

Identification and Characterization of Useful Fungi with α -Amylase Activity from the Korean Traditional *Nuruk*

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The objective of this study was to find useful fungi with α -amylase activity from the Korean traditional *nuruk* for the quality of traditional Korean alcoholic beverage. In this study, 165 samples of traditional *nuruk* were collected from 170 regions throughout Korea and the fungi were isolated to a total of 384 strains. In order to investigate the effect of microflora on *nuruk*, α -amylase activity, saccharogenic power (SP), starch hydrolysis activity and acid producing activity were evaluated. Ten strains were selected by α -amylase activity, which ranged from 458.47 to 1,202.75 U/g. The size of the discolored zone for the starch hydrolysis activity of each fungus ranged from 0.3 to 2 cm. The SP of the 10 strains ranged from 228.8 to 433.4 SP. Of the 10 stains, three were identified as *Aspergillus oryzae*, two as *Aspergillus flavus*, two as *Lichtheimia* sp., one as *Rhizopus oryzae* and two as other strains. The total aflatoxins present in the *nuruks* were examined using enzyme-linked immunosorbent assay. The 10 *nuruks* had less than 1.11 ppb of aflatoxins.

KEYWORDS : Aflatoxins, α -Amylase activity, Fungi, *Nuruk*, Saccharogenic power

Introduction

Traditional Korean liquor is obtained through a parallel fermentation process in brewing [1]. The fermentation involves an initial stage, in which the microorganisms, usually fungi, produce a number of enzymes that break down substrate components. Following this, yeast species produce alcohol from the sugar that was previously produced by activity of mold on starch [2, 3]. The use of *nuruk* in this process is responsible for many of the characteristic features of this traditional Korean liquor. In contrast, *koji*, which is comparable to malts used for beer brewing, is used for the saccharification of starch and the decomposition of the protein contained in the raw material, rice grains [4]. *Nuruk* and *koji* are often confused in the literature, with “*koji*” sometimes called “modified *nuruk*” [5]. *Koji*, however, is artificially inoculated, while *nuruk* combines both a saccharification and starter by yeast, and so is used to fabricate the liquor without pre-fermentation. In addition, this process can be done at a low temperature due to the slightly lower saccharogenic power (SP) and fermentative activity of *nuruk*; as a result, various flavors of the liquor produced by a number of these microorganisms are retained [6].

During the production of the *nuruk*, it is possible to produce secondary metabolites of the fungi, including *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius*, that contain aflatoxins B₁, B₂, G₁, and G₂ [7-9]. Aflatoxins are both acutely and chronically toxic to animals and humans, and are classified as hazardous substances [10]. Hence, the development of traditional Korean liquor must be improved to minimize contamination with the aforementioned pathogenic bacteria, and to produce modified *nuruk* that has excellent SP and fermentative activity.

In this study, fungi samples were collected from various regions throughout Korea and the samples with outstanding α -amylase activity and SP were isolated and identified. The presence of aflatoxins in these identified strains was examined by an enzyme-linked immunosorbent assay (ELISA).

Materials and Methods

Preparation of *nuruk*. The samples used in this study were collected from 170 regions throughout Korea. Then Three hundred eighty four strains of fungi were isolated from 165 *nuruks*. The samples used in this study were collected at traditional markets in 23 regions of

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Chungcheongnam-do, 30 regions of Gyeongsangbuk-do, 18 regions of Gyeongsangnam-do, 12 regions of Chungcheongbuk-do, 20 regions of Gyeonggi-do, 13 regions of Jeollanam-do, 16 regions of Jeollabuk-do, 34 regions of Gangwon-do and four regions of Jeju-do. The collected *nuruk* from these 170 regions included 165 different varieties that were isolated and kept at 4°C. Fungi were grown on potato dextrose agar medium (0.4% [w/v] potato starch, 2% [w/v] dextrose; Difco, Sparks, MD, USA) at 30°C for 4 days.

α -Amylase activity and SP. The samples were made from the wheat using selected strains of *nuruk*. The *nuruk* was inoculated in spore suspension (1.0×10^7 spores/mL) and shaken in 0.5% (w/v) NaCl at 20°C for 3 hr. The extraction solution was centrifuged at 8,000 rpm for 10 min at 4°C. A clear supernatant was used in the enzyme solution. The substrate solution contained an acetate buffer (40 mM, pH 5.0) and 1% (w/v) soluble starch (preheated at 40°C for 5 min). The enzyme solution (0.1 mL) was added, and the mixture was incubated at 40°C for 30 min. Thereafter, a 0.00025 N iodine solution was added, and the transmittance (%T) was determined at 670 nm. Sample, substrate, and α -amylase blank determinations were undertaken under the same conditions. One unit of enzyme activity was defined as the amount of glucose released from 1 g of *nuruk* in 30 min [11]. SP of the final *nuruk* was measured using a 2% (w/v) soluble starch solution at a substrate, according to the methods of National Tax Service [12].

Starch hydrolysis and acid producing activity. To measure the starch hydrolysis activity of the isolated fungi in the *nuruk*, the starch medium was made using 0.1% (w/v) yeast nitrogen base without amino acids (Difco) as a nitrogen source and 0.2% (w/v) soluble starch (Difco) as a carbon source. Isolated strains were cultured on the starch medium, after which the strains were stained with an iodine solution (2% [w/v] KI, 1% [w/v] I₂, and distilled water) and the size of their clear zone was measured [13]. To measure the acid-producing activity of the isolated fungi, a Czapek solution agar medium (Difco) was made using rose bengal (0.005% [w/v]; Kanto Chemical, Tokyo, Japan), bromocresol green (0.004% w/v as a pH indicator; Sigma-Aldrich, St. Louis, MO, USA), and filter-sterilized chloramphenicol (0.1% v/v; Sigma-Aldrich). The isolated colonies were then cultured and selected according to the size of their visible discolored zones [14]. Unless otherwise specified, all the chemicals were of analytical grade.

Microorganism identification. The microorganisms were sent to Macrogen (Seoul, Korea) for identification via PCR. PCR was performed using a model PTC-225 peltier thermal cycler (MJ Research, Reno, NV, USA) after DNA

extraction (99°C, 10 min). The primers used were: ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [15]. The amplification conditions were as follows: 95°C for 5 min, 35 cycles at 94°C for 45 sec, 75°C for 1 min, 72°C for 10 sec; and 72°C for 5 min. PCR fragments were purified using ABI PRISM BigDye™ terminator cycle sequencing kits (Applied Biosystems, Foster City, CA, USA). 18S rRNA sequencing was performed using an ABI PRISM 3730XL DNA analyzer (Applied Biosystems) with the same PCR primers. The sequences were identified using the BLAST program (<http://www.ncbi.nlm.nih.gov>).

Total aflatoxins. ELISA analysis was performed according to the instructions in the Neogen Veratox aflatoxin procedure. Using a blender, 5 g of a ground sample was shaken vigorously for 3 min in 25 mL of 70% methanol. The extract was filtered using Whatman No. 1 filter paper (Whatman, Maidstone, England), and the filtrate was collected. The concentration of total aflatoxins in parts per billion was recorded from a 650 nm-filter ELISA reader (Molecular Devices, Sunnyvale, CA, USA) that was calibrated using aflatoxin standards, after which all the data were read and calculated using Neogen's Veratox software ver. 2.3.4 (Neogen, Lansing, MI, USA).

Results and Discussion

Isolation of useful fungi. Three hundred and eighty four strains of fungi were isolated. Of these, 40 strains were isolated according to their morphology and cultured on starch media, after which the samples were stained with an iodine solution. Finally, 10 strains were selected according to their α -amylase activity, which ranged from 458.47 ± 48.1 to $1,202.75 \pm 97.7$ U/g (Table 1). α -Amylase activity of *nuruk* made with strain N262-1 was the highest, and *nuruk* made with strain N220-1 was the lowest. The 10 selected strains, were N16 (2.0 cm), N36-1 (0.3 cm), N83 (0.3 cm), N109-2 (0.3 cm), N159-1 (0.8 cm), N220-1 (0.6 cm), N241-2 (0.3 cm), N252-1 (0.4 cm), N262-1 (0.5 cm), and N278-1 (0.3 cm), according to the

Table 1. Sources of the selected fungi from traditional *nuruk*

Fungal strain	Sources (region of Korea where collected)
N16	Gyeongsangbuk-do, Andong I
N36-1	Gangwon-do, Wontong
N83	Jeollabuk-do, Gimje
N109-2	Gyeongsangnam-do, Hapcheon V
N159-1	Gyeongsangbuk-do, Geochang II
N220-1	Gyeongsangbuk-do, Yeongyang
N241-2	Gyeongsangbuk-do, Goryeong
N252-1	Busan, Gijang
N262-1	Chungcheongnam-do, Seocheon
N278-1	Jeollanam-do, Gwangcheon

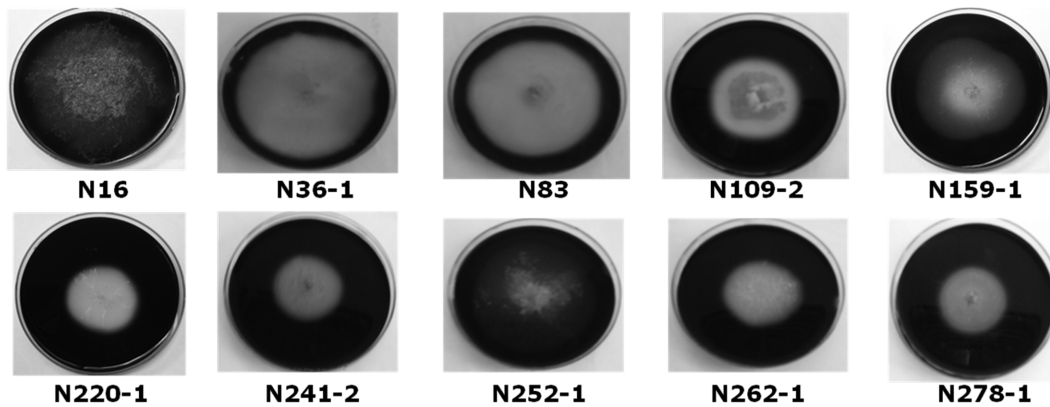


Fig. 1. Starch hydrolysis of selected fungi from traditional *nuruk*. N16, *Rhizopus oryzae*; N36-1, *Talaromyces spectabilis*; N83, *Paecilomyces variotii*; N109-2 and N278-1, *Lichtheimia* sp.; N159-1, N241-2, and N252-1, *Aspergillus oryzae*; N220-1 and N262-1, *Aspergillus flavus*.

Table 2. Characteristics of the selected fungi from traditional *nuruk*

Fungal strain	α -Amylase activity (unit/g <i>nuruk</i>)	Saccharogenic power	Acid producing activity (cm)	Total aflatoxins ^a (ppb)
N16	763.73 \pm 43.6	228.8 \pm 0.54	0.1	0.71 \pm 1.00
N36-1	569.91 \pm 12.0	303.9 \pm 2.80	0.1	0.00 \pm 0.00
N83	721.12 \pm 48.1	239.9 \pm 4.64	0.2	0.00 \pm 0.00
N109-2	615.08 \pm 82.6	290.7 \pm 0.79	0.3	0.00 \pm 0.00
N159-1	708.37 \pm 28.5	408.7 \pm 0.18	3.0	0.69 \pm 0.98
N220-1	458.47 \pm 48.1	433.4 \pm 0.27	1.2	1.11 \pm 1.57
N241-2	770.21 \pm 32.4	324.9 \pm 0.13	1.4	0.00 \pm 0.00
N252-1	563.97 \pm 33.2	332.0 \pm 0.12	1.1	0.55 \pm 0.78
N262-1	1202.75 \pm 97.7	313.5 \pm 0.68	1.0	0.65 \pm 0.92
N278-1	891.33 \pm 37.0	326.5 \pm 0.12	0.9	0.00 \pm 0.00

^aTotal aflatoxins (B₁, B₂, G₁, G₂).

size of the radius of the visible discolored zone, which ranged from 0.3~2.0 cm (Fig. 1). The SP of the 10 strains ranged from 228.8 \pm 0.54 to 433.4 \pm 0.27 SP (Table 2). The SP of *nuruk* made with N220-1 was the highest, and *nuruk* made with N16 was the lowest. The existence of acid-producing activity in fungi is important to prevent contamination with various microorganisms and abnormal fermentation in alcohol beverages made using *nuruk*. In this study, 10 fungi were examined using the size of their visible discolored zone via acid production. The visible discolored zones of the isolated strains ranged from 0.1~3.0 cm (Table 2). The systematic manufacture of liquor using useful strains with high acid-producing and starch hydrolysis activities must be carried out to globalize these traditional liquors, including *makgeolli*.

Identification of selected fungi. The 10 selected fungi were identified. N159-1 (KCTC 11927BP), N241-2, and N252-1 were *Aspergillus oryzae*; N220-1 and N262-1 were *A. flavus*, N36-1 was *Talaromyces spectabilis*, N83 was *Paecilomyces variotii*, and N109-2 and N278-1 were *Lichtheimia* sp. (Table 3). *A. oryzae* has previously been

isolated from traditional Korean *nuruk* [6, 7]. This strain shows high amylase activity for starch degradation [16, 17] and also has a high level of acid-producing activity and a unique flavor [14, 18]. *A. flavus* has also been reported in Korean *nuruk* and was reported to produce aflatoxin [19].

Total aflatoxins. Aflatoxin was detected in the *nuruk* made with 10 strains, *A. oryzae* (three strains), *A. flavus* (two strains), and others (five strains) at less than 1.11 \pm 1.57 ppb (Table 2). The results were tested in triplicate via an ELISA. The current maximum levels set by the European commission are 2~8 mg/kg for aflatoxin B₁ and total aflatoxins for groundnut, nuts, dried fruits, cereals, and processed products [20]. The action levels of the United States Food and Drug Administration [21] are 20 mg/kg of total aflatoxins in foods, peanuts, peanut products, and pistachio nuts. In the Codex Alimentarius [22], the guideline levels of total aflatoxins are 15 mg/kg for peanuts intended for further processing, almonds, hazelnuts, and pistachios. The current regulations in Korea for aflatoxin B₁ are 10 mg/kg for cereals, bean products,

Table 3. Identification of the selected fungi from traditional *nuruk*

Fungal strain	Nearest neighbor strain	Similarity (%)	Data base	Accession No.
N16	<i>Rhizopus oryzae</i> strain NA1 18S ribosomal RNA gene, partial sequence	95	gb	GU138496.1
N36-1	<i>Talaromyces spectabilis</i> strain CBS 121583 18S ribosomal RNA gene, partial sequence	98	gb	EU037060.1
N83	<i>Paecilomyces variotii</i> strain XSBN13 18S ribosomal RNA gene, partial sequence	100	gb	GU966517.1
N109-2	<i>Lichtheimia ramosa</i> strain CNRMA/F 07-69 18S ribosomal RNA gene, partial sequence	100	gb	FJ719387.1
N159-1	<i>Aspergillus oryzae</i> strain SV/09-09 18S ribosomal RNA gene, partial sequence	95	gb	FJ654483.1
N220-1	<i>Aspergillus flavus</i> isolate ucb032 18S ribosomal RNA gene, partial sequence	99	gb	EF409793.1
N241-2	<i>Aspergillus oryzae</i> strain 1 18S ribosomal RNA gene, partial sequence	98	gb	HQ380782.1
N252-1	<i>Aspergillus oryzae</i> strain FH4 internal transcribed spacer 1, partial sequence	95	gb	EU409806.1
N262-1	<i>Aspergillus flavus</i> strain MSSRF-IS2 18S ribosomal RNA gene, partial sequence	98	gb	HQ010119.1
N278-1	<i>Lichtheimia corymbifera</i> strain AX01 18S ribosomal RNA gene, partial sequence	99	gb	GU980967.1

gb, genebank.

peanuts, processed products, *meju*, *doenjang*, and *gochujang* [23, 24]. Kim *et al.* [25] reported that *Aspergillus* sp., *Penicillium* sp. and *Rhizopus* sp. isolated from *nuruk* cultured in raw wheat bran were devoid of aflatoxins. Yang *et al.* [26] noted that *A. flavus*, *A. parasiticus*, *A. niger*, and *A. oryzae* in *doenjang* were negative for aflatoxins, using multiplex PCR and direct-competitive-ELISA assays. Zheng *et al.* [27] compared the results of ELISA and high-performance liquid chromatography in the detection of total aflatoxins. ELISA was effective in measuring total aflatoxins ($B_1 + B_2 + G_1 + G_2$) in several appropriate commodities in quantitative ranges of 4~40 ppb. The ELISA method, due to its high variation in replicates, was found to be applicable as a screening method [8]. Therefore, the results indicated that ELISA is suitable for comparison and screening of a large number of samples.

It was verified that the useful fungi that were identified and isolated from *nuruk* produced only a small amount of aflatoxins (0~1.11 ppb). These may be a good material for manufacturing traditional Korean liquor in the near future.

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