and isoamyl acetate, having the fruit aroma characters than those from the thermal treatment (GC3). The volatile components

having the oily and fatty characters, such as ethyl tetradecanonate, ethyl linoleate, and ethyl oleate, were detected more from the thermal treatment (GC3) than from the HSH (GC2). It might imply that the non-thermal treatment using HSH could minimize the quality changes of FRW while could reduce the yeast level in FRW.

3.5. References

1. Arroyo-López FN, Querol A, Bautista-Gallego J, Garrido-Fernández A. Role of yeasts in table olive production. International journal of food microbiology. 2008;128:189-196.

2. Bevilacqua A, Cibelli F, Corbo MR, Sinigaglia M. Effects of high-pressure homogenization on the survival of *Alicyclobacillus acidoterrestris* in a laboratory medium. Letters in applied microbiology. 2007;45(4):382-386.

3. Bevilacqua A, Costa C, Corbo MR, Sinigaglia M. Effects of the high pressure of homogenization on some spoiling micro-organisms, representative of fruit juice microflora, inoculated in saline solution. Letters in applied microbiology. 2009;48:261-267.

4. Cho IH, Choi HK, Kim YS. Difference in the volatile composition of pine-mushrooms (Tricholoma matsutake Sing.) according to their grades. Journal of Agricultural and Food Chemistry. 2006;54:4820-4825.

5. Corbo MR, Bevilacqua A, Campaniello D, Ciccarone C, Sinigaglia M. Use of high pressure homogenization as a mean to control the growth of foodborne moulds in tomato juice. Food Control. 2010;21(11):1507-1511.

6. Engler CR. Disruption of microbial cells in comprehensive biotechnology. In M. Moo-Young, & C. L. Cooney (Eds.). 1985;Vol. 2 (pp. 305–324). Pergamon, UK.

7. Engler CR, Robinson CW. Disruption of *Candida utilis* cells in high pressure flow devices. Biotechnology and Bioengineering. 1981;23(4):765-780.

8. Engler CR, Robinson CW. Effects of organism type and growth conditions on cell disruption by impingement. Biotechnology Letters. 1981;3(2):83-88.

9. Gatfield IL. Bioreactors for industrial production of flavours: Use of enzymes. In R. L. S. Patterson, B. V. Charlwood, G. MacLeod, & A. A. Williams (Eds.). Bioformation of flavours. 1992;(pp. 171-185). The Royal Society of Chemistry, Great Britain.

10. Geciova J, Bury D, Jelen P. Methods for disruption of microbial cells for potential use in the dairy industry - a review. International Dairy Journal. 2002;12:541-553.

11. Gómez AH, García P, Navarro L. Elaboration of table olives. Grasas Y Aceites. 2006;57:86-94.

12. Gurbuz O, Rouseff JM, Rouseff RL. Comparison of aroma volatiles in commercial merlot and Cabernet Sauvignon wines using gas

chromatography–olfactometry and gas chromatography–mass spectrometry. Journal of Agricultural and Food Chemistry. 2006;54: 3990-3996.

13. Halim R, Harun R, Danquah MK, Webley PA. Microalgal cell disruption for biofuel development. Applied Energy. 2012;91:116-121.

14. Hernández A, Martín A, Aranda E, Pérez-Nevado F, Córdoba MG. Identification and characterization of yeast isolated from the elaboration of seasoned green table olives. Food Microbiology. 2007;24:346-351.

15. Hetherington PJ, Follows M, Dunnill P, Lilly MD. Release of protein from baker's yeast (*Saccharomyces cerevisiae*) by disruption in an industrial homogenizer. Transactions of the Institution of Chemical Engineers. 1971;49:142–148.

16. Jeong JW, Park KJ, Kim MH, Kim DS. Quality characteristics of takju fermentation by addition of chestnut peel-powder. The Korean Society of Food Preservation. 2006;13:329-336.

17. Joung EJ, Paek NS, Kim YM. Studies on Korean *takju* using the by-product of rice milling. The Korean Society of Food and Nutrition. 2004;17:199-205.

18. Kelemen MV, Sharpe JE. Controlled cell disruption: a comparison

of the forces required to disrupt different microorganisms. Journal Cellular Science. 1979;35:431-441.

19. Keshavarz-Moore E, Hoare M, Dunnill P. Disruption of baker's yeast in a high-pressure homogenizer: New evidence on mechanism. Enzyme and microbial technology. 1990;12:764-770.

20. Kim JY, Kim D, Park P, Kang H, Ryu EK, Kim SM. Effects of storage temperature and time on the biogenic amine content and microflora in Korean turbid rice wine, *Makgeolli*. Food Chemistry. 2011;128:87-92.

21. Lee CH, Tae WT, Kim GM, Lee HD. Studies on the pasteurization conditions of *Takju*. Korean journal of food science and technology. 1991;23:44-51.

22. Lee HS, Lee TS, Noh B.S. Chemistry/Analysis : volatile flavor components in the mashes of *takju* prepared using different yeasts. Korean Journal of Food Science and Technology. 2007;39:593-599.

23. Lee JH, Yu TS. Identification and characterization of lactic acid bacteria isolated from *Nuruk*. Korean Journal of Biotechnology and Bioengineering. 2000;15:359-365.

24. Lee SM, Lee TS. Effect of roasted rice and defatted soybean on the quality characteristics of *takju* during fermentation. J. Natural Science.

2000;12:71-79.

25. Lee SR. Korean Fermentative Food. Ewha Women's University publishing department. 1986;pp. 224-294.

26. Moroni O, Jean J, Autret J, Fliss I. Inactivation of lactococcal bacteriophages in liquid media using dynamic high pressure. International Dairy Journal. 2002;12:907-913.

27. Park CS, Lee TS. Quality characteristics of *takju* prepared by wheat flour *nuruks*. Korean Journal of Food Science and Technology. 2002;34:296-302.

28. Pathanibul P, Taylor TM, Davidson PM, Harte F. Inactivation of *Escherichia coli* and *Listeria innocua* in apple and carrot juices using high pressure homogenization and nisin. International journal of food microbiology. 2009;129:316-320.

29. Seo DH, Jung JH, Kim YR, Ha SJ, Kim YC, Park CS. Identification of lactic acid bacteria involved in traditional Korean rice wine fermentation. Food Science and Biotechnology. 2007;16:994-998.

30. Song JC, Park HJ. *Takju* brewing using the uncooked germed brown rice at second stage mash. Journal of the Korean Society of Food Science and Nutrition. 2003;32:847-854.

31. Taylor TM, Roach A, Black DG, Davidson PM, Harte F.

Inactivation of *Escherichia coli* K-12 exposed to pressures in excess of 300 MPa in a high pressure homogenizer. Journal of Food Protection. 2007;70:1007-1010.

32. Updegrove K. Human Milk Banking in the United States. Newborn and Infant Nursing Reviews. 2005;5:27-33.

33. Vachon JF, Kheadr EE, Giasson J, Paquin P, Fliss I. Inactivation of food borne pathogens in milk using dynamic high pressure. Journal of Food Protection. 2002;65:345-352.

34. van Den Dool H, Dec Kratz P. A generalization of the retention index system including linear temperature programmed gas - liquid partition chromatography. Journal of Chromatography A. 1963;11:463-471.

35. Wuytack EY, Diels AMJ, Michiels CW. Acterial inactivation by high pressure homogenization and high hydrostatic pressure. International journal of food microbiology. 2002;77:205-212.

36. Yang JY, Lee KH. Shelf-life and microbiological study of *Sansung Takju*. Korean Journal of Food Science and Technology. 1996;28:779–785.

Chapter 4.

Effects of High Shearing Process on the Fluid Stability of a Colloidal Dispersion from Coarse Filtered *Makgeolli*

Abstract

We studied the effect of high shearing process (HSP) on the flow behavior of a colloidal dispersion obtained from the coarse filtered rice fermentation. The translucency, a key major characteristic of the dispersion, is kinetically changed due to the settling of particles. To delay the phase separation of particles, HSP was applied to the colloidal dispersion. The applied pressure of HSP was varied from 7000 psi to 25000 psi, and for 7000 psi and 25000 psi the process was repeated. The mean particle sizes were dropped from 129.69 μm to about 5.24 μm when HSP was applied. In addition to the particle size, viscosity, volume fraction, settling velocity, turbidity and ζ-potential were measured to understand the flow behavior of the dispersion. The viscosity and the volume fraction were inversely proportional to the particle size. The settling velocities were reduced by applying HSP from ~4 x 10^{-2} m/s to ~3 x 10^{-7} m/s. The phase separation from solid particles in the dispersion was significantly delayed by applying HSP.

Key words: dispersion, microfluidization, settling velocity, particle size, phase separation

4.1. Introduction

A colloidal system consists of two separate phases: a dispersed phase (or internal phase) and a continuous phase (or dispersion medium) in which the colloid is dispersed. Colloidal dispersion is found in many beverages. For example, milk is an emulsified colloid of liquid butter fat globules dispersed within a water-based solution. The translucency, commercially called 'milky color', observed in the colloidal beverage is an important quality factor for consumer preference. The particles in the dispersed phase scattering light mainly contribute to the translucency. However, the solid, or solid-like, particles in the dispersed phase generally show the density differences from the continuous phase, and eventually it will cause a phase separation during the storage and/or serving.

Rice is a globally consumed agricultural product. The nutritional benefits from rice have been well studied (2), and rice is widely applied in the processed food, such as bakery, confectionary, candy, rice cake, and alcoholic and non-alcoholic beverage. Most of the rice based beverages, such as rice milk and rice wine, have translucent milky color which is very favorable to consumers. A coarse filtration after fermentation is applied to many rice based alcoholic beverage to

obtain the translucency. Recently, fermented rice wine (FRW) is world widely distributed. *Takju* in Korean or *Nigorijake* in Japanese, is the most popular type of rice wine currently distributed world widely. Most of FRW contain 6 to 7 % of alcohol brewed by the yeast *Saccharomyces cerevisiae*. The manufacturing process is similar to grape wine but most of FRW use a coarse filtration (4). In addition, the difference from the grape wine-making process, in which yeast converts natural sugars of the grapes into alcohol and carbon dioxide that bubble and then dissipate, is that the brewing process for FRW involves the use of a mold known as *nuruk* (7), which saccharifies the rice starch during fermentation. *Nuruk* is a starter culture made from the wheat flour/grits (6). Due to the coarse filtration, the yeast and starches in the final products were not separated and not decomposed completely during the manufacturing process, and the yeast is still alive in the final products. Such different processes produced high amount of solid contents which mainly consists of yeast, fibers, and starch.

The solid particles dispersed in the coarse filtered fermented rice wine (CFFRW) plays a major role to the translucent attribute of CFFRW, which is strongly related to the sensory perception. However, due to the density difference between the solid particles and the

medium, the solid particles are precipitated and caused a phase separation during the storage. In the worst case, during serving or drinking of CFFRW, the phase separation occurs in the bottle or in the drinking glass. Thus, it is necessary to shake the bottle or the wine glass before serving or during drinking to maintain a favorable translucent milky characters and its unique mouth perception (15).

According to the submerged principle, high shearing occurs when a fluid passes through a narrow gap with a constant pressure difference (17). Microfluidization (MF) is a technology for the high shearing process (HSP), which is based on the submerged jet principle. Due to possibility of obtaining very small particles or droplets, the use of HSP was investigated and proposed in many different fields, such as homogenization of milk (8) and production sterile-filterable blood substitutes (1). When the size of particles is reduced, the sedimentation rate decreased. Consequently, maintaining a constant mass of particles in a suspension while reducing the particle size of the solid phase leads to an increase in the number of particles in the system, and the viscosity of suspension increased as a result.

There have been very few studies to solve such phase separation problems in the CF fermented beverage. Xanthan was added

in the dispersion to delay the phase separation (5), but the weak crosslinks from xanthan change the viscosity of beverage and generally drops the hedonic rate in the sensory evaluation. In addition, adding ingredients to the beverage may cause a negative impression to consumers. The HSP using MF is a continuous process and its entire system is maintained at the room temperature. Applying HSP might reduce the size of the solid particle so that the phase separation of dispersion from the coarse filtered fermentation maybe delayed. In this study, the colloidal dispersion from the coarse filtered fermentation using rice and *Saccharomyces cerevisiae* was produced according to the standard manufacturing process in the pilot product scale and the sample fluid was used to investigate the effect of high shearing process on the particle size and flow behavior of a colloidal dispersion.

4.2. Materials and Methods

4.2.1. Preparation of a colloidal dispersion

The colloidal dispersion from coarse filtered rice fermentation was prepared by CJ Cheiljedang (Seoul, Korea). Making process of CFFRW was based on Seo et al. (14). The alcohol content in the samples was adjusted to 6% by dilution with distilled water. Samples were stored at 4 °C during the experiment.

4.2.2. High shearing process (HSP)

High shearing process (HSP) was conducted for the colloidal dispersion samples using the high shear fluid processor (Picomax-MN400, Micronox, Gyeonggi-do, Korea). The schematic diagram of HSP system was described in Fig. 1. The applicable pressure of this equipment is ranged from 3000 psi to 40000 psi. The interaction chamber contains a tube (diameter 35 mm, length 90 mm) and the fluid passes through the tube while maintaining the same pressure drop. The HSP can be repeated by continuously circulating the fluid in the system. In this study, the number of circulation (N) and the applied pressure (P) were varied to investigate the effect of HSP on the flow behavior and the microbiological characterization. Different P (7000, 10000, 15000,

20000, and 25000 psi) were applied, To investigate the effect of circulation, N was varied 1, 2, and 3 for the minimum (7000 psi) and the maximum (25000 psi), and for other applied pressures N was remained to 1.

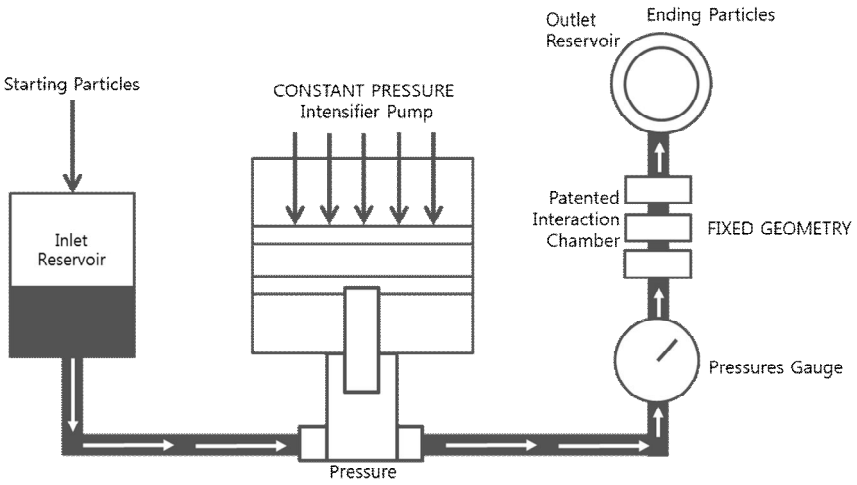


Figure 1. Schematic diagram of the experimental system used for high shearing process (HSP).

4.2.3. Analysis of the particle size

All the particle sizes were measured using laser diffraction particle size analyzer (Masterisizer 2000, Malvern Instrument Ltd., Worcestershire, UK) in Central Laboratory Kangwon National University. The samples after HSP were stored at 5 ℃ for 5 days to investigate the change of particle size during storage. The particle sizes of all the treatments were measured at 5 days of storage.

4.2.4. Zeta potential

ζ-potential was measured by the electrophoretic light scattering spectrophotometer(ELS-8000, Otsuka electronics Co. Ltd., Osaka, Japan). The samples were diluted by adding distilled water 1000 times of the sample amount in volume. The temperature during measuring was maintained at 20 ℃.

4.2.5. Viscosity

Rotational viscometer (DV-II+ Pro LV, Brookfield Engineering Laboratories, Inc., Stoughton, MA, USA) was used to measure the viscosity of the colloidal dispersion samples. The cup geometry used in this study was 85 mm of diameter and 121 mm of height. The sample

height was 108 mm. The spindle LV1 No. 6 (diameter 1.8 mm) was used for all viscosity measurements. Viscosity was measured at 5 °C at 2000 rpm.

4.2.6. Turbidity

Changes of the turbidity of the colloidal dispersion samples at rest were measured. LAB-Pro turbidity sensor (Vernier Software & Technology, Beverton, OR, USA) was used to measure the turbidity every 30 seconds. The system was calibrated using StabiCal® Formazin Standard Cuvette (100 NTU) (Vernier Software & Technology, Beverton, OR, USA). The sample was diluted with distilled water, and the dilution ratio of the dispersion sample to distilled water is 1:32. The diluted sample was poured into the 16.5 mL of cuvette and measured the turbidity for 3 hrs.

4.2.7. Video recording and image analysis

The sedimentation of dispersion samples was recorded by a digital camcorder (HDR-XR100, Sony, Tokyo, Japan) for 11 hrs after stirring. The videos were converted to the digital files, and encoded every 10 min to obtain the snap shots. The height of the interface

between the clear fluid and a slurry of higher solid concentration was measured by both visually and the image analysis software (Matlab 2011b, MathWorks, MA, USA). The recording system was shown in Fig. 2A and Fig. 2B. The encoded digital images were imported to Matlab and the ‘rgb gray’ function was applied to convert the color image to the black and white image. The background and the object were separated by the ‘gray thresh’ function. The binary code to estimate the interface of suspension was made by applying ‘im2hw’ function (16).

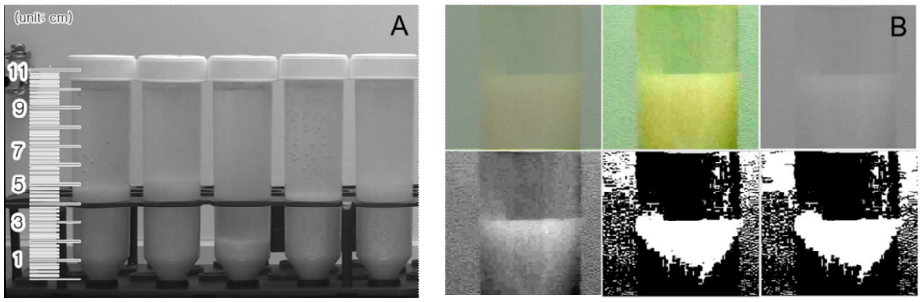


Figure 2. A) Picture captured during video recording, B) Converting to estimate the clear liquid interface during settling using Matlab.

4.2.8. Volume fraction

The dispersion samples was poured into 50 mL of test tube and centrifuged at 4000 x g for 10 min in the centrifuge (UNION 32R Plus, Hanil science Industrial, Incheon, Korea). The supernatant was separated and the volume was measured by the mass cylinder. The volume of the solid part was estimated by subtracting the volume of supernatant part from the initial volume of the dispersion sample.

4.2.9. Settling velocity

The free settling velocity of hard sphere particle in the Newtonian fluid in the low Reynolds number region ($N_{Re} < 1$), also called Stoke's law region, is

$$V_t = \frac{g D_p^2(\rho_p - \rho)}{18 \mu} \quad (1)$$

where D_p = the average particle diameter, ρ_p = the density of particle, ρ = the density of fluid, μ = viscosity (3).

When a large number of particles are present, the surrounding particles interface with the motion of individual particles. The velocity gradients

surrounding each particle are affected by the close presence of other particles. In this case, called hindered settling, the hindered settling velocity is

$$V_t = \frac{g D_p^2(\rho_p - \rho)}{18 \mu} (\varepsilon^2 \varphi_p) \quad (2)$$

$$\varphi_p = \frac{1}{10^{1.82(1-\varepsilon)}} \quad (3)$$

where ε = volume fraction of the slurry mixture, φ_p = empirical correction factor (3).

In this study, the Reynolds numbers were calculated to assure the settlement is in the Stoke's law region.

$$\frac{D_p V_t \rho}{\mu} \quad (4)$$

$$\frac{D_p V_t \rho_m}{\mu_m^{\varepsilon}} \quad (5)$$

The Reynolds numbers for free settling velocity and hindered settling velocity were calculated by equation (4) and (5), respectively.

4.2.10. Statistical analysis

All the measurements were repeated three times. The significant difference was evaluated by ANOVA using MS-Excel 2010 at a level of $p<0.05$.

4.3. Results & Discussion

4.3.1. Effect of HSP on the particle size

The effect of HSP on the mean particle size of solid in the dispersion samples was shown in Table 1. There was a large decrease in the particle size when the coarse suspension state of fluid was passed once through the HSP system at all the levels of pressures applied. The mean particle size decreased from around 129.69 μm to 6.20, 6.01, 5.94, 6.04, and 5.54 μm after single pass at the applied pressure of 7000, 10000, 15000, 20000, and 25000 psi, respectively. Single pass of HSP decreased the particle size about 95%. The particle size is inversely proportional to the applied pressure except at 20000 psi. These results indicated that the HSF was particularly efficient to reduce the size of particles suspended in the dispersion (10) found that the droplet size of soya oil and lecithin emulsion was strongly dependent on the applied pressure. The solid particles in the sample showed similar pressure dependence to those of the droplets in the emulsion of soya oil.

At the minimum (7000 psi) and the maximum (25000 psi) pressure, HSP was repeatedly applied to the sample fluid for three times, and the particle sizes at different number of cycles (N) were shown in Table 1. The particle size continuously decreased as the fluid

passed through the HSP system, but the further reductions were fairly modest. At 7000 psi, as N increased to 3, the particle size decreased significantly ($P < 0.05$), but at 25000 psi the average particle size decreased as N increased but not statistically significant. Previously, a few studies were conducted to figure out the effects of the number of cycles on the droplet size of emulsion (10, 18). Most of the emulsions discussed in the previous studies showed a similar dependence of N on the droplet size. The droplet size did not change appreciably after about 4 to 6 passes. The particle size in the dispersion samples showed a similar pattern to those of emulsions.

	Pressure (psi)	Number of passes	Particle size (μm)
Control	-	-	129.69 ± 54.00
	7000	1	6.20 ± 0.01[a]
	10000	1	6.01 ± 0.03[e]
	15000	1	5.94 ± 0.01[f]
	20000	1	6.04 ± 0.70[g]
	25000	1	5.54 ± 0.04[d]

	Pressure (psi)	Number of passes	Particle size (μm)
	7000	1	6.20 ± 0.01[a]
	7000	2	5.91 ± 0.02[b]
	7000	3	5.75 ± 0.02[c]

	Pressure (psi)	Number of passes	Particle size (μm)
	25000	1	5.54 ± 0.04[d]
	25000	2	5.33 ± 0.75[d]
	25000	3	5.24 ± 0.50[d]

Table 1. Effect of HSP on the particle size of the dispersion samples from the coarse filtered rice fermentation.

4.3.2. Effect of HSP on the viscosity and the volume fraction

Changes of viscosity of the sample fluid upon the applied pressure and the number of cycles were shown in Table 2. The viscosity of control was 8.57 (±0.06) cP. As HSP was applied, the viscosity changed to 9.51, 9.84, 10.43, 10.42, and 10.61cP at the applied pressure of 7000, 10000, 15000, 20000, and 25000 psi, respectively. The viscosity increased after applying the HSP, but at higher than 15000 psi, the viscosity was not influenced by the applied stress. Generally, viscosity of suspension increases with increase in the solid concentration. This is mainly because of the increased interaction between particles at higher concentration (13). Viscosity slightly increased by increase of the number of cycles. The effect of the number of cycles on the viscosity was greater at the low pressure than at the high pressure. The increase of the viscosity after three repetition of HSP was about 7% and 2% at 7000 and 20000 psi, respectively. Similar observation was found in the previous session discussing on the effect of the number of cycles on the particle size. The decrease of particle size was less at the high applied pressure. As shown in Fig. 3, viscosity of the fluid was inversely proportional to the particle size ($R^2 = 0.72$) in the range of 5 to 6 μm of the particle size. According to Senapati et al.

(13), the viscosity of suspension of calcium carbonate was greatly affected by not only the surface properties but also the particle size. Our study showed a similar dependence of viscosity on the particle size.

In general, when the particle size in suspensions is reduced, the volume fraction of the solid parts in the suspension is increased. Theoretically, as the size of spherical shape of particles decreases the surface area and volume increased, but it is difficult to estimate the volume fraction of solid particles in the suspension analytically. Changes of volume fraction of solid particles in the dispersion samples were experimentally measured and shown in Table 3. The volume fraction of control was 5%, and as HSP applied the volume fractions increased up to 7.63%. The volume fraction of the fluid proportionally increased as the applied pressure increased. In addition, as the number of passes increased the volume fraction increased significantly at both 7000 and 25000 psi. In general, when the volume fraction of suspension is less than 7%, the movements of particles in the suspension follow the Stoke's law and usually the fluid is a Newtonian.

	Pressure (psi)	Number of passes	Viscosity (cP)
Control	-	-	8.57 ± 0.06
	7000	1	9.51 ± 0.06[a]
	10000	1	9.84 ± 0.09[b]
	15000	1	10.43 ± 0.22[c,d,e]
	20000	1	10.42 ± 0.06[d]
	25000	1	10.61 ± 0.08[e]

	Pressure (psi)	Number of passes	Viscosity (cP)
	7000	1	9.51 ± 0.06[a]
	7000	2	9.84 ± 0.06[b]
	7000	3	10.17 ± 0.08[c]

	Pressure (psi)	Number of passes	Viscosity (cP)
	25000	1	10.61 ± 0.08[e]
	25000	2	10.99 ± 0.02[f]
	25000	3	10.89 ± 0.09[f]

Table 2. Effect of HSP on the viscosity of the dispersion samples.

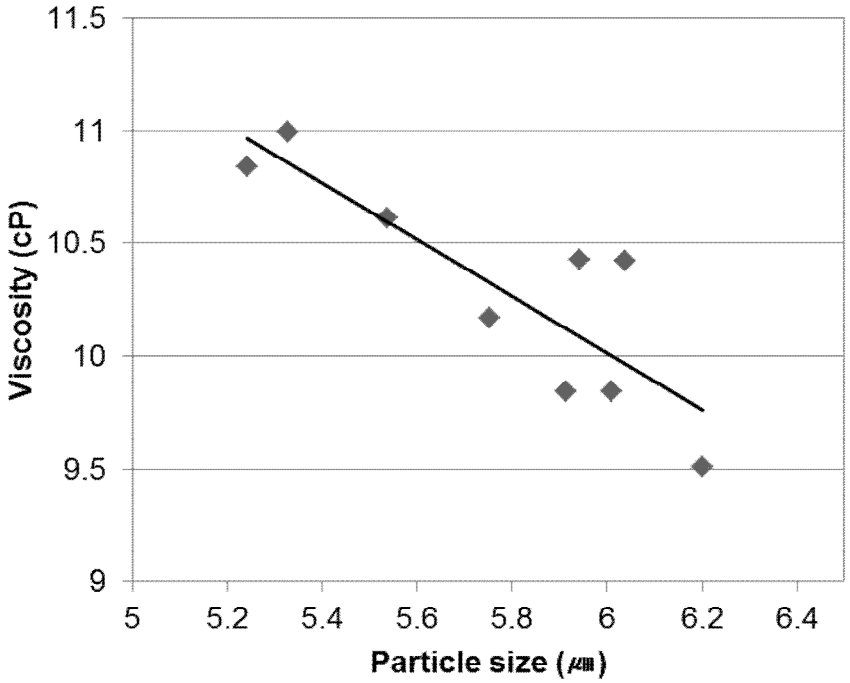


Figure 3. Relationship between viscosity and particle size in the dispersion samples prepared by coarse filtered rice fermentation.

	Pressure (psi)	Number of passes	Volume fraction (%)
Control	-	-	5.00 ± 0.00
	7000	1	6.50 ± 0.14[a]
	10000	1	6.88 ± 0.07[g]
	15000	1	6.38 ± 0.07[h]
	20000	1	7.50 ± 0.00[i]
	25000	1	7.63 ± 0.07[d]

	Pressure (psi)	Number of passes	Volume fraction (%)
	7000	1	6.50 ± 0.14[a]
	7000	2	7.00 ± 0.00[b]
	7000	3	7.13 ± 0.21[c]

	Pressure (psi)	Number of passes	Volume fraction (%)
	25000	1	7.63 ± 0.07[d]
	25000	2	8.00 ± 0.14[e]
	25000	3	8.13 ± 0.07[f]

Table 3. Effect of HSP on the volume fraction of the dispersion samples.

4.3.3. Effect of MF on the sedimentation of particles

Video recording and image analysis were conducted to measure the settling velocity of solid particles in the sample fluids. The height of settlement was determined based on the clear-liquid interface introduced in the Geankoplis (3). To monitor the changes of the height of the clear-liquid interface during settling, the video clips were analyzed every 10 min for 600 min. In this study, the height before settling, z_0, was 10 cm. Changes of the height of the clear-liquid interface at different applied pressure were plotted versus time (Fig. 4). The height of the control dropped to 4 cm in 150 min with nearly a constant velocity. The change of the height, $dz=z_0-z_t$, z_t= the height at an arbitrary time during settling, of the control sample during settling showed a prototypical behavior of a batch settling of particles. In a batch settling, the velocity of settling, which is the slope of the curve, is constant initially, then the slope is certainly changed. The point where the slope begins to change was defined as a critical point of free settling (3). The settling velocity is graphically determined by drawing a tangent to the curve, dz/dt, before the critical point. As shown in Fig. 4, the HSP strongly affected the settling velocity of particles in the sample fluids. When the fluid is a Newtonian and in the Stoke's region, the

settlement velocity of particles in the suspension is estimated by equation (1) and (2) (Rhode, 11). All HSP treatments dramatically reduced the settling velocity. The clear-liquid interface of HSP treated samples maintained the heights at the initial state until 600 min. The velocity of the control was 4 x 10^{-2} m/s while those of HSP treated samples were between 2.8 x 10^{-7} and 3.75 x 10^{-7} m/s. The velocity drop by HSP was mainly due to the decrease of the particle size and the increase of viscosity. Both free settling and hindered settling velocity were calculated by the equation (1) and (2), respectively, and compared to the velocity estimated by the data from the video recording and image analysis (Fig. 5). The free settling velocities of each treatment were close to the velocity data analyzed by the video recording. The hindered settling velocity values of all treatments were lower than those of the free settling, and showed a gap from the velocity estimated by the video recording. It might be because the volume fractions (< 7%) showed a Stoke's flow region and the interference between particles are limited in the fluid. The velocities decreased about 12%, as the applied pressure increased from 7000 psi to 25000 psi.

Settling velocities at different cycles at 7000 psi and 25000 psi were in Fig. 6. At the low applied pressure, 7000 psi, increasing the

number of cycles decreased the settling velocities, but at the high applied pressure, 25000 psi, the changes were very modest. It might be due to the effect of the number of cycles on the particle size was less at the high applied pressure than at the low applied pressure.

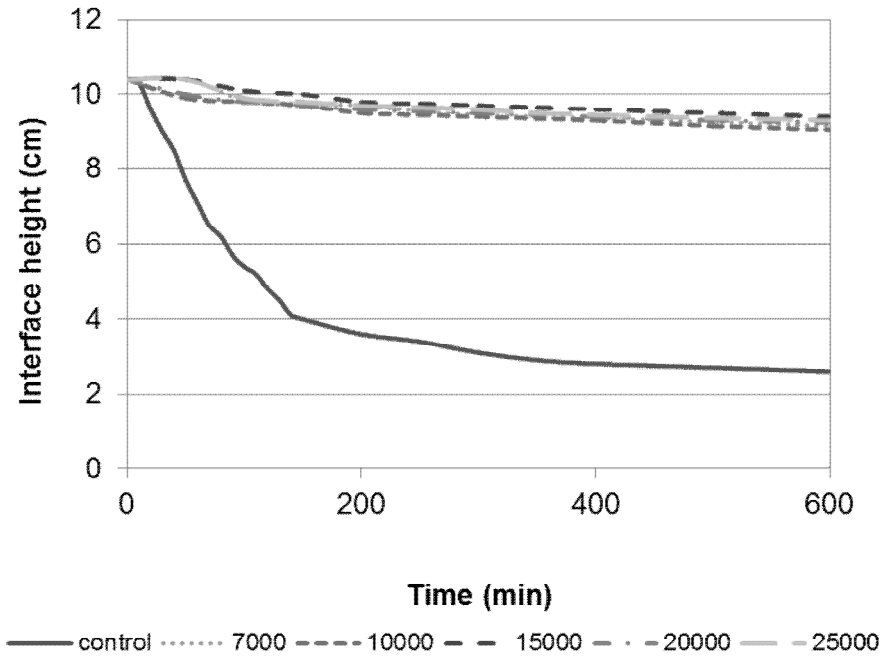


Figure 4. Changes of the height of the clear liquid interface upon the applied pressure (in psi).

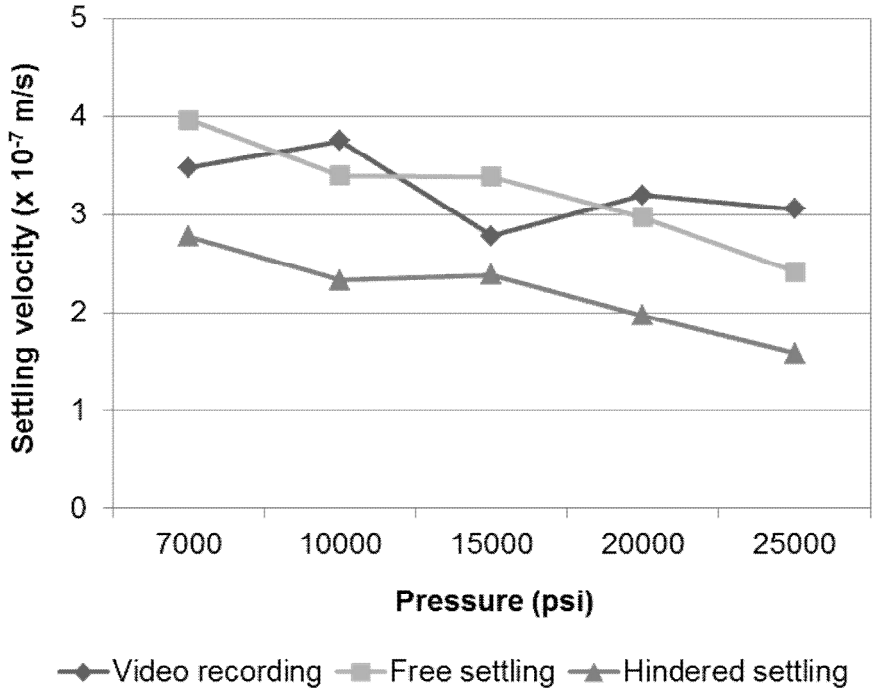


Figure 5. Effect of the applied pressure on the settling velocity of the dispersion samples. Settling velocities were calculated by the video recording method, free settling, and hindered settling.

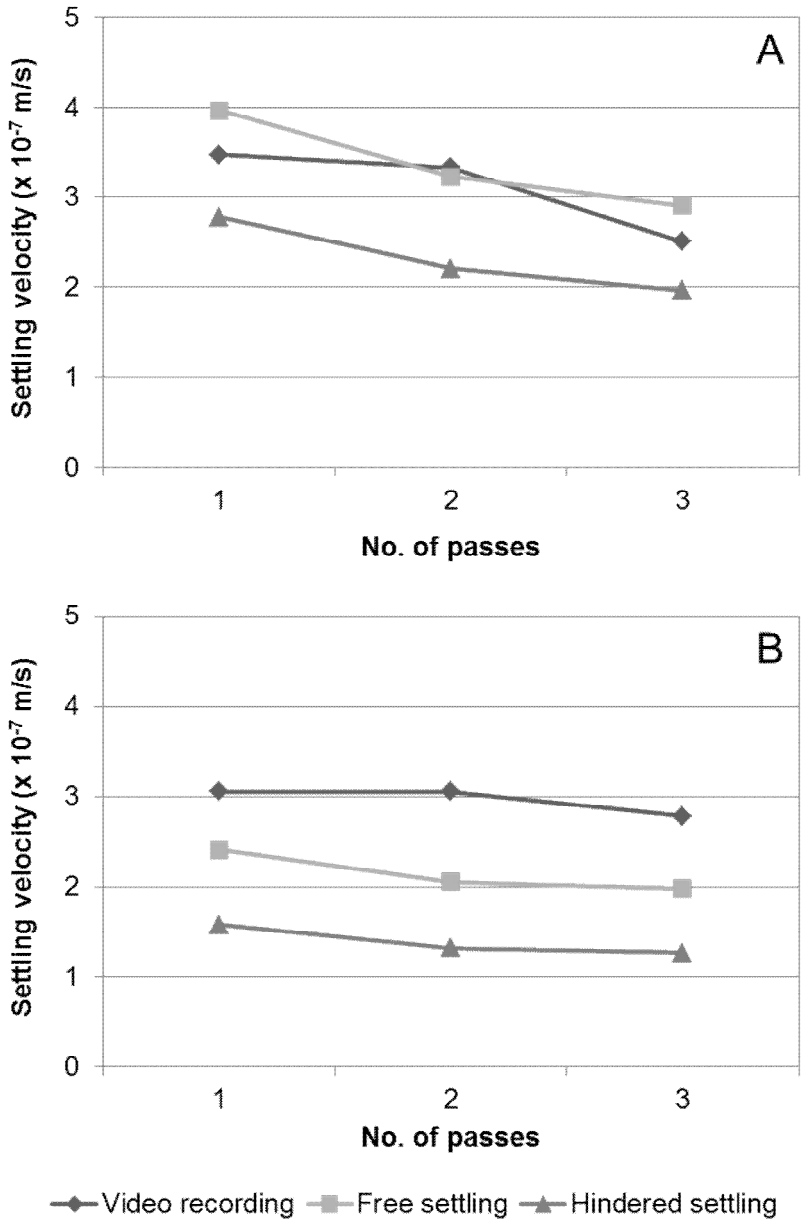


Figure 6. Effect of the number of passes of microfluidization on the settling velocity of the dispersion samples at A) 7000 psi, B) 25000 psi.

4.3.4. Effect of MF on the turbidity

The dispersion samples were diluted by adding the distilled water before measuring the changes of turbidity because the turbidity sensor can detect the turbidity under 600 NTU. The dilution ratio of dispersion samples to distilled water was 1:32. By the dilution, the ratio of the amount of solution to that of particle was changed over 400 times before dilution. At such a low concentration of particle, the influence of the particle size maybe less than before dilution. Turbidity results should be interpreted to understand the effect of HSP on the fluid behavior in a qualitative manner. The changes of turbidity at different applied pressure were described in Fig. 7. Clearly, the HSP treated samples showed a slow decrease of the turbidity during settling. The initial values of turbidity of HSP treated samples were higher than the control because the larger number of particles after HSP scattered the light more than the control sample. The turbidity of the control decreased immediately, while for those HSP treated samples the turbidity was constantly remained their initial values until 30 to 34 min.

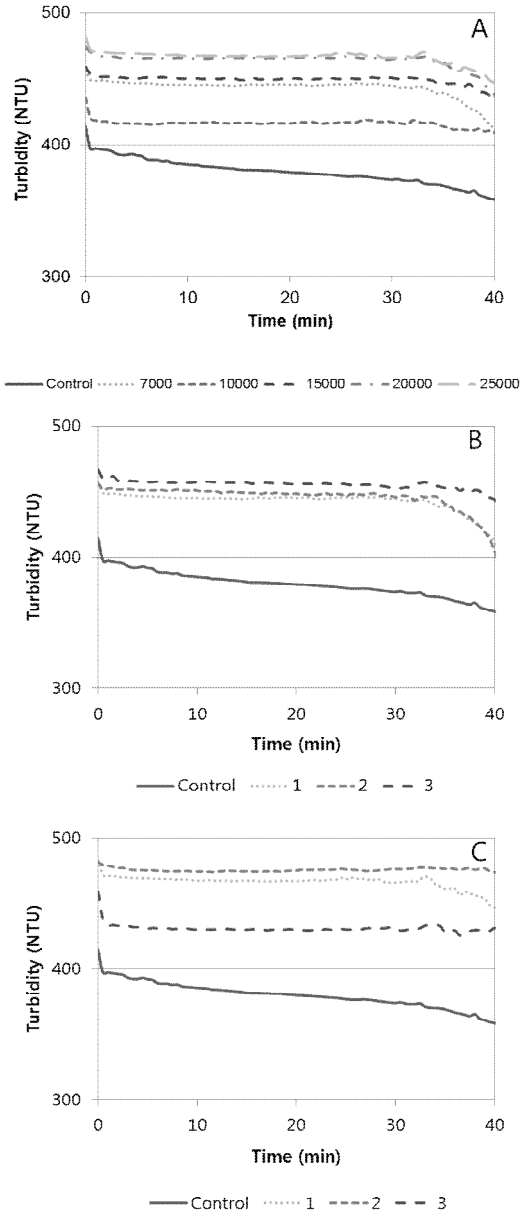


Figure 7. Time evolution of turbidity of the dispersion samples. A) effect of applies pressures, B) effect of the number of pass at 7000 psi, C) effect of the number of pass at 25000 psi.

4.3.5. Effect of HSP on the Zeta potential

The absolute values of ζ-potential of sample fluids after applying HSP were in the range of 3.79 to 12.45 mV (Table 4). The ζ-potential is the potential at the surface of shear, i.e. a plane between the solid (particle) and liquid (dispersion media) interface. In general, the absolute value of the ζ-potential in suspension increases with increase in particle size (9, 12). This increase in ζ-potential has helped to increase the stability of the suspension solution as stable suspension solutions are having the ζ-potential values in the range of 20–50 mV. Higher ζ-potential values are desirable for stable suspension solutions (9, 12). The absolute values of ζ-potential of sample fluids were lower than 20 mV for all treatments, and showed a minimum value at 15000 psi. It implies that the solid particles after applying the HSP are less stable and have a tendency to aggregate. It should be noted here that the practical objective of the use of HSP in this study was to extend the sedimentation time of the solid particles during storage. In this study, we clearly demonstrated that when the HSP applied to reduce the particle size in the suspension, the phase separation of the suspension is significantly delayed. But, the reduced particles might have more chances to be aggregated during the storage of the dispersed samples.

	Pressure (psi)	Number of passes	ζ-potential (mV)
Control	-	-	-12.45 ± 2.26
	7000	1	-16.24 ± 0.76[a]
	10000	1	-17.87 ± 4.63[b]
	15000	1	-3.79 ± 0.01[c]
	20000	1	-7.22 ± 0.03[d]
	25000	1	-15.14 ± 2.39[e]

Table 4. Effect of HSP on the zeta potential of the dispersion samples

4.3.6. Changes of the settling velocity and the particle size during storage

The settling velocities of HSP treated samples after 5 days of storage were measured by the video recording (Fig. 8). As the storage day increased, the settling velocities increased dramatically. After 5 days of storage, the settling velocities of all treatments increased more than 3 times than 0 day and showed between 15 x 10^{-7} and 16 x 10^{-7} m/s. But the settling velocities after 5 days were still very low compared to that of control, 4 x 10^{-2} m/s.

The particle sizes of HSP treated samples after 5 days of storage were measured by the particle size analyzer. During the storage, we clearly observed with naked eyes that the particle size of the HSP treated samples increased. However the particle sizes measured by the particle size analyzer showed there were no significant changes during storage (Fig. 9). It might be due to the sonication process required before using the particle size analyzer. In our case, the particle size analyzer requires the sonication process as a pretreatment to disassemble the particles in the agglomerates. The measurements implied that weak interactions between particles were mainly attributed to the aggregation of particles found in the HSP treated samples during

storage. Such aggregation might affect the settling velocity, but the aggregation was weak enough to be disassembled by the sonication before measuring the particle size using the laser diffraction particle size analyzer.

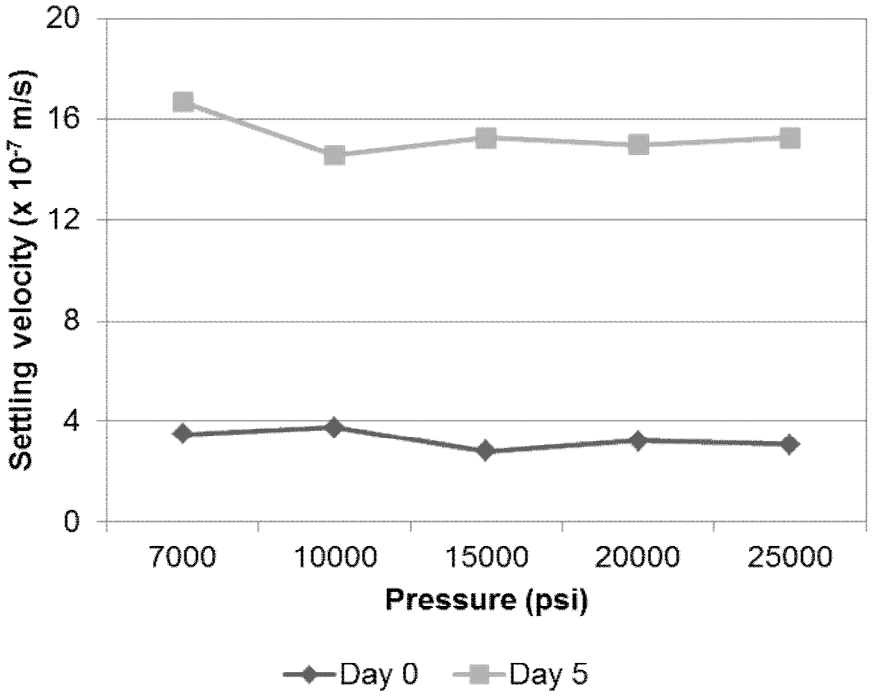


Figure 8. Effect of storage time on the settling velocity of the dispersion samples HSP treated at different pressure.

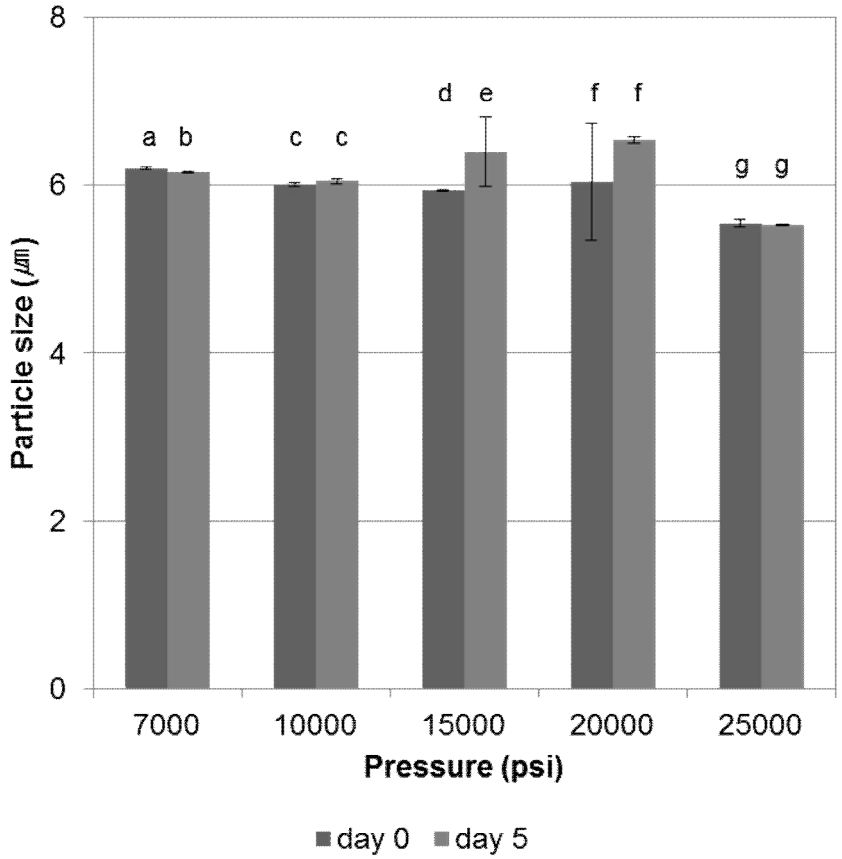


Figure 9. Comparison of particle size in the dispersion samples at 0 and 5 days of storage.

4.4. Conclusion

HSP was applied to delay the settlement of solid particles in the colloidal dispersion from the coarse filtered rice fermentation. The high shearing associated with HSP significantly decreased the particle size in the dispersion. As the applied stress increased up to 25000 psi, the particle size was inversely dependent on the applied stress. The repeat cycle also affected the particle sizes. The viscosity increased as the applied stress increased. The settling velocity was significantly reduced by applying HSP so that the phase separation between solid particles and solution was dramatically delayed by applying HSP. The reduced size of particles showed low ζ-potential and it implies the suspension after HSP may be unstable. After 5 days of storage, the settling velocity increased about 5 times compared to the first day, but the settling velocity was still quite lower than that of control. This study clearly showed that the HSP is a suitable method to delay the phase separation of the CFFRW without adding food ingredients.

4.5. References

1. Cliff RO, Kwasiborski V, Rudolph AS. A comparative study of the accurate measurement of endotoxin in liposome-encapsulated hemoglobin. Artificial Cells Blood Substitutes and Biotechnology. 1995;23:331-336.

2. Dawe D, Robertson R, Unnevehr L. Golden rice: what role could it play in alleviation of vitamin A deficiancy. Food Policy. 2002;27:541-560.

3. Geankoplis CJ. Transport process and separation process principles 2003;(4th ed., pp. 919-932). New York: Prentice Hall.

4. Kim IH, Min BY, Lee SK. Effect of lysozyme and glycine on the Mageoly brewing process. Journal of Korean Agricultural Chemical Society. 1990;33:252-256.

5. Lee JG. Dispersing agent for protecting organic material precipitation. Korea patent. 2010;10-2010008-2534.

6. Lee JH, Yu TS. Identification and characterization of lactic acid bacteria isolated from *Nuruk*. Korean Journal of Biotechnology and Bioengineering. 2000;15:359-365.

7. Lee SM, Lee JH. The isolation and characterization of simultaneous saccharification and fermentation microorganisms for *Laminaria*

japonica utilization. Bioresource technology. 2011;102:5962-5967.

8. Lemay A, Paquin P, Lacroix C. Influence of microfluidization of milk cheddar cheese composition, color, texture, and yield. Journal of Dairy Science. 2004;77:2870-2879.

9. Muñoz-Guerrero H, Gutiérrez MR, Vidal-Brotons D, Barat JM, Gras ML, Alcaina MI. Environmental management of the residual brine of cod desalting. Quantification of mass transfer phenomena and determination of some parameters on the residual brine important for its treatment by membrane technology. Journal of Food Engineering. 2010;99:424-429.

10. Qian C, McClements DJ. Formation of nanoemulsions stabilized by model food-grade emulsifiers using high-pressure homogenization: factors affecting particle size. Food Hydrocolloid. 2011;25:1000-1008.

11. Rhode MJ. Introduction to particle technology. 2008;(2nd ed., pp. 53-63). New York: Wiley.

12. Sejersen MT, Salomonesen T, Ipsen R, Clark R, Rolin C, Engelsen SB. Zeta potential of pectin-stabilized casein aggregates in acidified milk drinks. International Dairy Journal. 2007;17:302-307.

13. Senapati PK, Mishra BK, Parida A. Modeling of viscosity for power plant ash slurry at higher concentrations: effect of solids volume

fraction, particle size and hydrodynamic interactions. Powder Technology. 2010;197:1-8.

14. Seo DH, Jung JH, Kim YR, Ha SJ, Kim YC, Park CS. Identification of lactic acid bacteria involved in traditional Korean rice wine fermentation. Food Science and Biotechnology. 2007;16:994-998.

15. Shin KR, Kim BC, Yang JY, Kim YD. Characterization of *Yakju* prepared with yeasts from fruits. 1. Volatile components in *Yakju* during fermentation. Journal of the Korean Society of Food Science and Nutrition. 1999;28:794-800.

16. Shin SH. A method for determining the coiling ratio and classifying species of fossil planktonic foraminifera using digital image analysis. Journal of the Korean Earth Science Society. 2004;25:799-811.

17. Washington C. Emulsion production by microfluidizer. Laboratory Equipment Digest. 1987;25:69-71.

18. Washington C, Davis SS. The production of parenteral feeding emulsions by microfluidizer. International Journal of Pharmaceutics. 1988;44:169-176.

국문초록

국내 전역으로부터 다양한 누룩을 수집하여 미생물 종(種) 수준에서의 천연 균총을 분석하였다. 총 42종의 누룩을 분석한 결과 박테리아, 곰팡이, 효모, 유산균의 평균 균체수는 7.21, 7.91, 3.49 그리고 4.88 log CFU/10g 수준이었다. 지역별로 균총의 큰 차이는 없었으며 우점종인 박테리아는 *Bacillus amyloliquefaciens*와 *B. subtilis*로 관찰되었으며 특히 13종의 누룩에서 식품에서 유해 균주인 *B. cereus* 혹은 *Cronobacter sakazakii*가 발견되었다. 또한 다양한 유산균이 검출되었는데 *Enterococcus faecium, Pediococcus pentosaceus* 등이 해당된다. 막걸리 제조시 당화에 가장 주요한 역할을 하는 *Aspergillus oryzae*의 경우 13종에서만 검출된 반면에 이보다 폭넓게 *Lichtheimia corymbifera*가 발견되었다. 효모 중 우점종으로 검출된 것은 *Pichia jadinii*였으며 알코올 발효에 가장 대표적인 *Saccharomyces cerevisiae*는 18종에서만 발견되었다. 누룩에 존재하는 균총의 종합적인 분석 결과를 보면 박테이라 64종, 곰팡이 39종, 15종이었는데 이 중에서 박테리아 37종, 곰팡이 20종, 효모 8종은 0.1% 미만으로 검출되었다. 누룩으로부터 분리된 효모 중 여러 단계의 선별 과정을 거쳐 *Saccharomyces fibuligera* CJN1020, *Pichia jadinii* CJN1287, *Saccharomyces cerevisiae* CJN1514 3종에 대해 실제 막걸리 제조를 통한 적용 가능성을 고찰하였다. 백미(증자법), 백미-현미-밀가루(WBW, 복합법)로 막걸리를 제조한 결과 발효에는 이상 없음을 확인하였다. 이화학적 분석을 통해서 보면 백미 막걸리와 WBW 막걸리에서는 최종 알코올 농도에

별다른 차이를 보이지 않았으나 효모별로는 차이를 나타냈다. *Saccharomyces fibuligera*, *Pichia jadinii* 는 16%, *Saccharomyces cerevisiae* 는 18% 수준을 나타냈다. 관능 검사 결과를 WBW 막걸리가 전체 기호도에서 백미보다 선호도가 우수하였다. GC/MS 결과에서도 WBW가 백미 막걸리보다 향미 성분이 더 풍성함을 보여주었으며 이를 통해서 볼 때 현미, 밀가루를 활용하여 복합법으로 제조한 막걸리가 좋은 특성을 나타냄을 알 수 있었다. 고압 균질 (High shearing homogenization, HSH) 처리는 저온살균을 대체해서 생막걸리에서의 효모를 제어할 수 있는 방안이다. 생막걸리의 유통 기한은 매우 중요한데 이는 유통 중 품질 변화가 계속 일어나기 때문이다. 전통적으로 저온살균법이 사용되었으나 이는 영양소 손실 및 맛품질 저하의 문제를 지니고 있다. HSH 처리를 25000 psi에서 5회전 수행한 결과 효모가 10^4 이상 제어됨을 볼 수 있었으며 향미는 생막걸리 대비 거의 변화를 보이지 않는 특성을 나타냈다. 생막걸리에서 효모의 D-value는 50℃에서 28분, 65℃에서 4.18분인데 HSH 25000psi, 5회전은 80℃, 3분 가열 살균한 것과 동일한 수준의 효과를 나타내는 것이다. 처리 후 향미 분석 결과 총 34종의 휘발성 물질이 검출 되었는데 알코올 8종, 에스터 18종, 산 2종, 카보닐 화합물 1종, 탄화수소 3종, 기타 2종이었다. HSH 처리 샘플에서는 과일향이 가열 살균 샘플에서는 지질 유래의 오일향이 보다 많이 검출되는 특징을 보였다. 추가적으로 생막걸리에 고압 균질 처리 (high shearing process) 를 했을 때 콜로이드 분산성을 측정하였다. 투명성은 분산 용액에서 중요한

요소인데 입자의 침전 속도에 영향을 받기 때문이다. 입자의 침전을 늦추기 위해 HSP 공정을 막걸리 콜로이드 용액에 적용해 보았다. 압력은 7000~25000 psi 범위로 적용했는데 이를 통해 입자 크기가 평균 129.69㎛에서 5.24㎛로 감소함을 알 수 있었다. 이외에 분산 용액의 특성 파악을 위해 점도, 부피율 (volume fraction), 침전 속도, 탁도, 제타 (ζ) 포텐셜을 측정하였다. 점도와 부피율은 입자 크기에 반비례 했으며 침전 속도는 처리 후 평균 4×10^{-2} m/s 에서 3×10^{-7} m/s으로 느려짐을 알 수 있었다. 이를 통해 HSP 적용은 막걸리에서의 상 분리를 유의적으로 감소시켜 주는 것으로 평가되었다.

감사의 글

만 6년이 걸린 박사 학위 과정을 통하여 무엇인가를 시작하고 마치는 것에 대한 중요성, 인내하고 때를 기다리는 것, 더불어 함께 하는 이들에 대한 고마움을 배웠습니다. 91년 대학원 생활을 시작할 때부터 작년 8월 퇴임하실 때까지 한결 같은 모습으로 제자들을 아끼시고 삶으로 가르침을 보여 주신 이형주 교수님의 마지막 박사 제자가 된 것에 대한 의미를 되새겨 보면 더할 나위 없이 영광스러움과 감사함을 느끼게 됩니다. 많이 부족한 가운데서도 논문 심사를 할 수 있도록 기회를 주셨기에 지금 이 순간이 있다고 생각됩니다. 선생님 정말 감사 드립니다. 바쁘신 중에도 시간을 내서 논문 심사에 임해 주신 강동현 교수님, 박영서 교수님, 이기원 교수님, 강남주 교수님께도 감사의 인사 드립니다.

하지만 지금 언급하는 4분이 안 계셨다면 저의 학위 취득은 불가능했을 것입니다. 최준봉 박사님, 제가 많이 어려울 때에 막걸리 팀으로 흔쾌히 불러 주시고 발효를 잘 모르면서 실험차 지방 출장 다니며 돌아다니던 저에게 박사 논문 준비를 할 수 있도록 연구 과제를 기획해 주시고 토대를 닦을 수 있도록 해 주신

점 감사 드립니다. 박영서 교수님, 누룩 연구부터 함께 해 주시면서 어렵고 힘든 실험도 마다하지 않으시고 좋은 결과로 만들어 주시고 아울러 논문 작성에도 세세한 부분까지 도움 주신 점 감사 드립니다. 윤원병 교수님, 균주와 발효 연구만으로는 다소 내용이 부족하던 때에 공정 연구 및 물성 부분을 제안해서 실험할 수 있도록 해 주시고 아울러 마지막 관문까지 끝까지 함께 고민해 주신 점 감사 드립니다. 김영석 교수님, 학교 동기이자 30년 친구이면서 이번 논문에 음으로 양으로 지원해 주고 특히 향미 성분 분석에서 결과 도출해 준 점 감사합니다.

막걸리 연구팀에 합류하면서 인연을 맺게 된 조규종 부장, 이호우 대리, 박재승 대리에게도 감사함을 전합니다. 앞으로도 함께 할 소중한 후배들 입니다. 아울러 누룩 연구 초기 단계에서 스크리닝 작업부터 수고해 주신 박정민 소장님, 막바지 논문 실험 및 자료 정리에서 애써 준 강원대 이명기 학생, 엄주태 학생에게도 고마움을 전합니다. 마지막으로 부족한 아들을 항상 지켜봐 주신 양가 부모님들, 밝은 얼굴로 사람들을 평안하게 만드는 신비한 힘을 가진 아내 우경신, 퉁명스러워 보이지만 사람에 대한 따뜻한 관심을 가진 큰아들 주현, 숫자와 과학에 관심이 많고 항상 자신감에 넘치는

작은아들 주현, 큰누나 내외, 작은누나 내외, 그리고 동생 혜선이에게도 고마움을 전합니다.

어느덧 우리 나이로 이제 49살이 되었습니다. 새롭게 시작하는 마음으로 원점에서 나를 다시 돌아보는 한 해가 되고자 합니다. 그 시작점에 너무나도 값진 선물을 받았기에 겸허한 마음으로 주변에 있는 이들에게 베풀고 돕는 마음으로 살아가고자 합니다. 이 자리가 있기까지 함께 해 주신 모든 분들께 다시 한 번 진심으로 감사 드립니다.

2014년 1월에

송상훈 드림