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Antimicrobial Efficiency in the Fermented Slurry of Unpolished Rice

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Unpolished rice (UR) is considered to be a healthy alternative to white rice when coping with chronic diseases. In the present study, the fermented slurry of unpolished rice (FSUR) was evaluated with respect to its antimicrobial activities and biochemical characteristics, including the quantities of sugar, total soluble sugar, organic acids, free amino acids, pH, and physiological activity. The antimicrobial efficiency of FSUR was assessed using the paper disc-agar diffusion method. FSUR exhibited strong antimicrobial activity against six pathogenic bacterial strains (*Staphylococcus aureus, Escherichia coli, Listeria monocytogenes, Pseudomonas aeruginosa, Salmonella typhimurium*, and *Yersinia enterocolitica*) and two fermentation strains (*Gluconacetobacter intermedius* and *Lodderomyces elongisporus*). The antimicrobial activity of FSUR was higher than the commercial antibiotics, carbenicillin (50 μg/ml) and tetracycline (50 μg/ml) against *S. aureus*, *E. coli*, *L. monocytogenes*, *P. aeruginosa, S. typhimurium*, *Y. enterocolitica*, and *L. elongisporus.* Also FSUR had a high antioxidant activity. The microorganisms were isolated from FSUR using tryptic soy broth and yeast extract-peptone-dextrose agar media. The isolated microorganisms were characterized using physiological and biochemical analyses as well as by 16S rRNA gene sequencing and phylogenic analysis. 16S rRNA gene sequence analysis showed that the isolated microorganisms had a high similarity to *G. intermedius, Lactobacillus casei, Lactobacillus plantarum*, and *Acetobacter peroxydans*.

Keywords: Fermented slurry of unpolished rice, antimicrobial activity, antioxidant activity, human pathogenic microorganisms, 16S rRNA gene

Introduction

Bacterial infection is one of constitute one of the greatest global challenges facing public healthcare today. Extensive efforts have been dedicated to the development of therapies for bacterial infections, including the continued development of antimicrobial materials, such as antibiotics, silver particles, photosensitizers, antimicrobial peptides, and hydrogels [9]. There has been a recent focus on producing natural medicines and other natural products. Several fruits and fruit extracts as well as arrowroot tea extract and caffeine exhibit antimicrobial activity against *Escherichia coli* O157:H7. Plants with relatively high levels of antimicrobial activity may be sources of the compounds that inhibit the growth of foodborne pathogens. Bacterial cells could be killed by rupture of cell walls and membranes and by irregular disruption of the intracellular matrix when treated with plant extracts [3].

Unpolished rice (UR), which is hulled directly from rough rice, consists of a bran layer (6–7% of its total weight), embryo (2–3%), and endosperm (approximately 90%). It is a better source of nutritional components, such as proteins, lipids, dietary fibers, vitamins, and minerals, than white rice. These nutrients exist mainly in the germ and bran layers of rice grains, which are mostly removed during the milling process that converts UR to white rice, the form that is typically consumed. UR is less desirable than white rice due to its poor cooking and eating qualities. Cooked brown rice is

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dark in appearance and unpalatable owing to its hard texture and chewiness, which are attributed to the tough fibrous bran layer. However, UR has a higher nutritional value [5]. Recently, human and animal studies have shown that UR consumption reduces the risk of type-2 diabetes, cardiovascular disease, and cancer, and these protective health effects have been linked to the presence of bioactive compounds such as polyphenols, γ-aminobutyric acid (GABA), acylated steryl β-glucoside, and γ-oryzanol [4, 6, 8, 12, 23].

In this study, we prepared a fermented slurry of unpolished rice (FSUR) and investigated its antimicrobial activity against pathogenic bacteria and yeast. The antioxidant activity and the organic components of the FSUR were analyzed. Additionally, we performed microbial screening during the fermentation process.

Materials and Methods

FSUR Preparation

The mother brew (Mitsul) was prepared as follows: Polished rice (4 kg) was rinsed three times to remove impurities and soaked in water for 4 h until saturation. Excess water was removed, and the soaked rice was then steamed for about 40 min to allow full gelatinization. The steamed rice was cooled to 25°C, mixed with 2 kg of yeast leavening agent (Nuruk powder), and incubated at 32°C for 2–3 days to allow saccharification.

Steamed rice cakes (Baekseolgi) were prepared as follows: UR (20 kg) was rinsed 3 times to remove impurities and soaked in water for 8 h until saturation; then, water was drained for 1 h. For the instant rice cakes, the crushed powder was steamed to prepare Baekseolgi.

A mixture (20 kg of Mitsul, 20 kg of Baekseolgi, 2 kg of Nuruk, and 50 L of water) was prepared and then incubated at 32°C to reach an alcohol content of 12% [7]. The supernatant was removed and the precipitate was filtered using a 60-mesh sieve after dilution in 90 L of water. The FSUR was boiled for 10 min at 100°C.

Alcohol, pH, and total soluble solid content

The FSUR samples were subjected to various chemical analyses. The pH was measured using an Orion 420A pH Meter (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The total soluble solid content was measured using a Brix Refractometer HI 96811 (Hanna Instruments, Woonsocket, RI, USA). The alcohol content was determined using a vinometer after distillation. One hundred milliliters of each sample was run through a distiller until a volume of approximately 70 ml was collected. The collected sample volume was adjusted to 100 ml by adding distilled water.

Free amino acid and componential analysis of FSUR

The components (sugar, organic acid, moisture, ash, and crude protein content) of FSUR were determined by a proximate composition analysis. Three types of sugar were measured (fructose, glucose, and sucrose) and four types of organic acid were measured (oxalic acid, lactic acid, acetic acid, and propionic acid). Free amino acids were analyzed using liquid chromatography tandem mass spectrometry with positive electrospray ionization and selected ion monitoring mode. This analysis was performed at the Biotechnology Industrialization Center (BIC; Dongshin University, Naju, Korea).

Culture conditions and isolation of microbial strains

To isolate microbial strains, 100 μl of FSUR was spread onto yeast extract-peptone-dextrose (YPD) agar medium (20.0 g/l peptone, 10.0 g/l yeast extract, 20.0 g/l glucose, and 20.0 g/l agar) and tryptic soy broth (TSB) agar medium (BD 211825; Becton, Dickinson and Co., Franklin Lakes, NJ, USA). The plates were incubated at 30°C for 2 days. Single colonies on the plates were purified by transferring them to fresh plates, followed by re-incubation [20]. Morphological characteristic was observed using a Gram Staining Kit (Fluka-77730, Sigma-Aldrich, St. Louis, MO, USA). Catalase activity was examined by measuring the production of oxygen bubbles in an aqueous hydrogen peroxide solution. To identify the carbon sources, the bacteria were grown on basal salt media [2] with maltose, mannitol, cellobiose, D-mannose, D-glucose, lactose, fructose, and D-arabinose at a final concentration of 2% [2].

Polymerase chain reaction (PCR) amplification and sequencing of 16S rRNA gene

PCR was performed in a total reaction volume of 20 ml containing 14.2 μl of ddH_2O, 2 μl of PCR buffer, 2 μl of dNTPs (2 mM), 0.5 μl of each primer (27F, 5'-AGAGTTTGATCMTGG-CTCAG-3' and 1492, 5'-TACGGYTACCTTGTTACGACTT-3' [22]), 1 μl of extracted DNA, and 0.3 μl of Taq DNA polymerase (5 U/μl) (Roche Diagnostics, Basel, Switzerland). PCR was performed using an MJ Research PTC

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225 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with the following PCR conditions: initial denaturation for 5 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C; extension of incomplete products for 10 min at 72°C; and cooling at 4°C. The sizes and quantities of the PCR products were determined using 1.5% (w/v) agarose gel electrophoresis. The PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Limburg, Netherlands) according to the manufacturer's instructions. The 16S rRNA gene sequence analysis was performed using an ABI PRISM BigDye Terminator Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA, USA) and ABI 310 DNA Sequencer (Applied Biosystems, Inc.).

Free radical scavenging activity of FSUR

The FSUR was screened for antioxidant activity using a 2,2-diphenyl-picryl-hydrazyl (DPPH) scavenging assay according to the methods of previous studies [21] with a few modifications. DPPH is a stable free radical that loses its absorbance at 517 nm when it is reduced. DPPH stock was prepared by dissolving 0.4 mM DPPH in 100 ml of absolute ethanol. FSUR samples (20 μl) were added to 180 μl of DPPH solution. The absorbance of the mixture was measured at 517 nm after 30 min of incubation at 37°C in the dark using a microplate spectrophotometer (Eon, BioTek, Winooski, VT, USA). A low absorbance of the reaction mixture indicated a high free radical scavenging activity. Ascorbic acid (1 mg/ml, 5 mM) was used as a positive control. The relative DPPH scavenging effect (estimated as a percentage) was calculated as follows [13, 14]:

$$\text{DPPH scavenging effect (\%)} = A_{cont} - A_{test} / A_{cont} \times 100$$

Antimicrobial activity of FSUR

Antimicrobial activity of FSUR was estimated using the agar well diffusion method. TSB and YPD agar were used throughout the investigation. The pathogens (*S. aureus*, *E. coli*, *L. monocytogenes*, *P. aeruginosa, S. typhimurium, Y. enterocolitica, G. intermedius*, and *L. elongisporus*) were grown in TSB and YPD for 24 h. A 100-μl aliquot of each bacterial suspension was spread on a TSB agar plate and *L. elongisporus* was inoculated on the YPD agar plate [20]. FSUR and four organic acids (lactic acid, oxalic acid, acetic acid, and propionic acid; 25 μg/ml, Sigma-Aldrich) were added to paper discs (8 mm in diameter), which were placed on the surfaces of the inoculated agar plates and incubated at 37°C for 18 h. The total diameter (mm) of the inhibition zone was measured for each microorganism [18]. Tetracycline (50 μg/ml) and carbenicillin (50 μg/ml) was used as a positive control. In order to clarify the effect of changes in acidity of FSUR on antimicrobial acitity, 100 mM KCl buffer (pH 3.0) was used as a control.

Results

Alcohol, pH, total soluble solid content, and component analysis of FSUR

The total soluble solid content of FSUR increased during fermentation, while the pH did not change. The alcohol content decreased (Table 1). Based on a component analysis of FSUR, three sugars, i.e., fructose, glucose, and sucrose, and three organic acids, i.e., lactic acid, acetic acid, and propionic acid, were detected (Table 2).

Table 2. Component analysis of FSUR.

		Contents (mg/l)
pH		3.20 ± 0.24
Sugar content (Brix)		16.5
Moisture (%)		95.54 ± 0.08
Ash (%)		0.04 ± 0.01
Crude protein (%)		1.36 ± 0.03
Sugar (mg/l)	Fructose	0
	Glucose	0.582 ± 0.052
	Sucrose	0.052 ± 0.046
Organic acid (mg/l)	Oxalic acid	0.034 ± 0.004
	Lactic acid	4.775 ± 0.122
	Acetic acid	42.253 ± 0.048
	Propionic acid	7.391 ± 0.046

Table 1. Change of Alcohol, pH and total soluble sugar content during the fermentation of FSUR.

	3 days	7 days	10 days	15 days	22 days	29 days
Sugar content (Brix)	8.1	8.4	8.8	8.8	9.2	9.1
pH	3.6	3.87	3.63	3.64	3.65	3.6
Alcohol contents (%)	9	7	5	4	2	0

Free amino acid analysis of FSUR

Amino acids are naturally occurring compounds that exist in a variety of food products, such as fish, alcoholic beverages, cheeses, and meat products. They play an important role in human metabolism as the building blocks of proteins, growth factors, or stabilizers of DNA and RNA [10]. The free amino acid content of FSUR was analyzed and the results are summarized in Table 3. A total of 18 amino acids were assessed. The total free amino acid content of FSUR was 922.4 ± 115.8 mg/ml. The total free amino acid contents of FSUR, except for glycine, tyrosine, and lysine, were higher than those reported by Joo *et al.* [11]. The levels of GABA in FSUR were similar to those reported by Caceres [4].

Table 3. Amino acid analysis of FSUR.

	Contents (mg/l)
Glycine	47.7 ± 7.0
Alanine	153.5 ± 33.4
Serine	44.8 ± 7.3
Proline	63.9 ± 6.0
Valine	49.0 ± 4.6
Threonine	21.2 ± 2.6
Leucine	60.8 ± 5.4
Isoleucine	124.4 ± 13.0
Aspartic acid	37.0 ± 4.7
Lysine	13.4 ± 1.2
Glutamic acid	57.8 ± 6.2
Methionine	48.8 ± 5.4
Histidine	21.7 ± 2.2
Phenylalanine	29.5 ± 2.5
Arginine	62.6 ± 5.3
Tyrosine	20.2 ± 1.2
Cystine	9.9 ± 1.5
γ-aminobutyric acid	56.2 ± 6.3

Identification of microorganisms isolated from FSUR

Four microorganisms were isolated from FSUR and were named fermented slurry of unpolished rice strain (FS)-1, -2, -3, and -4. FS-1 and -4 were gram-negative and rod-shaped. FS-2 and -3 were gram-positive and rod-shaped. None of the four strains sporulated. Based on peptidoglycan types and a 16S rRNA gene sequence analysis, FS-1, FS-2, FS-3, and FS-4 were homologous to *G. intermedius, L. casei, L. plantarum*, and *A. peroxydans* with 100, 100, 100, and 98% similarities (Fig. 1). The carbon source usage tests for the four strains were carried out using basal salt media (Table 4).

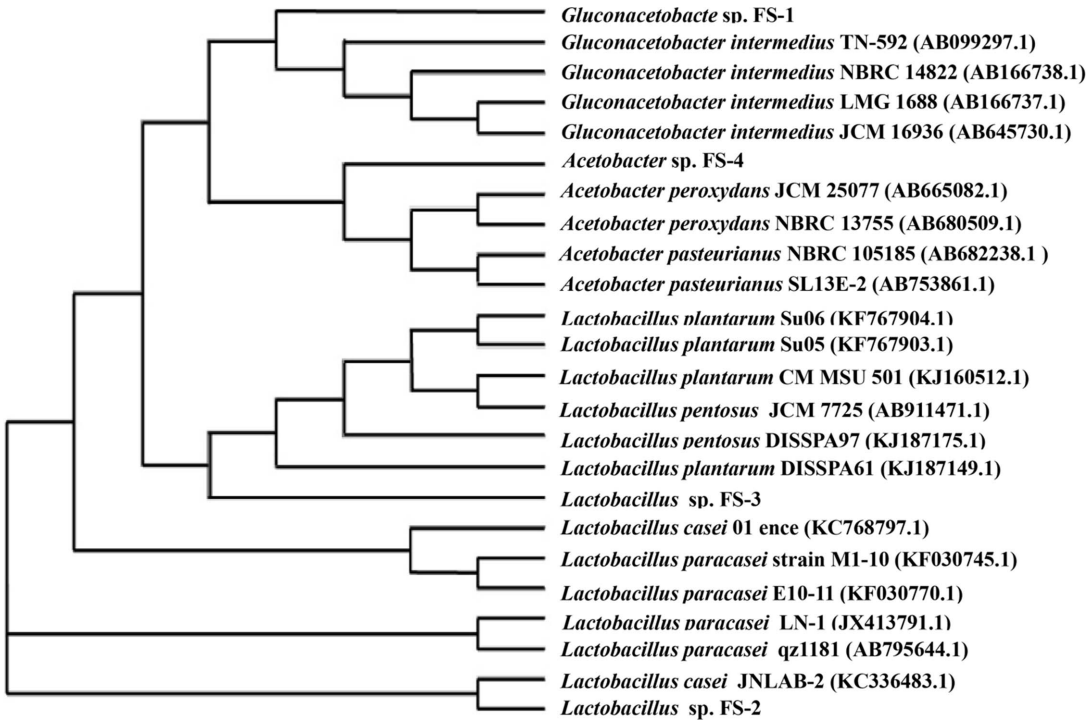

Fig. 1. Phylogenic tree based on 16S rRNA gene sequences of four isolated microorganisms from FSUR. The tree was based on an alignment of 1,318 bp of 16s rRNA gene sequences, and constructed by the neighbor-joining method.

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Table 4. Carbon source usage of three microorganisms isolated from fermented slurry of unpolished rice (FSUR).

	Maltose	Fluctose	Lactose	Arabinose	Cellobiose	Mannose	Mannitol	Glucose
Gluconacetobacter sp. FS-1	-[a]	+	-	-	-	-	-	-
Lactobacillus sp. FS-2	-	-	-	-	-	-	-	-
Lactobacillus sp. FS-3	-	-	-	-	-	-	-	-
Acetovacter sp. FS-4	-	-	-	-	-	-	-	-

[a]+; growth or presence, -; no growth or absence.

Free radical scavenging activity

Reactive oxygen species are generated via many pathways. Generally, free radicals are beneficial to cell immune systems. However, increasing evidence suggests that many uncontrolled reactions generating reactive oxygen species and oxygen-derived free radicals contribute to a variety of chronic diseases such as cancer, diabetes mellitus, and arteriosclerosis [15]. As a result of DPPH assay, the radical scavenging activity of FSUR was 88.46 ± 1.30%. The antioxidant activity of FSUR determined was similar to that of ascorbic acid (1 mg/ml) (Fig. 2).

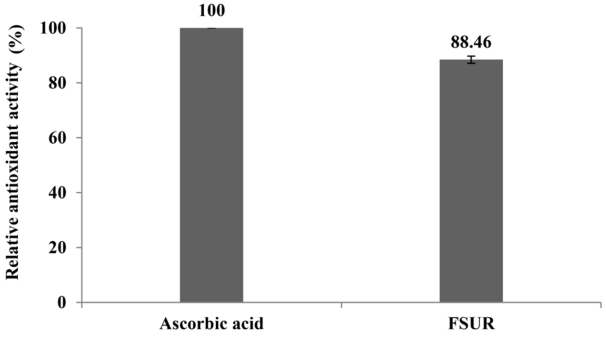

Fig. 2. Antioxidant activities of FSUR. The overall antioxidant activity of FSUR was assessed by the DPPH method. As a positive control, ascorbic acid (1 mg/ml) was used.

Antimicrobial activity of FSUR

The antimicrobial activities of FSUR and organic acids against eight microorganisms were examined using the paper disc diffusion method; the results of this analysis are summarized in Table 5. FSUR effectively inhibited the two gram-positive bacterial strains (*S. aureus* and *P. aeruginosa*), five gram-negative strains (*E. coli*, *L. monocytogenes*, *S.*

Table 5. Antimicrobial activity of fermented slurry of unpolished rice (FSUR) by paper disc assay (mm).

	KCl buffer pH 3	Carbenicillin (50 μg/ml)	Tetracycline (50 μg/ml)	FSUR	Propionic acid
Staphylococcus aureus	0	15	12	8	15
Escherichia coli	0	12	11	12	17
Listeria monocytogenes	0	14	11	21	19
Pseudomonas aeruginosa	0	17	16	18	17
Salmonella typhimurium	0	13	11	15	22
Yersinia enterocolitica	0	0	12	32	28
Gluconacetobacter intermedius	0	19	18	12	ND
Lodderomyces elongisporus	0	11	11	11	ND

Paper disk loaded with each sample (50 μl) was placed on agar plate which was inoculated with each test microorganism. ND; not determined

Table 6. The effect of pH on antimicrobial activity of FSUR.

	pH 3	pH 4	pH 5	pH 6	pH 7
Staphylococcus aureus	17	12	10	0	0
Escherichia coli	19	16	12	0	0
Listeria monocytogenes	20	16	11	0	0
Pseudomonas aeruginosa	17	13	11	0	0
Salmonella typhimurium	21	16	10	0	0
Yersinia enterocolitica	17	16	13	0	0

typhimurium, Y. enterocolitica, and *G. intermedius*), and one fungal strain (*L. elongisporus*). FSUR produced a zone of inhibition ranging from 11 to 32 mm in diameter.

Most organic acids had no inhibitory activity against the strains. However, propionic acid showed reasonably strong activity against all strains (15–28 mm zone of inhibition). For neutral pH ranges, FSUR did not show antimicrobial activity, as shown in Table 6.

Discussion

The purpose of this study was to evaluate the antimicrobial activity of FSUR and determine its antioxidant activity, pH, sugar, total soluble solid, total acid, and free amino acid content. FSUR had a free amino acid content of 922.4 ± 115.8 mg/ml, organic acid content of 6.524 ± 0.075 mg/ml, and a sugar content of 2.877 ± 0.289 mg/ml. The observed total free amino acid content was higher than one that reported by Joo *et al.* [11]. Four strains were isolated from FSUR, an acetic acid bacterium (*Acetobacter* sp. FS-1), two lactic acid bacteria (*Lactobacillus* sp. FS-2 and *Lactobacillus* sp. FS-3), and *Gluconacetobacter* sp. FS-4. Acetic acid bacteria are important in the food and beverage industry owing to their ability to oxidize ethanol to acetic acid. These bacteria are the key microorganisms in vinegar production [17]. Lactic acid bacteria have beneficial effects as probiotics in the gut. *L. casei* and *L. plantarum* have health benefits against rotavirus diarrhea, reduce the recurrence of superficial bladder cancer, modulate immune responses, relieve irritable bowel syndrome, and reduce LDL-cholesterol [19].

As a result of DPPH assay, the radical scavenging activity of FSUR was 88.46 ± 1.30%. The observed antioxidant activity was comparable to that of ascorbic acid.

FSUR inhibited the growth of *S. aureus*, *E. coli*, *L. monocytogenes*, *P. aeruginosa, S. typhimurium*, *Y. enterocolitica*, and *L. elongisporus*. The inhibitory activity was higher than those of the commercial antibiotics carbenicillin (50 μg/ml) and tetracycline (50 μg/ml). In particular, FSUR strongly inhibited the growth of *Y. enterocolitica* and *Listeria monocytogenes. Y. enterocolitica* is a zoonotic pathogen that is widely distributed in nature and can cause acute gastroenteritis and mesenteric lymphadenitis mimicking appendicitis [16]. *Listeria monocytogenes* is a major concern for food producers, health regulatory officials, and consumers because it is a highly virulent foodborne pathogen. The incidence of listeriosis is rare compared to illnesses caused by other foodborne pathogens, such as *E. coli* O157:H7, *Campylobacter jejuni*, or *Salmonella* spp. [1]. Propionic acid in the FSUR may have affected the antimicrobial activity.

In conclusion, FSUR has a high free amino acid content, high antioxidant activity, and higher antimicrobial activity than that of antibiotics against human pathogenic microorganisms. Therefore, FSUR consumption may have various beneficial effects with respect to gut health.

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국문초록

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현미는 도정한 백미보다 이로운 영양분을 더 많이 함유하고 있다. 본 연구에서는 현미를 이용하여 만든 현미 발효 슬러리의 이화학적 특성과 항균활성에 대해 시험하였으며, 현미발효슬러리의 항균활성은 paper disc-agar diffusion 방법을 이용하여 6가지 병원성 균주(*Staphylococcus aureus, Escherichia coli, Listeria monocytogenes, Pseudomonas aeruginosa, Salmonella typhimurium* and *Yersinia enterocolitica*)와 2가지 발효균주(*Gluconacetobacter intermedius* and *Lodderomyces elongisporus*)에 대해 항균성을 조사하였다. 특히, *Staphylococcus aureus*, *E. coli*, *Listeria monocytogenes*, *P. aeruginosa*, *Salmonella typhimurium*, *Y. enterocolitica* 그리고 *Lodderomyces elongisporus*에 대해서는 시판 항생제인 카베니실린과 테트라사이클린보다 더 높은 항균활성을 보였다. 항산화활성은 2,2-diphenyl-1-picrylhydrazyl (DPPH) 라디칼 소거능을 이용하여 측정하였을 때, 대표되는 항산화제인 아스코르빅 산과 비슷한 활성을 나타내었다. 현미발효슬러리의 발효중에 나타나는 균주를 동정하기 위해 TSB 고체배지와 YPD 고체배지에 현미발효슬러리를 도포하였을 때, 분리된 콜로니를 16S rDNA sequence 분석을 통하여, 네가지 균주를 분리하였으며, phylogenic tree 분석법을 이용하여 조사하였을 때, 각각 *G. intermedius, Lactobacillus casei, Lactobacillus plantarum* 그리고 *Acetobacter peroxydans*와 유사하였다.