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Solid Fungi Starters Using *Aspergillus* spp. under Different Manufacturing Conditions

Su Jeong Lee, Han Byul Kang, Sun Hee Kim, Woo Soo Jeong, So-Yeong Kim and Soo-Hwan Yeo *

Fermented and Processed Food Science Division, Department of Agrofood Resource, NIAS, RDA, Wanju 55365, Republic of Korea; leesooj@korea.kr (S.J.L.); khb9608@gmail.com (H.B.K.); sunheekim00@korea.kr (S.H.K.); wjddntnek@korea.kr (W.S.J.); foodksy@korea.kr (S.-Y.K.)
* Correspondence: yeobio@korea.kr; Tel.: +82-63-238-3609; Fax: +82-63-238-3843

Abstract: The generation of royalties on the use of foreign-made inoculum strains or starters as a result of the implementation of the 'Nagoya Protocol' has led to efforts and healthy competition to secure useful biological resources in each country. In this study, we recognized the necessity and importance of securing useful strains in Korea and produced starters using five strains of fungi (*Aspergillus oryzae* and *Aspergillus niger*). Specifically, the quality characteristics exhibited by different strain inoculum concentrations (1, 3, 5% (*v*/*w*)) and drying conditions (low-temperature air drying at 35 °C for 24 h and high-temperature hot air drying at 45 °C for 18 h) during the starter manufacturing process were analyzed. Parameters such as enzyme activity and free amino acid and volatile flavor compound content, which may vary based on production conditions, are expected to produce basic data for the production of Korean-type starters.

Keywords: fungi; starter; *Aspergillus oryzae*; *Aspergillus niger*; solid-state fermentation

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1. Introduction

The fermentation industry uses microorganisms for manufacturing fermented foods and products, such as alcoholic beverages and fermented sauces. This industry is expanding and overlaps with various fields, such as pharmaceuticals, and contributes to manufacturing other high-value-added products [1]. However, since the '*Nagoya Protocol*' came into effect, the generation of royalties on foreign-made inoculum strains or starters has meant that there has been an increase in efforts and healthy competition to secure useful biological resources in each country. Therefore, various studies are necessary to contribute to the development of the bio-industry [2,3].

Most countries with developed brewing industries have national or association-level management systems to provide custom fermentation strains, also called starters. However, starters used in the Korean brewing industry are mainly imported, which does not suit the intended purpose nor satisfy various demands [4]. Therefore, securing indigenous resources for microbial fermentation and developing basic source technologies to produce and utilize domestically produced strains or starters that can replace imported strains is crucial.

Most *Nuruk*, which is used as a fermenting agent in the production of Korean alcoholic beverages such as *Takju* and *Makgeolli*, is produced using traditional methods, meaning that the manufacturing process is inefficient due to the influx of unpurified bacteria exposed to the manufacturing environment and unregulated temperature and humidity conditions [1]. In order to improve the inconsistent quality and the difficulty of stable supply due to such a manufacturing environment, we investigated the manufacturing conditions of the Korean-type solid starter using a single strain with excellent characteristics as a substitute.

Aspergillus spp., which is mainly isolated from Korean fermented products such as *Nuruk* and *Meju*, is one of the most useful species in the Asian fermentation industry [5–9]. A representative fermentation starter is *Koji* with a single strain (*Aspergillus* spp.), which

affects saccharification and proteolytic activity because of enzyme action; the effects are also dependent on the manufacturing technology and strain characteristics, which determine the quality of the final fermentation product [10–13].

In this study, we recognized the necessity and importance of securing useful strains in our country and produced each starter using five strains of fungus (*Aspergillus* spp.) available within our research team. The quality characteristics exhibited using different strain inoculum concentrations and drying conditions during the starter manufacturing process were analyzed. In addition to adding useful industrial value through research results, this study contributes to attaining uniformity and safety in Korean traditional fermented products, which are relatively unstable under different process conditions.

2. Materials and Methods

2.1. Materials and Manufacturing Method of Solid-State Fungi Starter

The rice cultivar *Samgwang*, a hard-textured rice harvested in Chungcheong-do, Korea, was used as a raw material in this study. The strains used were *Aspergillus oryzae* 81-2 (AO 81-2), *A. oryzae* 82-3 (AO 82-3), *A. oryzae* 2-6 (AO 2-6), *A. niger* IF 13-3 (AN IF 13-3), and *A. niger* 4-3-2 (AN 4-3-2). This was a selection of strains from the research team that had good enzyme activity and could be used for food. To use each of these fungi, 5% (*w*/*v*) bran was added to distilled water and the medium thus prepared was sterilized at 121 °C for 15 min; the medium was inoculated with the same spore count (10^7 spores/mL), cultured with shaking at 120 rpm at 28 °C for 5 days, and then filtered to obtain the supernatant. This supernatant was used as the pre-cultured culture broth.

The *Samgwang* rice was washed, soaked for 3 h, drained for 1 h, steamed for 40 min at 110 °C, and cooled to 40 °C. The liquid starter (pre-cultured culture broth, 10^8 spores/mL) was inoculated at various concentrations (1, 3, 5% (*v*/*w*)) and cultured at 36 °C for five days using a *Koji*-making machine (Mini-15, Yaegaki Food Co., Hyogo, Japan). The manufactured solid-state fungal starter (SSFS) was dried using two methods under non-sterile conditions: low-temperature blast drying (L.T. drying) at 35 °C for 24 h and high-temperature hot air drying (H.T. drying) at 45 °C for 18 h, to suppress contamination due to moisture content. We compared the quality characteristics of the solid starters obtained using the two methods and chose the optimum drying conditions for the industrial solid fungal starter inoculum.

2.2. Microbial Contamination and Spore Count

To test for contamination produced during the process from bacteria other than the single inoculated strain during SSFS manufacturing, the dried starter was checked for general bacteria with aerobic count (AC) petrifilm (3M, MN, USA), and *Penicillium* spp. fungus with asymmetric broom abstract shapes was examined under a microscope (DM500, Leica, Wetzlar, Germany) using malt extract agar (MEA) medium (Difco, NJ, USA). The number of spores, which indicates the activity of the starter, was measured using a hemocytometer after suspending 1 g of the starter in 10 mL of 5% Tween 80 solution, with 2 to 3 drops of 1% methylene blue solution [14].

2.3. Enzyme Activity Analysis

For testing enzyme activity, 10 g of each SSFS was extracted with 50 mL of 10 mM acetate buffer containing 0.5% NaCl. The α-amylase, glucoamylase, and carboxyl peptidase were activated using a Kikkoman Brew Analyzing Kit (Kikkoman Co., Tokyo, Japan) according to the manufacturer's instructions. The kit was constructed with reference to the laws prescribed by the National Tax Agency of Japan, and was calculated according to the regression formula applied for the rice sample, *koji*.

2.4. Analysis of Free Amino Acid Content

The free amino acid content of the SSFS was analyzed by modifying the method of a previous study as follows [11]. After 0.1 g of the SSFS was diluted 10-fold with ethanol and

stirred, only the supernatant was filtered through a 0.2 µm membrane and used. Next, to measure the free amino acid content, 1 mL of filtrate was diluted with 5% trichloroacetic acid (TCA) and again filtered through a 0.2 µm membrane. A free amino acid analyzer (Sykam S7130 amino acid reagent organizer, Eresing, Germany) and an S5200 sample injector were used, along with an S2100 solvent delivery system. A cation separation column (LCA K06/NA 250 mm × 4.6 mm) was used; the mobile phase flow rate was set to 0.45 mL/min, and ninhydrin was set to 0.4 mL/min.

2.5. Analysis of Volatile Flavor Compounds

To analyze the volatile flavor compounds, 0.5 g of SSFS was saturated with 1 g NaCl for each manufacturing condition and placed into vials (20 mL) containing 1 mL tertiary distilled water. The vials were equilibrated at 50 °C for 20 min with stirring. Volatile compounds in the headspace were absorbed by solid phase microextraction (SPME) fiber (50/30 um Divinylbenzene/Carboxen/Polydimethylsiloxane DVB/CAR/PDMS, Supelco Inc., PA, USA) for 15 min, and then desorbed via fiber injection into gas chromatography/mass spectrometry (GC/MS) apparatus. A GC-2010 Plus (Shimazu, Tokyo, Japan) equipped with a DB-WAX column (30 mm × 0.25 mm i.d., 0.25 µm film thickness, J&W, CA, USA) was used to analyze the volatile compounds. The SPME fiber absorbed the volatile compounds, and a 1 µL aliquot of derivatized sample was injected into the GC apparatus. Helium was used as the column carrier gas at a constant flow rate of 1 mL/min. The injector temperature was set at 250 °C, and the oven temperature was maintained at 40 °C for 3 min, increased to 90 °C at 5 °C/min, increased to 230 °C at 19 °C/min, and then held at 230 °C for 5 min. The GC column effluent was analyzed on a GCMS-TQ 8030 (Shimazu, Tokyo, Japan) using the electron impact ionization mode. The ion source temperature was 230 °C for the volatile compounds. The interface temperature was 280 °C, and the detector voltage was 0.1 kV. The volatile compounds were identified through the online databases of the NIST 11 and Wiley 9 mass spectral libraries [15,16].

2.6. Statistical Analysis

All the experiments were conducted in triplicate, and the mean values are reported. Processed GC/MS data sets were statistically analyzed through multivariate statistical analysis using SIMCA-P+ (ver. 16.0.2, Umetrics, Umea, Sweden) and through one-way analysis of variance (ANOVA) with Duncan's test ($p < 0.05$) using SPSS (ver. 27.0, SPSS Inc., IL, USA). Partial least squares discriminant analysis (PLS-DA) was performed to visualize the separation among samples and to find volatile compounds contributing to the separation. Furthermore, volatile compounds contributing to the differences between groups ($p < 0.05$) were visualized in the heat map drawn using R-project with the color representing the z-score-transformed raw data of the volatile compounds.

3. Results

3.1. Manufactured Solid Fungal Starter

The appearance of the SSFS obtained using each of the five inoculated strains of fungi (*A. oryzae* 81-2, *A. oryzae* 82-3, *A. oryzae* 2-6, *A. niger* IF13-3, and *A. niger* 4-3-2) is shown in Table 1. Color confirmations appeared as green for *A. oryzae* and black for *A. niger*. Single-strain dominance occurred on rice grains.

3.2. Microbial Contamination and Spore Count

Various types of microbes that cause safety problems have been detected in *Nuruk*, a Korean indigenous starter. Therefore, hygienic treatment methods are required during the manufacturing process and distribution [17,18]. The degree of contamination was judged based on whether or not fungi of the *Penicillium* spp. and general bacteria were detected in the solid fungi starter [10,19]. Most of the general bacteria were detected (or not) under both drying conditions, depending on the liquid starter inoculation concentration (Table 2). It is presumed that the inhibitory effect on the growth of spoilage bacteria resulted from

the superiority of using a single strain [4]. The presence of *Penicillium* spp. was considered positive (+) when blue fungi with an asymmetrical broom abstract shape were detected under microscopy, and considered negative (-) when they were not detected (Table 2). No fungi of *Penicillium* spp. were detected under any of the conditions, indicating their safety to use as a solid fungal starter.

Table 1. Morphology of solid-state fungal starters depending on manufacturing conditions.

Strain [1]	Liquid Starter (*v*/*w*)(%)		
	1	3	5
AO 81-2			
AO 82-3			
AO 2-6			
AN IF13-3			
AN 4-3-2			

[1] AO, Aspergillus oryzae; AN, Aspergillus niger.

To confirm the activity of the SSFS depending on the inoculum concentration of the liquid starter, spore counts were obtained. All treatment conditions were measured at a level of 10^8 spores/g and judged to exhibit similar activity when compared with that reported in previous studies [20]. The spore count of SSFS was an indicator of its activity as a starter if it remained active under both drying conditions. The condition of L.T. drying and inoculation with 5% liquid starter showed a two- to three-fold increase in spore count, but the difference was small. The AO 2-6 strain showed the highest sporulation (4.2×10^8 spores/g) regardless of the drying conditions, confirming its excellent growth activity.

Table 2. Quality analysis of solid-state fungal starters under different manufacturing conditions.

Strain [1]	Drying Condition		Liquid Starter (*v*/*w*)(%)	Microbial Contamination		Spores of Aspergillus spp. ($\times 10^8$ Spores/g)
				General Bacteria (CFU/g)	*Penicillium* spp. (-), (+)	
AO 81-2	L.T. [2]	35 °C 24 h	1	9.0×10^2	-	2.7 ± 0.3
			3	2.0×10^3	-	2.2 ± 0.2
			5	2.0×10^3	-	2.3 ± 0.3
	H.T.	45 °C 18 h	1	1.0×10^3	-	2.4 ± 0.4
			3	3.6×10^3	-	2.0 ± 0.2
			5	4.4×10^3	-	2.1 ± 0.2
AO 82-3	L.T.	35 °C 24 h	1	4.1×10^2	-	0.9 ± 0.2
			3	3.8×10^2	-	1.7 ± 0.1
			5	8.0×10^1	-	1.8 ± 0.3
	H.T.	45 °C 18 h	1	1.5×10^3	-	0.7 ± 0.2
			3	2.2×10^2	-	1.5 ± 0.2
			5	1.2×10^2	-	1.9 ± 0.3
AO 2-6	L.T.	35 °C 24 h	1	2.3×10^2	-	2.7 ± 0.2
			3	1.0×10^1	-	3.4 ± 0.4
			5	1.0×10^1	-	4.2 ± 0.6
	H.T.	45 °C 18 h	1	9.3×10^2	-	2.6 ± 0.4
			3	3.5×10^2	-	3.1 ± 0.3
			5	1.0×10^1	-	4.0 ± 0.6
AN IF13-3	L.T.	35 °C 24 h	1	8.8×10^2	-	1.3 ± 0.1
			3	6.4×10^2	-	2.3 ± 0.1
			5	1.7×10^2	-	2.8 ± 0.2
	H.T.	45 °C 18 h	1	1.1×10^3	-	1.2 ± 0.3
			3	9.7×10^2	-	2.1 ± 0.2
			5	2.3×10^2	-	2.6 ± 0.2
AN 4-3-2	L.T.	35 °C 24 h	1	3.9×10^2	-	1.2 ± 0.0
			3	1.0×10^1	-	2.1 ± 0.3
			5	N.D.[3]	-	2.3 ± 0.2
	H.T.	45 °C 18 h	1	4.2×10^2	-	0.8 ± 0.4
			3	4.0×10^2	-	1.8 ± 0.2
			5	N.D.	-	2.1 ± 0.3

[1] AO, *A. oryzae*; AN, *A. niger*. [2] L.T., low-temperature blast drying; H.T., high-temperature hot air drying. [3] N.D., not detected. Values are mean ± standard deviation (n = 3).

3.3. Enzyme Activity

The activities of the three types of enzymes (α-amylase, glucoamylase, carboxyl peptidase) were quantified (Figure 1, Table S1). The α-amylase activity, which indicates liquefying power, of AO 82-3, AO 2-6, AN IF13-3, and AN 4-3-2 was higher when the liquid starter concentration was 3% under the L.T. than the H.T. drying condition. AO 82-3 had the highest measured α-amylase activity at 858.6 U/g (BS3), approximately five-fold higher than the lowest measured activity using AN IF13-3 of 148.7 U/g (DS3). This result was consistent with the reported liquefaction power of 100–735 U/g in single-strain culture [21].

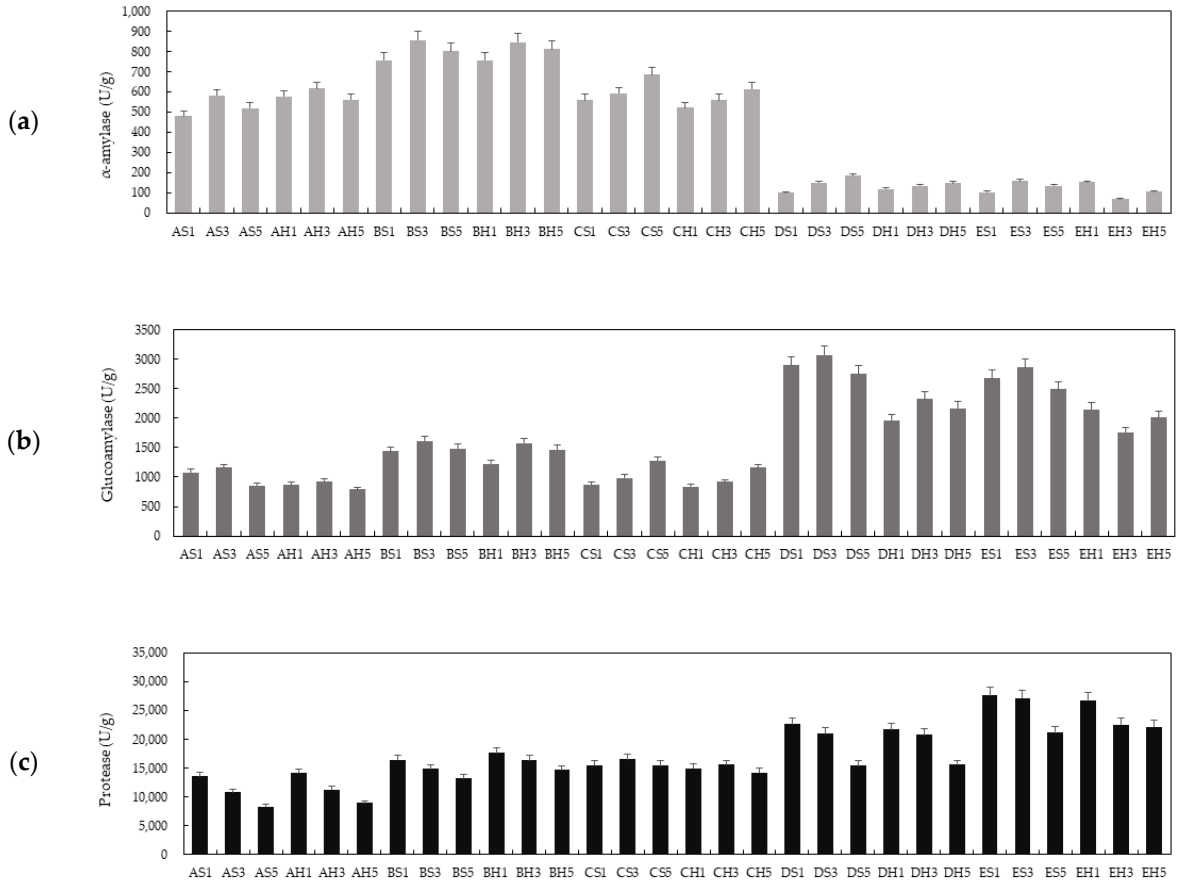

Figure 1. Enzyme activity properties for various solid-state fungal starters (SSFS): (**a**) α-amylase, (**b**) glucoamylase, and (**c**) protease activity of SSFS by manufacturing conditions. A, *A. oryzae* AO 81-2; B, *A. oryzae* AO 82-3; C, *A. oryzae* AO 2-6; D, *A. niger* AN IF 13-3; E, *A. niger* AN 4-3-2; S, low-temperature blast drying; H, high-temperature hot air drying; 1, 3, 5, concentration of liquid starter (%). Error bars indicate standard deviation ($p < 0.05$).

The glucoamylase activity, which indicates the saccharogenic power, an important function of SSFS, was elevated for all strains when dried under L.T. drying conditions. Of all the strains, except AO 2-6, the highest glucoamylase activity was exhibited when inoculated with 3% liquid starter, indicating the optimum condition for starter production. These results are consistent with those reported previously, wherein an excessively increased liquid starter concentration lowered glucoamylase activities [4,22]. In addition, it was confirmed that AN strains exhibited higher glucoamylase activity than AO strains. After inoculation with a liquid starter at a concentration of 3%, the AO 82-3 in the L.T. drying condition produced 1,611.4 U/g (BS3), a difference of approximately two-fold over that of AN IF13-3 (3,072.3 U/g; DS3) under the same conditions, confirming that the AN strains had higher saccharogenic power. A previous study reported that *A. niger* had better glucoamylase activity than *A. oryzae* under the same culture conditions [23,24]. In addition, it has been reported that the establishment of optimal culture conditions for each strain is necessary because the enzyme activity appears differently when *A. niger* isolated from various origins are cultured under different conditions [25].

Protease activity analysis confirmed that, like for the glucoamylase activities, AN strains exhibited higher proteolytic activity than the AO strains. The highest measured activity was 27,668.8 U/g when the L.T. drying condition was used after 1% inoculation with AN 4-3-2 (ES1), indicating a difference between the α-amylase and glucoamylase results. Based on these results, the characteristics of the inoculum strain, concentration of the liquid starter, and drying conditions can be adjusted to select the optimum conditions

for producing excellent enzyme activity. This contributes to the technology for producing high-quality SSFS.

3.4. Free Amino Acid Content

The free amino acid content of SSFS by manufacturing conditions are listed in Tables S2–S6. A total of 26 types of free amino acids were detected, of which glutamic acid, which produces umami flavor, was the highest under all test conditions [26,27]. The AO 81-2 inoculated with liquid starter 1% and L.T.-dried produced 1953.8 μg/g (AS1) and AN IF 13-3 inoculated with 3% produced 1952.1 μg/g (DS3). Another highly detected amino acid was aspartic acid, which imparts a weak sweetness, at 944.8 μg/g on BS1. Alanine, leucine, and arginine were usually measured in abundance. This was consistent with a previous study in which glutamic acid was the highest when rice was used as the raw material for *Nuruk*, and high leucine and arginine contents were also detected [28].

When comparing the free amino acid content of each SSFS, the 1% inoculated liquid starter generally showed a higher content. However, the difference in the free amino acid content depended on the drying conditions for each inoculum and was very small. The total free amino acid content appeared in the order BS1 10,563.2 μg/mL > DH1 10,491.7 μg/mL > EH5 10,406.9 μg/mL > AS1 9722.7 μg/mL > CH1 8713.3 μg/mL (Figure 2). Therefore, the use of an inoculum containing amino acids that affect taste should contribute to the production of various fermentation products [29,30].

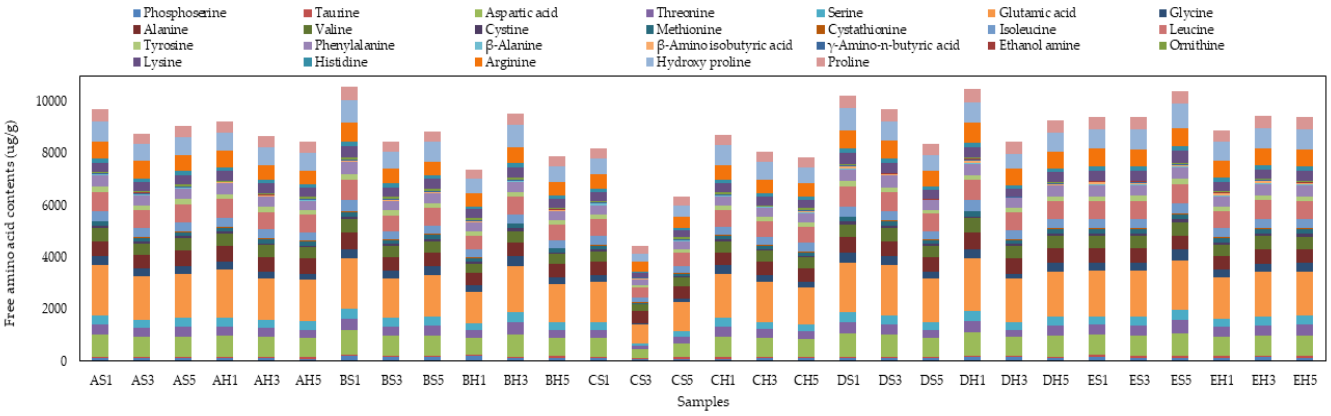

Figure 2. Free amino acid content of solid-state fungal starters (SSFS) by manufacturing conditions. A, *A. oryzae* AO 81-2; B, *A. oryzae* AO 82-3; C, *A. oryzae* AO 2-6; D, *A. niger* AN IF 13-3; E, *A. niger* AN 4-3-2; S, low-temperature blast drying; H, high-temperature hot air drying; 1, 3, 5, concentration of liquid starter (%).

3.5. Volatile Flavor Compounds

The detected substances were identified by measuring the volatile flavor compounds depending on the drying conditions of the SSFS (Table 3). The PLS-DA (partial least square discriminant analysis) multivariate statistical analysis (Figure 3) indicates the correlation between samples in a heatmap (Figure 4).

A total of 33 compounds were detected in AN IF 13-3 and AN 4-3-2, 24 compounds in AO 81-2, and 27 compounds in AO 82-3 and AO 2-6. The AN strains exhibited more diverse volatile flavor compounds than the AO strains. During the production of the SSFS with the AN strains, there was a tendency to produce more aldehydes (including nonanal and decanal), which was not seen using the AO strains. Moreover, the higher the concentration of the liquid starter inoculum, the more volatile flavor compounds were detected, but the difference due to drying conditions was small.

Table 3. Identification of volatile flavor compounds of solid fungal starters.

Compound \ Stain	AO [1] 81-2		AO 82-3		AO 2-6		AN IF 13-3		AN 4-3-2	
	p-Value [2]	VIP [3]	*p*-Value	VIP	*p*-Value	VIP	*p*-Value	VIP	*p*-Value	VIP
Esters										
Benzeneacetic acid, 2-tridecyl ester	3.5×10^{-1}	0.91	1.5×10^{-1}	0.79	2.6×10^{-1}	0.78	1.8×10^{-1}	0.65	3.5×10^{-1}	0.91
Methyl salicylate	9.0×10^{-1}	0.49	1.2×10^{-7}	0.97	6.0×10^{-3}	1.09	7.4×10^{-2}	0.54	9.0×10^{-1}	0.49
Alcohols										
Ethanol	2.9×10^{-2}	0.60	3.7×10^{-1}	0.45	1.5×10^{-1}	0.35	7.8×10^{-7}	1.47	2.9×10^{-2}	0.60
2-Methyl-1-propanol	1.1×10^{-1}	0.50	4.7×10^{-1}	0.62	4.1×10^{-1}	1.10	3.6×10^{-1}	0.46	1.1×10^{-1}	0.50
2-Methyl-1-butanol	6.5×10^{-2}	0.77	4.5×10^{-3}	1.15	1.6×10^{-1}	1.63	4.4×10^{-1}	0.45	6.5×10^{-2}	0.77
3-Methyl-1-butanol	1.7×10^{-2}	0.94	4.3×10^{-2}	0.93	1.7×10^{-1}	1.61	8.3×10^{-2}	0.93	1.7×10^{-2}	0.94
1-Hexanol	6.4×10^{-1}	0.71	1.2×10^{-2}	1.09	1.1×10^{-4}	1.18	6.2×10^{-2}	1.18	6.4×10^{-1}	0.71
3-Octanol	-	-	1.2×10^{-6}	1.37	5.5×10^{-2}	1.34	3.6×10^{-2}	1.16	6.0×10^{-3}	1.22
1-Octen-3-ol	2.3×10^{-6}	1.52	1.1×10^{-3}	1.26	3.9×10^{-2}	0.62	7.6×10^{-8}	1.62	2.3×10^{-6}	1.52
1-Heptanol	-	-	6.0×10^{-5}	1.34	2.1×10^{-2}	1.25	6.7×10^{-1}	0.78	3.9×10^{-2}	1.19
2-Ethylhexanol	3.6×10^{-1}	0.87	2.4×10^{-3}	1.11	1.1×10^{-2}	0.95	1.1×10^{-1}	1.12	3.6×10^{-1}	0.87
1-Octanol	5.0×10^{-3}	1.34	1.6×10^{-1}	0.94	1.4×10^{-2}	0.89	5.1×10^{-1}	1.00	5.0×10^{-3}	1.34
(E)-2-Octen-1-ol	1.0×10^{-3}	1.42	1.1×10^{-2}	1.19	1.4×10^{-1}	0.77	1.0×10^{-3}	1.45	1.0×10^{-3}	1.42
1-Nonanol	-	-	-	-	-	-	9.0×10^{-3}	1.29	4.8×10^{-2}	1.10
Benzeneethanol	4.8×10^{-2}	0.40	3.6×10^{-3}	0.78	1.6×10^{-2}	1.13	6.2×10^{-2}	1.30	1.5×10^{-1}	0.40
Aldehydes										
3-Methylbutanal	-	-	2.5×10^{-4}	0.96	1.2×10^{-2}	1.62	3.4×10^{-5}	1.49	3.0×10^{-3}	1.14
Hexanal	-	-	-	-	-	-	7.9×10^{-2}	1.16	6.0×10^{-3}	1.18
Nonanal	-	-	-	-	-	-	2.4×10^{-2}	1.28	1.1×10^{-2}	1.27
Decanal	-	-	-	-	-	-	1.7×10^{-2}	0.81	1.1×10^{-1}	1.04
Ketones										
2,4-Dimethylheptane	8.2×10^{-2}	1.10	6.9×10^{-2}	1.09	7.4×10^{-1}	0.50	2.1×10^{-1}	0.86	8.2×10^{-2}	1.10
3-Octanone	2.8×10^{-6}	1.49	2.5×10^{-3}	1.10	2.9×10^{-2}	0.78	1.1×10^{-4}	1.45	2.8×10^{-6}	1.49
6-Methyl-5-hepten-2-one	1.3×10^{-1}	1.01	2.4×10^{-4}	0.21	3.0×10^{-1}	0.29	2.7×10^{-1}	1.05	1.3×10^{-1}	1.01
Miscellaneous										
Dodecane	1.5×10^{-1}	1.02	3.0×10^{-2}	1.07	1.3×10^{-1}	0.87	4.3×10^{-1}	0.45	1.5×10^{-1}	1.02
1,3,5-Trimethylbenzene	8.2×10^{-1}	0.11	1.4×10^{-2}	1.22	4.4×10^{-1}	0.72	3.4×10^{-1}	0.99	8.2×10^{-1}	0.11
1,4-Dimethyl-2-ethylbenzene	2.2×10^{-1}	0.85	9.2×10^{-3}	0.85	1.7×10^{-1}	0.61	1.9×10^{-1}	0.58	2.2×10^{-1}	0.85
1,2-Dimethyl-4-ethylbenzene	3.0×10^{-1}	0.52	7.0×10^{-3}	1.20	2.6×10^{-2}	1.04	1.5×10^{-1}	0.67	3.0×10^{-1}	0.52
1,2,4,5-Tetramethylbenzene	9.9×10^{-1}	0.05	3.0×10^{-1}	0.58	8.0×10^{-3}	1.02	1.8×10^{-1}	0.37	9.9×10^{-1}	0.05
1,3-Di-tert-butylbenzene	4.4×10^{-1}	0.73	6.6×10^{-2}	1.06	3.2×10^{-2}	0.95	6.8×10^{-2}	0.28	4.4×10^{-1}	0.73
1,2,3,4-Tetramethylbenzene	3.7×10^{-1}	0.88	7.5×10^{-3}	1.15	5.2×10^{-2}	0.9	3.7×10^{-2}	0.79	3.7×10^{-1}	0.88
Linalool oxide	-	-	-	-	-	-	5.3×10^{-9}	1.5	1.6×10^{-2}	1.06
2,2,6-Trimethyl-3-keto-6-vinyltetrahydropyran	1.0×10^{-3}	1.43	3.4×10^{-3}	0.99	2.4×10^{-2}	0.99	1.7×10^{-5}	1.52	1.0×10^{-3}	1.43
Caryophyllene	-	-	-	-	-	-	1.1×10^{-7}	1.57	3.3×10^{-4}	1.43
Butylated hydroxyltoluene	1.0×10^{-3}	1.48	1.3×10^{-4}	1.12	5.0×10^{-3}	1.51	1.0×10^{-2}	0.67	1.0×10^{-3}	1.48

[1] AO, *A. oryzae*; AN, *A. niger*. [2] *P*-values were analyzed using one-way analysis of variance (ANOVA). [3] Variable importance in the projection (VIP) values were determined using partial least squares discriminant analysis (PLS_DA).

Among the volatile flavor compounds commonly detected under all conditions, 1-octen-3-ol was mainly observed and was detected at particularly high levels in the AO 81-2 starter. This compound was previously reported as the flavor compound in mushrooms and was detected at very high levels in *A. niger* and *A. oryzae* in a study of volatile flavor compounds produced by molds [31]. Methyl salicylate was also detected and belongs to esters, which are the main flavor components of alcoholic fermentation products, such as *Yakju* and *Makgeolli* (Korean traditional rice wine) [32,33]. 3-methyl-1-butanol is also known as a *Deonjang* (soybean paste) and traditional liquor flavor component; it is classified as having a banana scent, and is reported as a high-quality alcohol produced by *Nuruk* filamentous fungi [34–36]. Based on these results, we expect the possibility of using this SSFS for the production of Korean alcoholic beverages and fermented foods such as *Deonjang* and *Gochujang* (red pepper paste), etc.

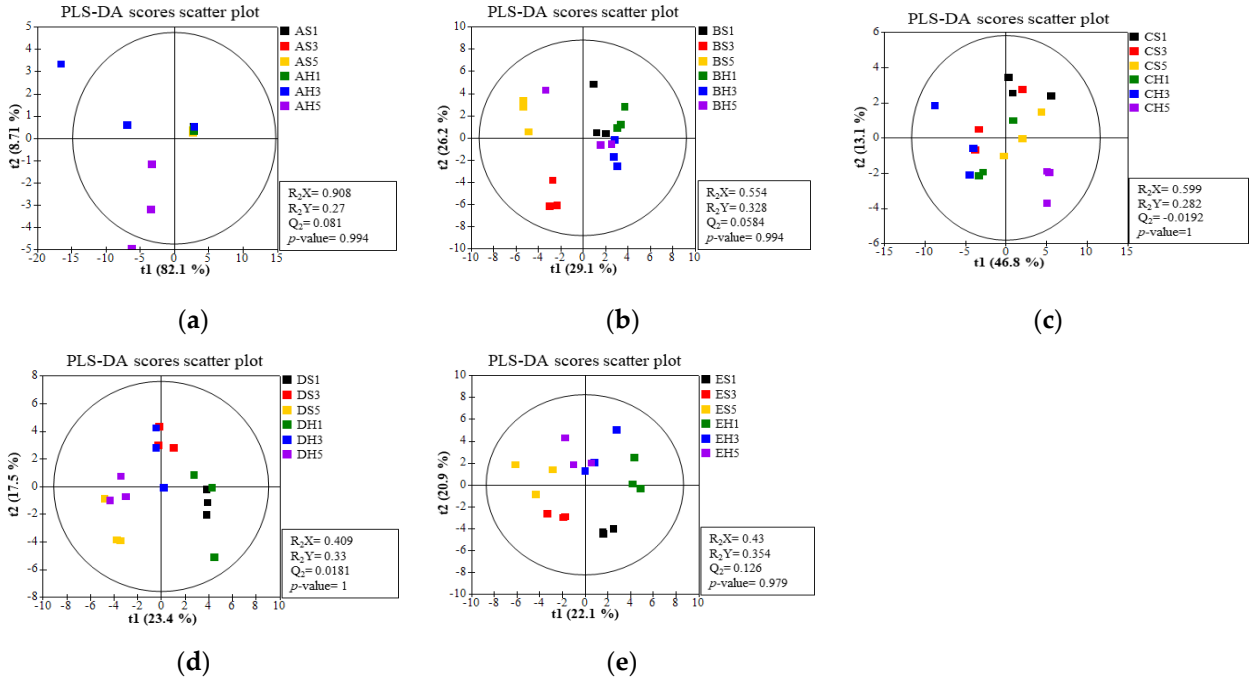

Figure 3. PLS-DA score plots of volatile flavor compounds. (**a**) A, *A. oryzae* AO 81-2; (**b**) B, *A. oryzae* AO 82-3; (**c**) C, *A. oryzae* AO 2-6; (**d**) D, *A. niger* AN IF 13-3; (**e**) E, *A. niger* AN 4-3-2; S, low-temperature blast drying; H, high-temperature hot air drying; 1, 3, 5, concentration of liquid starter (%).

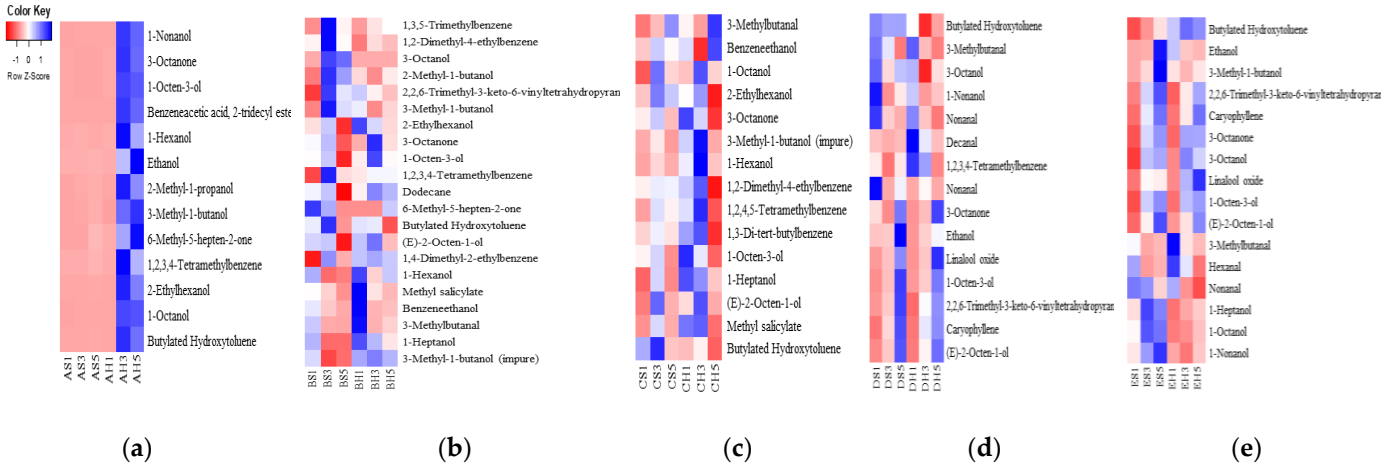

Figure 4. Heatmap of volatile flavor compounds. (**a**) A, *A. oryzae* AO 81-2; (**b**) B, *A. oryzae* AO 82-3; (**c**) C, *A. oryzae* AO 2-6; (**d**) D, *A. niger* AN IF 13-3; (**e**) E, *A. niger* 4-3-2; S, low-temperature blast drying; H, high-temperature hot air drying; 1, 3, 5, concentration of liquid starter (%).

The detection of hexanal, which is an aldehyde selectively detected in the AN strain-manufactured starters, was also consistent with the results of studies of volatile compounds in commercial fermentation starters [37]. In a previous study, the results showed that the activity of *A. flavus* inhibits the production of aldehydes such as nonanal and decanal [38]. This was similar to the results that were not generated in SSFS using *A. oryzae* (AO81-2, AO 82-3, AO 2-6) in this study, based on the results of previous studies that revealed that *A. oryzae* and *A. flavus* are the same species [39]. From this, it is expected that the commercial fermentation starters in the previous study were dominated by a fungus similar to the AN strains, since the solid fungal starter was produced with a single fungus in this study.

Compared to the other four strains, relatively few volatile flavor compounds were detected in AO 81-2. The PLS-DA and heatmap show the correlation between samples; 3 and

5% liquid starter inoculation under the H.T. drying condition clearly produced volatile flavor compounds, unlike other conditions. Based on these results, it was confirmed that AN strains contained more diverse volatile flavor compounds than AO strains. Unlike the enzyme activity and free amino acid contents, almost no difference due to drying conditions was observed in the volatile flavor compound analysis. Therefore, further studies are considered necessary to explain that such variation in results is due to differences in enzymes that are produced through various microbial mechanisms among fungal species [40,41].

4. Conclusions

This study aimed to contribute to the production of high-quality domestic SSFS by investigating the quality characteristics of five fungi with different inoculum concentrations and drying conditions. Glucoamylase activity, which indicates the saccharogenic power, an important function of SSFS, was relatively high when most of the strains used were inoculated with 3% liquid starter and dried using L.T. drying. A total of 26 types of free amino acids were detected, of which glutamic acid (which produces umami flavor) was the highest under all test conditions. Differences in the amounts of volatile flavor compounds produced depended on the fungi used and the manufacturing conditions. More volatile flavor compounds were detected when the AN strain was inoculated, but the difference due to drying conditions was small. Based on these results, we expect that the optimum manufacturing conditions can be selected according to the quality characteristics of solid fungi starters for each fungus, and that it will be useful to produce uniform, Korean-type, solid starters.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9050487/s1, Table S1: Enzyme activity properties for various SSFS. Tables S2–S6: Free amino acid content of SSFS by manufacturing conditions.

Author Contributions: This work was carried out in collaboration with all authors. S.-H.Y. conceived the study idea, verified the analytical methods, and analyzed the data; S.J.L. conducted the experiments and wrote the initial and final draft; H.B.K., S.H.K., W.S.J. and S.-Y.K. performed data curation, review, and editing. All authors have read and agreed to the published version of the manuscript.

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