International Journal of Food Microbiology 307 (2019) 108270

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Comparative study on metabolic changes of *Aspergillus oryzae* isolated from fermented foods according to culture conditions

Min Kyung Park[a], Jeong-Ah Seo[b], Young-Suk Kim[a,*]

[a] Department of Food Science and Engineering, Ewha Womans University, Seoul 03760, Republic of Korea
[b] School of Systems Biomedical Science, Soongsil University, Seoul 06978, Republic of Korea

ARTICLE INFO

Keywords:
Aspergillus oryzae
Culture conditions
Enzymatic activity
Metabolomics

ABSTRACT

Aspergillus oryzae is an important microbial starter for making diverse fermented foods due to its high hydrolytic enzyme activities. In this study, two strains of *A. oryzae* (AOB/AOK) with different activities of hydrolytic enzymes, such as α-amylase, protease and lipase, were cultured under various conditions of temperature, fermentation time, and initial pH. Comparative mass spectrometry (MS) based metabolomic analysis was performed to obtain primary metabolites and secondary volatile metabolite data sets. In the results of partial least square-discriminant analysis (PLS-DA), fatty acids and volatile metabolites derived from fatty acids and amino acids mainly contributed to AOK with higher protease and lipase activities, whereas carbohydrate-derived volatiles, sugars and sugar alcohols were related to main metabolites of AOB with higher α-amylase activity. The temperature and initial pH were critical factors for the generation of primary metabolites and secondary volatile metabolites, such as organic acids, fatty acids-derived volatiles, and some amino acids, in both *A. oryzae* strains. This study demonstrated that the specific culture conditions were closely linked to the formation of primary metabolites and secondary volatile metabolites of *A. oryzae*.

1. Introduction

Aspergillus oryzae, a filamentous fungus, is an important microbial-starter that is widely used in the production of diverse fermented foods, such as soy sauces, fermented soybean pastes, rice wines, and vinegars (Kobayashi and Sugawara, 1999; Kim et al., 2010). During fermentation, *A. oryzae* mostly acts as an initial fermentation driver, degrading substrates, such as starches, proteins, and lipids. Its hydrolytic enzymes efficiently break down substrates to relatively low-molecular-weight metabolites, including sugars, amino acids, and free fatty acids, which are then further fermented by yeasts and lactic acid bacteria (Smit et al., 2005).

Several studies have demonstrated that *A. oryzae* could be associated with not only hydrolysis but also the generation of secondary volatile metabolites that contribute to the quality of fermented products. Kum et al. (2015) studied the effects of secondary volatile metabolite generations and enzyme activities according to *Aspergillus* strains in fermented soybean pastes. They demonstrated that the formation of long-chain fatty acid esters, which could affect the organoleptic properties of fermented soybeans product, was strongly correlated with lipase activity. Another study identified 1-octen-3-ol, octan-3-ol, (E)-2-octenal, di-epi-α-cedrene, benzaeneacetaldehyde, and 3-(methylthio)-propanal as the most important contributors to the sensory properties in *A. oryzae*-inoculated *koji* used in soy sauce (Feng et al., 2013). In particular, (E)-2-octenal, benzaeneacetaldehyde, and 3-(methylthio)-propanal were revealed to be positively related to musty and soy sauce-like odors.

However, the previous studies on secondary volatile metabolites induced by *A. oryzae* were subject to some important limitations. First, most studies observed the changes in generation of metabolites, including secondary volatile metabolites, only in fermented foods (Son et al., 2018; Xie et al., 2018). Fermented foods are produced using various manufacturing processes and have complex ingredients, and so it is possible that parameters other than the microbial starters affected the results. Second, many of the studies focused on changes in extracellular volatile metabolites, rather than those in the intracellular metabolites of *A. oryzae* (Kum et al., 2015; Kim et al., 2012). Since intracellular metabolites represent the reactants of a metabolic network, knowledge of changes therein could provide a comprehensive understanding of extracellular metabolite production (Buchholz et al., 2002). It is therefore necessary to study the changes in both intracellular metabolites and extracellular secondary metabolites in order to explain the metabolic expressions associated with certain variables.

The effects of culture conditions of pH variations (acidity or

* Corresponding author.
E-mail address: yskim10@ewha.ac.kr (Y.-S. Kim).

https://doi.org/10.1016/j.ijfoodmicro.2019.108270
Received 31 January 2019; Received in revised form 13 July 2019; Accepted 19 July 2019
Available online 20 July 2019

alkalinity), temperatures (high or low), and fermentation times (initial or later) on the formation of metabolites during fermentation were investigated in the present study. These responses of culture conditions were found to be specific and complicated (Cicotello et al., 2018; Kitano et al., 2002). The microbial starters for fermented foods were exposed to diverse culture conditions during fermentation processes and storage. A good understanding of the impact of culture conditions on the biosynthesis of metabolites could facilitate optimizing the production of microbial metabolites. The culture conditions can therefore determine the qualities of fermented products even when the culture is inoculated with the same microbial starter (Beltran et al., 2008; Chung et al., 2017).

The aim of this study was to investigate the changes of metabolites formation, including primary metabolites and secondary volatile metabolites of *A. oryzae* strains, according to culture conditions. Two strains of *A. oryzae*, *A. oryzae* SSU1102-08 (AOB) has a high activity of α-amylase compared to *A. oryzae* KCTC 6983 (AOB), along with superior protease and lipase activities. Glycolysis, proteolysis, and lipolysis are strongly related to characteristic qualities of fermented foods. Thus, different enzymatic activities can lead to the changes of metabolic generation during fermentation (Shin et al., 2017; Wu et al., 2019).

The both *A. oryzae* strains were cultured in chemically defined media under diverse culture conditions, such as temperatures, times, and initial pH, in order to understand the effects of culture conditions on any observed metabolite changes. A mass spectrometry (MS)-based metabolomics approach was applied to obtain a comprehensive understanding of the changes and patterns of intracellular and extracellular metabolism of *A. oryzae*. Gas chromatography-time of flight/mass spectrometry (GC-TOF/MS) and gas chromatography–mass spectrometry (quadrupole) (GC–MS) were combined with a partial least squares-discrimination analysis (PLS-DA) to investigate the intracellular (primary metabolites) and extracellular (secondary volatile metabolites) changes during fermentation. The metabolite indicators related to the changes in culture conditions [strain (enzyme activity), time, temperature, and pH] were also determined using calculation of the area under the receiver operating characteristics curve (ROC-AUC) analysis.

2. Materials and methods

2.1. Chemicals and reagents

L-Threitol was purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Sucrose, sodium nitrate, potassium chloride, potassium phosphate, and magnesium sulfate heptahydrate were obtained from Samchun Pure Chemicals Co. Ltd. (Pyeongtaek-si, Gyeonggi-do, Korea). Peptone and yeast extract were purchased from Becton Dickinson (Sparks, MD, USA). Potato dextrose agar was obtained from Difco Laboratories (Detroit, MI, USA). 1 M HCl was obtained from Showa Chemical Industry Co., Ltd. (Tokyo, Japan). All the other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation

A. oryzae SSU1102-08 (KCTC13033BP, AOB) was isolated from traditional fermented product, *nuruk*, and *A. oryzae* KCTC 6983 (AOK) was supported from Korean collection for type cultures (KCTC), respectively. In particular, *A. oryzae* SSU1102-08 was selected among fungi isolated from *nuruk* due to its higher amylase and glucoamylase activities. Chemically defined media, which contained the same amounts of most components, except for peptone and sodium nitrate, with those of a previous study (Chung et al., 2017), was used for cultivation of *A. oryzae* in this study. The contents of both peptone and sodium nitrate were increased twice than those used previously.

2.3. Cultivation conditions

One milliliter of 4×10^8 spores/ml suspension was added in 250 ml flask containing 40 ml of culture media before incubated at 180 rpm under various conditions using a shaking incubator (Vision Scientific Co., Ltd., Daejeon, Korea). The spores were prepared by scraping from culture grown on potato dextrose agar. The observed samples were attributed to the following states: (1) control (AOB/AOK_24), achieved by incubating at 25 °C for 24 h; (2) incubation time (AOB/AOK_32), achieved by sampling at 25 °C for 32 h; (3) high temperature (AOB/AOK_37), achieved by incubating at 37 °C for 24 h; (4) acidic condition (AOB/AOK_P4), achieved by adding 1 N HCl in order to obtain pH 4, initially, and cultivating at 25 °C for 24 h. The experiments were performed in three independent experiments.

2.4. Primary metabolites analysis

The lyophilized cell mass (50 mg) was dissolved in 10 ml of mixed solvent (acetonitrile:water = 1:1) with 0.5 g of glass beads (diam. 1.0 mm, Sigma-Aldrich). Then it was vortexed vigorously for 30 s at highest speed and rested for 20 s, repeating four times, and prior to centrifugation at 2054 ×*g* for 10 min at 4 °C. After centrifugation, 500 μl of supernatant was transferred into a 1.5 ml Eppendorf tube with internal standards, such as L-threitol (100 μg/ml in water, for sugars and sugar alcohols), 3-hydroxy-2-phenylpropanoic acid (100 μg/ml in water, for organic acids), L-norleucine (100 μg/ml in water, for amino acids), and heptanoic acid (100 μg/ml in hexane, for fatty acids). Then it was vacuum-dried in Centri-Vap (Labconco Co., Kansas City, MO, USA). The residue was derivatized with methoxyamine hydrochloride (20 mg/ml) in pyridine (Sigma-Aldrich) at 30 °C for 90 min, before being silylated with *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) with 1% trimethyl chlorosilane (TMCS) at 70 °C for 60 min.

An Agilent 6890N GC (Agilent Technologies, Palo Alto, CA, USA) coupled with PEGASUS III TOF/MS (Leco, St. Joseph, MI, USA) was applied. DB-5MS column (30 m length, 0.25 mm internal diameter, 0.25 μm film thickness, J&B Scientific, Folsom, CA, USA) was equipped and helium flowed at constant flow rate of 0.8 ml/min. 1 μl of the derivatized extract was injected in splitless mode. Oven temperature was maintained at 80 °C for 5 min and increased to 180 °C at a rate of 10 °C/min per 5 min, then it was raised to 240 °C at a rate of 8 °C/min and ramped to 290 °C at a rate of 10 °C/min and maintained for 10 min. Inlet and transfer line temperatures were 270 °C and 260 °C, respectively. The mass spectra data were collected in EI mode using 70 eV and obtained with mass scan range of *m/z* 35–400 at a rate of 20 spectra/s.

2.5. Secondary volatile metabolites analysis

Cultivated media was filtered through a nylon membrane filter (0.45 μm pore size, 47 mm diameter, LK LAB Korea Inc., Namyangju-si, Gyeonggi-do, Korea) under vacuum. Then, it was re-filtered using a 0.22 μm cellulose acetate syringe filter (Macherey-Nagel GmbH & Co., Düren, Germany) to be completely separated from mycelium residues. Eight milliliters of filtered culture media and 2 μl of (3*S*)-4,7,7-trimethylbicyclo[2.2.1]heptan-3-ol (an internal standard compound, 50 mg/L in methanol) were transferred into 10 ml glass vial (Agilent Technologies, Santa Clara, CA, USA) for analysis of secondary volatile metabolites. Stir bar sorptive extraction (SBSE) was applied, and polydimethylsiloxane coated stir bar (PDMS twister, 10 mm length, 1.0 mm film thickness, Gerstel GmbH, Mülheim and der Rühr, Germany) and ethylene glycol/silicone coated stir bar (EG/Silicon twister, 10 mm length, Gerstel GmbH) were agitated at 1000 rpm for 60 min. Then, volatiles were thermally desorbed by increasing the temperature of thermal desorption unit (TDU) from 35 °C (1 min) to 220 °C (5 min) at a rate of 60 °C/min. Cooled injection system (CIS) was kept at −60 °C (1 min) and increased to 250 °C (3 min) at a rate of 10 °C/s.

Volatile metabolites were analyzed using a HP 7890B gas chromatograph (GC) system connected to a 5977A mass spectrometer (MS) (Agilent Technologies) equipped with a Stabilwax® column (30 m length, 0.25 mm internal diameter, 0.25 μm film thickness, Restek, Bellefonte, USA). Volatile metabolites were injected in split-less mode. Oven program was started initially at 40 °C (5 min) to 160 °C at a rate of 4 °C/min, and then ramped to 220 °C (5 min) at a rate of 6 °C/min. Helium was used as a carrier gas at a constant flow rate of 0.8 ml/min. Mass scan range was 35 to 350 *m/z* and transfer line temperature was 250 °C. The identification of metabolites was positively confirmed by comparing mass spectral data and retention times to those of authentic standard compounds. Otherwise, they were identified with the basis of their mass spectral database (NIST08 and Wiley9n.1) and retention index (RI) values using the NIST Chemistry Webbook. Quantitative data were obtained by comparing their peak areas to that of the internal standard compound.

2.6. Enzyme activity assay

The assay of enzyme activities followed that of Kum et al. (2015) with some modification. α-Amylase activity was calculated to ability of conversion of 1% soluble starch to 1.0 mg of maltose in 1 min. Protease activity was defined as the amount of the enzyme that hydrolyses to 1 μg of tyrosine from 2% casein solution per min. Lipase activity assay was conducted using a coupled enzyme reaction, which results in a colorimetric (570 nm) using UV–Vis spectrometry (Thermo Scientific, Waltham, MA, USA) product proportional to the enzymatic activity present. The unit of lipase was the amount of generating 1.0 μmol of glycerol from triglycerides per minute at 37 °C.

2.7. Statistical analysis

Data processing was performed using the IMB SPSS Statistics for Windows, version 25.0 (IBM Corp., Armonk, N.Y., USA) to evaluate statistical differences in metabolites contents and enzymatic activities during fermentation. Analysis of variance (ANOVA) was performed to determine significant differences among group means in a sample. The result of Duncan's multi-range test was presented to show the significant different level at $p < 0.05$. Partial least squares discriminant analysis (PLS-DA) was conducted using SIMCA-P software (version 11.0, Umetrics, Umea, Sweden) to demonstrate differences according to cultivation conditions, such as strains, fermentation temperatures, fermentation times, and initial pH levels.

Fold change of each mass peak was calculated as the ratio of signal intensity in variables to that of control samples (AOB/AOK_24h). Then data transformation ($\log_2$ scale) was conducted to normalize the observed data sets. Heat map visualization based on the fold changes of other samples against controls. The area under the receiver operator characteristic curve (AUC) was performed using a pROC package (Robin et al., 2011) implemented in R environment (www.r-project.org, version 3.5.1).

3. Results

3.1. Enzyme activities of A. oryzae

The hydrolytic abilities of AOB and AOK were compared by measuring the activities of the hydrolytic enzymes, such as α-amylase, protease, and lipase. Table 1 indicates that the activities of all enzymes differed significantly with the strain and culture conditions. The α-amylase activity of AOB was superior to that of AOK, while the protease and lipase activities were lower in AOB than those in AOK, regardless of culture conditions. The variations in the enzyme activities of AOK were wider than those of AOB, implying that the hydrolyzing activities of AOK could be more dependent on the culture conditions. Furthermore, all of the enzymatic activities were strongly correlated with fermentation temperature in both *Aspergillus* strains. Higher temperature increased the α-amylase and lipase activities, whereas protease activities were decreased when the temperature was elevated. Furthermore, the α-amylase activity decreased at a lower pH.

3.2. Comparative evaluation of metabolite profiles according to different A. oryzae and culture conditions

GC-TOF/MS identified 46 primary metabolites, comprising 21 amino acids, 8 sugars and sugar alcohols, 11 organic acids, and 6 fatty acids, while GC–MS identified 76 secondary volatile metabolites (Tables S1 and S2). The PLS-DA score plots based on the GC-TOF/MS and GC–MS data sets in Fig. 1-(a) and (b), respectively, exhibit clustered strains and patterns correlated with the culture parameters for different *A. oryzae* fermentation conditions. The PLS-DA models for AOK and AOB groups explained (a) 50.6% and (b) 41.1% of total variance (PLS[1] + PLS[2] dimensions) in the score plot, respectively. Their internal cross-validations on PLS-DA yielded $R^2X = 0.948$, $R^2Y = 0.968$, and $Q^2 = 0.912$ for the primary metabolite profile and $R^2X = 0.894$, $R^2Y = 0.981$, and $Q^2 = 0.906$ for the secondary volatile metabolite profile. The AOB and AOK groups were located along opposite direction of their corresponding PLS-DA score plots. The two *A. oryzae* strains were clearly separated, but the distributions of the groups differed, with the AOK group being more scattered than the AOB group in both score plots. In order to identify the significant differences according to strains, a total of 58 significant variables (26 primary metabolites and 32 secondary volatile metabolites) contributing to the PLS [1] and PLS[2] dimension (p-value, 0.1 > or < −0.1) were selected on the basis of a threshold of 1.0 on the variable importance in the projection (VIP) test (Tables 2 and 3).

The most important indicator variables based on the results of the PLS-DA analysis for primary metabolites results were as follows (Table 2): the main primary metabolites contributing to the negative PLS[1] dimension, representing the AOB group, were benzoic acid (p = 0.30), methionine (0.25), glucose (0.24), histidine (0.24), arabitol (0.23), trehalose (0.22), erythritol (0.19), and so on. On the other hand, 9,12-octadecadienoic acid (−0.29), octadec-9-enoic acid (−0.27), hexadecanoic acid (0.26), homocysteine (0.12), valine (0.12), and so on were involved in the negative PLS[2] dimension, corresponding to the AOK group. The AOB group was characterized by the formation of sugars and sugar alcohols, while fatty acids were the main variants for discriminating the AOK group from the AOB group.

Table 3 presents the main loadings responsible for the separation of samples based on secondary volatile metabolite profiles. The main variables representing the AOK group were 3-methylbutan-1-ol (p = −0.20), ethyl acetate (−0.19), 1,4-dioxan-2-one (−0.19), ethyl 2-phenylaceate (−0.18), 3-methylbutanoic acid (−0.18), and so on. In particular, some branched-chain aliphatic volatiles and benzenes, such as 3-methylbutan-1-ol, 3-methylbutanoic acid, 2-methylpropan-1-ol, ethyl 3-methylbutanoate, ethyl 2-methyl-propanoate, ethyl 2-methylbutanoate, ethyl 2-phenylaceate, and 2-phenylethanol, were more strongly associated with the AOK group than with the AOB group. These compounds are involved in the Ehrlich pathway that converts free amino acids into aldehydes, alcohols, and acids according to the particular intracellular and extracellular conditions (Ravasio et al., 2014). On the other hand, 1-phenylbutan-2-one (0.16), 3,5-dimethylbenzaldehyde (0.15), 1-hydroxy-propan-2-one (0.14), (2-acetyloxy-3-hydroxypropyl) acetate (0.14), and other metabolites were main variables contributing to the negative PLS[1] dimension (the AOB group). Benzenes were the main metabolites discriminating between the AOB and AOK groups. The effects of the other culture parameters of temperature, fermentation time, and initial pH on the primary and volatile metabolism in *A. oryzae* are described below.

Tables S1 and S2 compare the relative intensities of primary metabolites and secondary volatile metabolites obtained for the two strains of *A. oryzae* cultured under different conditions. The contents of

Table 1
Effects of strains and culture conditions on enzyme activities.

Enzymes (E.C.)[a]	Strain	Enzymatic activities (mean ± SD, unit/mg)			
		Control (24 h)[d]	32 h[e]	37 °C[f]	P4[g]
α-Amylase (3.2.2.1)	AOB[b]	345.6 ± 36.1d[h],x[i]	297.6 ± 38.4c,x	432.0 ± 16.6e,y	358.4 ± 30.9d,x
	AOK[c]	80.0 ± 12.8b,yz	76.8 ± 12.8b,y	97.1 ± 6.7b,z	25.6 ± 5.5a,x
Protease (3.4.21.19)	AOB	64.8 ± 9.2c,z	57.9 ± 3.5bc,yz	42.7 ± 1.3a,x	51.0 ± 5.8ab,xy
	AOK	117.3 ± 2.9d,y	139.0 ± 8.1e,z	53.7 ± 2.3b,x	125.3 ± 6.9d,y
Lipase (3.1.1.3)	AOB	30.1 ± 2.9a,x	69.9 ± 4.8b,y	121.9 ± 25.2c,z	26.3 ± 0.5a,x
	AOK	25.0 ± 3.5a,x	90.3 ± 6.3b,y	192.3 ± 41.2d,z	39.0 ± 7.8a,x

[a] E.C.: Enzyme Commission number.
[b] AOB: *A. oryzae* SSU1102-08.
[c] AOK: *A. oryzae* KCTC 6983.
[d] Incubating at 25 °C for 24 h at initial pH 6.
[e] Incubating at 25 °C for 32 h at initial pH 6.
[f] Incubating at 37 °C for 24 h at initial pH 6.
[g] Incubating at 25 °C for 24 h at initial pH 4.
[h] Different letters mean significant differences ($p < 0.05$) between both *A. oryzae* fermentations varying with culture conditions by Duncan's multi range test.
[i] Different letters mean significant differences ($p < 0.05$) between each *A. oryzae* fermentation varying with culture conditions by Duncan's multi range test.

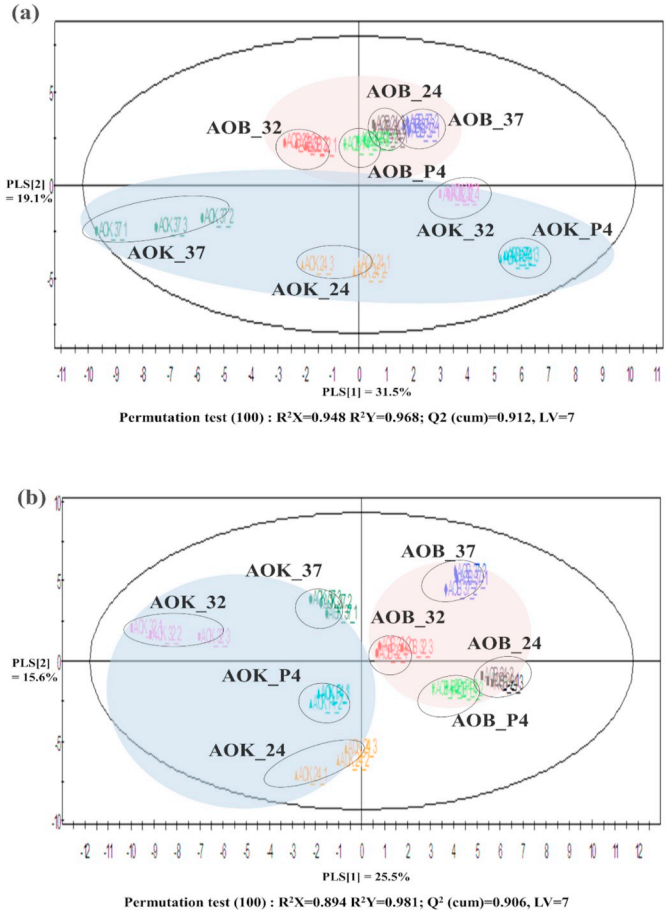

Fig. 1. PLS-DA score plot derived from (a) primary metabolic and (b) secondary volatile metabolic profiling of different *A. oryzae* fermentations according to strains and culture parameters.

amino acids changed significantly with fermentation temperature. The overall contents of amino acids were higher in sample AOB_37 than in the other AOB samples, and lowest in sample AOK_37. Alanine, aspartate, glutamate, glycine, ornithine, and proline were the most abundant amino acids in all samples, while, cysteine, methionine, lysine, homocysteine, and tryptophan were detected at lowest levels. Mannitol was a predominant sugar alcohol in both AOB and AOK, while fructose and glucose were the predominant sugars. The level of fructose was higher

Table 2
Main variables contributing to discrimination between primary metabolic profiles of different *A. oryzae* fermentations.

No.[a]	p-Value	Compound
Positive PLS[2]		
O5	0.30	Benzoic acid
A9	0.25	Methionine
C4	0.24	Glucose
A19	0.24	Histidine
C2	0.23	Arabitol
C8	0.22	Trehalose
C1	0.19	Erythritol
A17	0.19	Glutamine
C5	0.17	Mannitol
A15	0.16	Asparagine
C3	0.15	Fructose
O3	0.14	Pyruvic acid
A10	0.14	Aspartic acid
A4	0.14	Isoleucine
A5	0.13	Proline
O4	0.11	Malonic acid
C7	0.10	Maltose
Negative PLS[2]		
F3	−0.29	9,12-Octadecadienoic acid
F4	−0.27	Otadec-9-enoic acid
F2	−0.26	Hexadecanoic acid
A14	−0.12	Homocysteine
A2	−0.12	Valine
A12	−0.11	Glutamic acid
F6	−0.11	9,12,15-Octadecatrienoic acid
F1	−0.10	Hexadec-9-enoic acid
A1	−0.10	Alanine

[a] Numbered as in the retention time; C: carbohydrate, A: amino acid, F: fatty acid, O: organic acid.

in control (AOB and AOK_24), AOK_P4, and AOB_37, whereas, glucose was the main sugar in time-correlated samples (AOB and AOK_32), APK_37, and AOB_P4.

Malic acid, succinic acid, and citric acid were the main organic acids. A particularly interesting finding was that the contents of the organic acids, such as lactic acid, oxalic acid, succinic acid, fumaric acid, malic acid, citric acid, and glutaric acid, were significantly increased at high temperature in AOB. On the other hand, some organic acids, such as succinic acid, fumaric acid, and malic acid, were decreased in AOK at lower pH, while oxalic acid, benzoic acid, malic acid, mevalonic acid, glutaric acid, and citric acid increased with the fermentation time.

Among fatty acids, the levels of the unsaturated fatty acids, such as

Table 3
Main variables contributing to discrimination between secondary volatile metabolic profiles of different *A. oryzae* fermentations.

No.[a]	p-Value	Compound
Positive PLS[1]		
V54	0.16	1-Phenylbutan-2-one
V55	0.15	3,5-Dimethylbenzaldehyde
V28	0.14	1-Hydroxypropan-2-one
V63	0.14	(2-Acetyloxy-3-hydroxypropyl) Acetate
V34	0.13	2-Ethylhexan-1-ol
V71	0.13	Dodecanoic acid
V49	0.11	Naphthalene
V33	0.11	2-Ethylhexyl prop-2-enoate
V56	0.11	2-Hydroxy-3-methylcyclopent-2-en-1-one
V67	0.11	3,5-Dihydroxy-6-methyl-2,3-dihydropyran-4-one
Negative PLS[1]		
V22	−0.20	3-Methylbutan-1-ol
V4	−0.19	Ethyl acetate
V52	−0.19	1,4-Dioxan-2-one
V53	−0.18	Ethyl 2-phenylacetate
V48	−0.18	3-Methylbutanoic acid
V17	−0.17	Butyl acetate
V23	−0.17	Octan-3-one
V19	−0.16	2-Methylpropan-1-ol
V15	−0.16	Ethyl 3-methylbutanoate
V31	−0.16	Acetic acid
V12	−0.16	Ethyl butanoate
V39	−0.15	2-Methylpropanoic acid
V38	−0.15	Propanoic acid
V60	−0.15	2-[2-(2-Ethoxyethoxy) ethoxy]ethanol
V42	−0.14	Butanoic acid
V57	−0.14	2-Phenylethanol
V9	−0.13	Ethyl 2-methylpropanoate
V14	−0.13	Ethyl 2-methylbutanoate
V43	−0.13	2-Hydroxyethyl acetate
V21	−0.12	Butan-1-ol
V29	−0.12	Hexan-1-ol

[a] Numbered as in the order of retention indices (RI).

linoleic acid, oleic acid, and linolenic acid, were higher in AOK than in AOB. In particular, oleic acid was markedly increased in both AOB and AOK at high temperature, while palmitic acid and linoleic acid increased with fermentation time in AOK.

In the case of secondary volatile metabolites, temperature greatly affected the formation of both fatty acids-derived volatiles (straight aliphatic volatiles and furans) and amino acids-derived volatiles (branched-chain aliphatic volatiles) in AOK, as well as the generation of carbohydrate-derived volatile in both *A. oryzae* strains. Butan-1-ol was the most abundant straight aliphatic volatile in both groups, while it was dramatically decreased at higher temperature. On the other hand, the contents of ethyl tetradecanoate, ethyl hexadecanoate, decanoic acid, and ethyl octadecanoate were considerably increased at 37 °C. The contents of branched-chain aliphatic volatiles were significantly higher in AOK than in AOB. 3-Methylbutan-1-ol, a leucine-derived volatile, was the most abundant volatile, whereas 3-methylbutanoic acid and ethyl 3-methylbutanoate were detected only in AOK. On the other hand, 2-methylbutanoic acid (an isoleucine-derived compound) as found in all AOB samples, while it was detected only in AOK_P4.

3.3. Metabolite abundance changes in metabolic pathways of A. oryzae

Fig. 2 indicates the changes in the generated of primary metabolites and secondary volatile metabolites according to their possible metabolic pathways based on KEGG database (Kanehisa and Goto, 2000) and previous literature (Zhao et al., 2018). The expression levels of metabolites for different *A. oryzae* fermentation processes were expressed as the logarithmic changes relative to control samples (AOK and AOB for 24 h) (Tables S3 and S4). The metabolic pathways could be separated into three parts: carbohydrate metabolism (glycolysis and tricarboxylic acid (TCA) cycle), fatty acids metabolism (e.g. β-oxidation), and amino acids metabolism (Ehrlich pathway). The precursors (primary metabolites) and end-products (secondary volatile metabolites) generated in the corresponding three biochemical pathways were outlined to demonstrate how the metabolism changed according to culture conditions.

The two *A. oryzae* strains showed different metabolic patterns for the generation of primary metabolites and secondary volatile metabolites. For example, the formation of the sulfur-containing volatiles, such as methanethiol and (methyldisulfanyl)methane, was increased compared to control in the AOK group, while the opposite results were showed in the AOB group. On the other hand, some metabolites were strongly associated with the cultivation conditions, such as fermentation times, temperatures, and initial pH. Increasing the fermentation temperature increased the levels of medium- (C6–C12) and long- (> C14) chain fatty acid-derived volatile metabolites, and decreased those of short-chain-fatty-acid-derived volatile metabolites (< C4). Also, higher temperature altered the TCA cycle activities of AOB, with the levels of the organic acids, such as citric acid, succinic acid, fumaric acid, and malic acid, being enhanced compared to control, whereas, branched-chain aliphatic volatiles (especially valine (V)-derived and isoleucine (IL)-derived volatiles) were decreased.

The initial pH can also affect the formation of metabolites in *A. oryzae* strains. The formation of furans and arabitol were enhanced at a lower pH in both *A. oryzae* strains, and the organic acids, such as malic acid, fumaric acid, succinic acid, and lactic acid, were decreased in AOK. Branched-chain aliphatic volatiles, aromatic amino acids (phenylalanine, tyrosine, and tryptophan), and benzenes were decreased in AOB, while lactone (oxolan-2-one) was detected only at lower pH level.

4. Discussion

A. oryzae, which has higher hydrolytic enzyme activities than other bacteria or yeasts, can accelerate overall fermentation processes or certain metabolisms (Zapelena et al., 1999), and alter the related metabolic pathways (Scherlach and Hertweck, 2009). The volatile metabolic pathways of other microorganisms, such as lactic acid bacteria and yeasts, have been well studied, whereas there have been relatively few studies of *A. oryzae*. The present study applied an integrated analytical approach that included the analysis of primary metabolites (precursors) and secondary volatile metabolites (end products), and assays of some enzymatic activities (facilitators) with the aim of understanding how the metabolism of *A. oryzae* changes according to the culture conditions.

During fermentation, substrates, such as starches, proteins, and lipids, are degraded by specific enzymes and converted to micro molecules, including primary metabolites and secondary volatile metabolites, which can contribute to the sensory qualities of the fermented products (Bruna et al., 2001). In particular, the hydrolytic enzymes, including α-amylase, protease, and lipase, are secreted out from cells and catalyze the substrates, such as starch, proteins, and lipids, into sugars, amino acids, and fatty acids, respectively (Gurung et al., 2013). Then, the breakdown of larger macromolecules can pass through cell membrane and enter into the cell, further conversion to energy source and the formation of primary metabolites and secondary volatile metabolites directly or indirectly during fermentation (Borjesson et al., 1992; Dugelay et al., 1993). In the present study, we measured the activities of the following major hydrolytic enzymes of both *A. oryzae* strains, such as α-amylase, protease, and lipase. α-Amylase, is related to the metabolism of carbohydrates, and it catalyzes the breakdown of α-1,4-glucosidic linkages in polysaccharides, such as starches or glycogen, liberating low-molecular-weight sugars. On the other hand, protease can perform to be catabolized by hydrolysis of peptide bonds (Jakubke et al., 1985; Yoo et al., 1987). Lipases are a ubiquitous group of enzymes that catalyze the hydrolysis of triacylglycerols into diacylglycerols, monoacylglycerols, fatty acids, and glycerol (Thomson et al.,

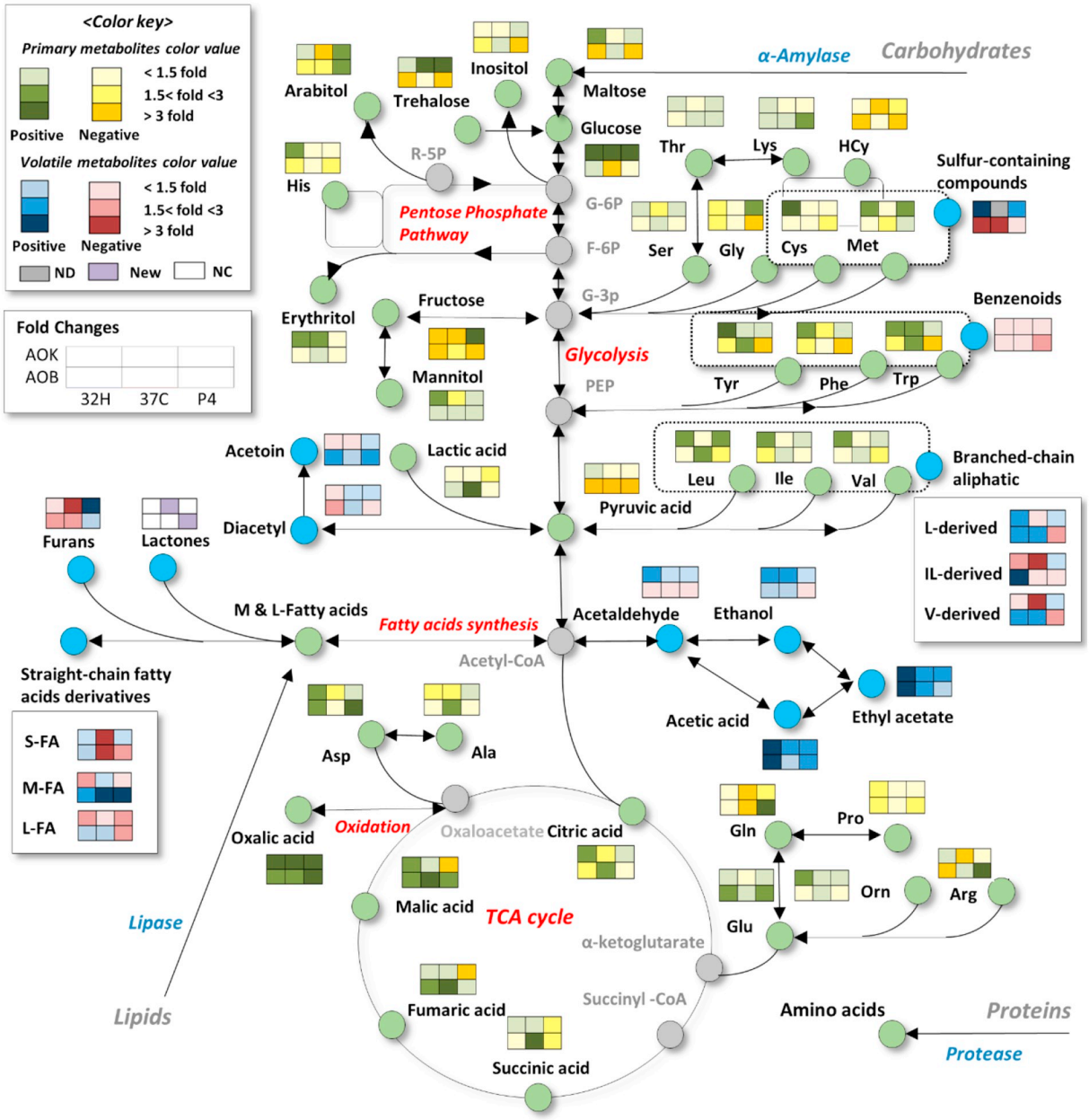

Fig. 2. The changes in the formations of primary metabolites and secondary volatile metabolites of both *A. oryzae* stains (AOB: *A. oryzae* SSU1102-08, AOK: *A. oryzae* KCTC 6983) according to culture conditions. The metabolic expressions comparing to control samples were visualized by heat maps according to culture parameters. Color dots represent as followings; green: primary metabolites, blue: secondary volatile metabolites, and gray: non-detected. All abbreviations are shown below; not detected (ND), newly detected (New), no change (NC), leucine-derived (L-derived), isoleucine-derived (IL-derived), valine-derived (V-derived), short-chain fatty acids-derived volatiles (S-FA), medium-chain fatty acids-derived volatiles (M-FA), and long-chain fatty acids-derived volatiles (L-FA). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2006). Our results indicated that AOB and AOK showed different enzyme activities, with AOB having higher yields of α-amylase, while its protease and lipase activities were lower than those of AOK. It can be assumed that AOB has a greater ability to generate carbohydrate-derived metabolites, while AOK is superior in forming proteins and fatty acids-derived metabolites. Regarding culture conditions, temperature strongly affected the α-amylase, protease, and lipase activities in both *A. oryzae* strains. Kitano et al. (2002) has been reported that the protease activity of *A. oryzae* varies with temperature, and that high temperature (especially > 38 °C) can inhibit the expression of mRNA involved in protease, which is consistent with the results of the present study. Also, the specific α-amylase production of *A. oryzae* peaks at around pH 6, and the optimal temperature for α-amylase was observed at 35 °C (Carlsen et al., 1996). It is therefore feasible for temperature changes to significantly affect the formation of metabolites.

PLS-DA analysis for primary metabolites and secondary volatile metabolite profiles revealed scattered samples that could be classified into two groups (the AOK and AOB group) according to PLS[1] and PLS[2] axes, respectively (Fig. 1). However, the distribution of each group differed, implying dependency on the culture conditions. These results were consistent with the differences found in the changes of enzymatic activities. AOB showed relatively smaller changes in metabolite profiles and enzyme activity compared to AOK. Moreover, the main variables contributing to discriminate the two groups differed in each group (Tables 2 and 3). Carbohydrates (sugars and sugar alcohols), benzenes, and carbohydrates-derived metabolites were related to the AOB group.

Table 4
Correlation between metabolites and strain/culture conditions using ROC analysis (> 0.8 AUC values were colored).

No.[a]	Metabolites	ROC-AUC				Remarks
		Strain[b]	Time[c]	Temp.[d]	pH[e]	
V4	Ethyl acetate	0.88	0.81	0.58	0.54	Carbohydrate-derived volatiles
V10	Butane-2,3-dione	0.45	0.69	0.52	0.92	
V27	3-Hydroxybutan-2-one	0.73	0.96	0.73	0.67	
V28	1-Hydroxypropan-2-one	0.99	0.46	0.57	0.64	
V31	Acetic acid	0.70	0.93	0.55	0.56	
V51	2-Hydroxycyclopent-2-en-1-one	0.83	0.58	0.70	0.92	
C2	Arabitol	0.75	0.54	0.88	0.92	Sugar & sugar alcohols
C4	Glucose	1.00	0.69	0.55	0.46	
C8	Trehalose	0.76	0.86	0.81	0.53	
O1	Lactatic acid (Lac)	0.58	0.47	0.86	0.91	Organic acids
O2	Oxalic acid (Oxa)	0.58	0.46	0.92	0.62	
O5	Benzoic acid (Ben)	1.00	0.58	0.59	0.64	
O6	Succinic acid (Suc)	0.69	0.56	0.91	0.91	
O7	Fumaric acid (Fum)	0.57	0.62	0.96	0.91	
O8	Malic acid (Mal)	0.63	0.67	0.90	0.83	
O9	Mevalonic acid (Mev)	0.58	0.89	1.00	0.60	
A5	Proline (Pro)	0.61	0.92	0.68	0.68	Amino acids
A9	Methionine (Met)	0.87	0.62	0.92	0.79	
A10	Aspartic acid (Asp)	0.66	0.83	0.99	0.83	
A11	Cysteine (Cys)	0.46	0.92	0.73	0.82	
A12	Glutamic acid (Glu)	0.72	0.80	0.87	0.79	
A19	Histidine (His)	0.87	0.65	0.51	0.73	
A21	Tryptophan (Try)	0.56	0.47	0.88	0.87	
V1	Methanethiol	0.52	0.62	1.00	0.69	Amino acids-derived volatiles
V6	2-Methylbutanal	0.94	0.48	0.48	0.62	
V9	Ethyl 2-methylpropanoate	0.49	0.97	0.58	0.59	
V14	Ethyl 2-methylbutanoate	0.56	1.00	0.50	0.75	
V22	3-Methylbutan-1-ol	0.96	0.69	0.53	0.61	
V24	Styrene	0.59	0.59	0.87	0.63	
V39	2-Methylpropanoic acid	0.75	0.71	1.00	0.72	
V48	3-Methylbutanoic acid	0.88	0.67	0.50	0.75	
V55	3,5-Dimethylbenzaldehyde	0.94	0.65	0.58	0.63	
V57	2-Phenylethanol	0.95	0.55	0.47	0.68	
V73	2-Phenylacetic acid	0.47	0.90	0.71	0.92	
F2	Palmitic acid (C16:0)	0.84	0.89	0.65	0.69	Fatty acids
F3	Linoleic acid (C18: 2)	0.98	0.76	0.61	0.51	
F4	Oleic acid (C18:1)	0.93	0.44	0.84	0.68	
F5	Stearic acid (C18:0)	0.67	0.85	0.90	0.77	
V3	Butanal	0.87	0.78	0.53	0.78	Fatty acids-derived
V12	Ethyl butanoate	0.94	0.75	0.49	0.64	
V18	Hexanal	0.57	0.75	0.75	0.91	
V21	Butan-1-ol	0.72	0.75	1.00	0.65	
V23	Octan-3-one	1.00	0.50	0.60	0.49	
V32	Furan-2-carbaldehyde	0.74	0.70	0.84	1.00	
V34	2-Ethylhexan-1-ol	0.49	0.85	0.64	0.76	
V35	Decanal	0.49	0.67	0.67	0.67	
V38	Propanoic acid	0.70	0.85	0.98	0.56	
V41	2-Hydroxyethyl formate	0.51	0.48	0.60	0.54	
V42	Butanoic acid	0.72	0.88	1.00	0.61	
V43	2-Hydroxyethyl acetate	0.88	0.56	0.52	0.75	
V46	Furan-2-ylmethanol	0.72	0.69	0.80	0.92	
V50	2H-Furan-5-one	0.62	0.65	1.00	0.76	
V61	Ethyl tetradecanoate	0.69	0.58	0.92	0.75	
V65	Ethyl hexadecanoate	0.59	0.60	1.00	0.73	
V69	Ethyl octadecanoate	0.47	0.67	1.00	0.67	
V71	Dodecanoic acid	0.98	0.44	0.72	0.54	
V72	5-(hydroxymethyl)Furan-2-carbaldehyde	0.51	0.68	0.99	0.97	

Sugars are generally consumed as carbon sources, yielding energy for proliferation and growth via carbohydrate metabolic pathways (Lee et al., 2016). It can facilitate intracellular metabolisms leading to formation of various volatile metabolites. On the other hand, in the AOK group, volatile metabolites derived from amino acids and fatty acids as well as the fatty acids themselves were the main contributors. Among them, unsaturated fatty acids are especially known to be precursors of diverse volatile metabolites, such as lactones (Haffner et al., 1996), straight aliphatic alcohols, aldehydes, acids, and esters (Schwab et al., 2008). The contents of unsaturated fatty acids were lower in AOK than those in AOB, implying higher contents of straight aliphatic volatiles in AOK compare to those in AOB.

The culture conditions also affected the metabolite profiles. Higher temperature led to increase of α-amylase and lipase along with a reduction in protease during fermentation, further inducing changes in formed primary metabolites and secondary volatile metabolites. In both *A. oryzae* strains, medium- and long-chain fatty acids-derived volatiles were increased at higher temperatures whereas short-fatty acids-derived volatiles were decreased. Lipids were not added to the cultures in this study, and so most of the fatty acids would have been generated from the degradation of polysaccharides and conversion of acetyl-CoA (glycolytic pathway) (Harwood, 2005). However, short-chain fatty acids-derived volatiles, such as fusel aldehydes, alcohols, acids, and esters, can be formed via the Ehrlich pathway (Hazelwood et al., 2008). Thus, short-chain fatty acids-derived volatiles could be formed more efficiently from the degradation of amino acids than from the degradation and oxidation of long-chain fatty acids. Temperature also affected AOK and AOB in different ways. Higher temperature increased the levels of the TCA cycle-derived organic acids, such as succinic acid, citric acid, fumaric acid, malic acid, and lactic acid, increased in AOB, while some amino acid-derived volatiles, including sulfur-containing volatiles and branched-chain aliphatic volatiles, were decreased in AOK. These are usually considered as characteristic metabolites with strong and unique odor notes (Park et al., 2007; Nguyen et al., 2010), indicating that temperature can influence the sensory qualities of fermented products.

The pH has been considered the most important regulator of glucose fermentation, influencing reductase activity and, accordingly, intracellular and extracellular microbial activity (Mohd-Zaki et al., 2016). Similar to the effects of temperature, the expressed metabolites varied with pH in this study. The Ehrlich pathway-derived metabolites, such as branched-chain aliphatic volatiles, benzenes, and aromatic amino acids, were decreased at lower pH in AOB, while, the contents of most organic acids were decreased in AOK.

Table 4 represents the correlation between the generated metabolites and culture parameters, including strains, temperatures, fermentation times, and initial pH values, calculating the ROC-AUC (AUC, > 0.8). RSV0049distribution across the total range than the statistical p-value (Broadhurst and Kell, 2006). The AUC measures the performance of a classifier and is frequently applied for method comparison (Robin et al., 2011). A higher AUC represents better sorting the classes. A total of 18, 24, 14, and 13 metabolites were strongly associated with the classification of metabolic alterations according to strains with different enzyme activities, temperatures, fermentation times, and pH, respectively.

The formations of organic acids, amino acids, and fatty-acid-derived volatiles were affected more strongly by temperature than by the other parameters analyzed in this study. On the other hand, time-correlated main metabolites were 3-hydroxybutan-2-one, acetic acid, ethyl 2-methylpropanoate, ethyl 2-methylbutanoate, and so on. Meanwhile, carbohydrate-derived volatiles and some organic acids, such as lactic acid, succinic acid, and fumaric acid, were main metabolites correlated with lower pH.

5. Conclusion

This study demonstrated the effects of culture conditions of temperature, fermentation time, and initial pH on the formation of primary metabolites and secondary volatile metabolites of two *A. oryzae* strains. The results revealed that primary metabolites (precursors), secondary volatile metabolites (end-products), and enzymatic activities (facilitators) were closely linked to each other. This study can provide culture condition-specific metabolite profiles, especially secondary volatile metabolites, and may be helpful for determining the optimal culture conditions of *A. oryzae*.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (NRF-2017R1A2B4002233) and d the High Value-added Food Technology Development Program (grant number 317035-03), funded by the Ministry of Agriculture, Food and Rural Affairs (MAFRA).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2019.108270.

References

Beltran, G., Novo, M., Guillamón, J.M., Mas, A., Rozès, N., 2008. Effect of fermentation temperature and culture media on the yeast lipid composition and wine volatile compounds. Int. J. Food Microbiol. 121, 169–177.
Borjesson, T., Stollman, U., Schnurer, J., 1992. Volatile metabolites produced by six fungal species compared with other indicators of fungal growth on cereal grains. Appl. Environ. Microbiol. 58, 2599–2605.
Broadhurst, D.I., Kell, D.B., 2006. Statistical strategies for avoiding false discoveries in metabolomics and related experiments. Metabolomics 2, 171–196.
Bruna, J.M., Hierro, E.M., de la Hoz, L., Mottram, D.S., Fernández, M., Ordóñez, J.A., 2001. The contribution of *Penicillium aurantiogriseum* to the volatile composition and sensory quality of dry fermented sausages. Meat Sci. 59, 97–107.
Buchholz, A., Hurlebaus, J., Wandrey, C., Takors, R., 2002. Metabolomics: quantification of intracellular metabolite dynamics. Biomol. Eng. 19, 5–15.
Carlsen, M., Spohr, A.B., Nielsen, J., Villadsen, J., 1996. Morphology and physiology of an α-amylase producing strain of *Aspergillus oryzae* during batch cultivations. Biotechnol. Bioeng. 49, 266–276.
Chung, H., Lee, N.K., Seo, J.-A., Kim, Y.-S., 2017. Comparative analysis of nonvolatile and volatile metabolites in *Lichtheimia ramosa* cultivated in different growth media. Biosci. Biotechnol. Biochem. 81, 565–572.
Cicotello, J., Wolf, I.V., D'Angelo, L., Guglielmotti, D.M., Quiberoni, A., Suárez, V.B., 2018. Response of *Leuconostoc* strains against technological stress factors: growth performance and volatile profiles. Food Microbiol. 73, 362–370.
Dugelay, I., Gunata, Z., Sapis, J.C., Baumes, R., Bayonove, C., 1993. Role of cinnamoyl esterase activities from enzyme preparations on the formation of volatile phenols during winemaking. J. Agric. Food Chem. 41, 2092–2096.
Feng, Y., Cui, C., Zhao, H., Gao, X., Zhao, M., Sun, W., 2013. Effect of *koji* fermentation on generation of volatile compounds in soy sauce production. Int. J. Food Sci. Technol. 48, 609–619.
Gurung, N., Ray, S., Bose, S., Rai, V., 2013. A broader view: microbial enzymes and their relevance in industries, medicine, and beyond. Biomed. Res. Int. 2013, 1–18.
Haffner, T., Nordsieck, A., Tressl, R., 1996. Biosynthesis of δ-jasmin lactone (=(Z)-dec-7-eno-5-lactone) and (Z, Z)-dodeca-6, 9-dieno-4-lactone in the yeast *Sporobolomyces odorus*. Helv. Chim. Acta 79, 2088–2099.
Harwood, J.L., 2005. Fatty acid biosynthesis. In: Murphy, D.J. (Ed.), Plant Lipids: Biology, Utilisation and Manipulation. Blackwell Publishing Ltd., Oxford, pp. 27–66.
Hazelwood, L.A., Daran, J.-M., van Maris, A.-J., Pronk, J.T., Dickinson, J.R., 2008. The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. Appl. Environ. Microbiol. 74, 2259–2266.
Jakubke, H., Kuhl, P., Könnecke, A., 1985. Basic principles of protease-catalyzed peptide bond formation. Angew. Chem. Int. Ed. Engl. 24, 85–93.
Kanehisa, M., Goto, S., 2000. KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic Acids Res. 28, 27–30.

Kim, T.W., Lee, J.-H., Park, M.-H., Kim, H.-Y., 2010. Analysis of bacterial and fungal communities in Japanese-and Chinese-fermented soybean pastes using nested PCR–DGGE. Curr. Microbiol. 60, 315–320.
Kim, A.-J., Choi, J.-N., Kim, J.Y., Kim, H.Y., Park, S.-B., Yeo, S.-H., Choi, J.-H., Liu, K.-H., Lee, C.H., 2012. Metabolite profiling and bioactivity of rice *koji* fermented by *Aspergillus* strains. J. Microbiol. Biotechnol. 22, 100–106.
Kitano, H., Kataoka, K., Furukawa, K., Hara, S., 2002. Specific expression and temperature-dependent expression of the acid protease-encoding gene (*pepA*) in *Aspergillus oryzae* in solid-state culture (Rice-Koji). J. Biosci. Bioeng. 93, 563–567.
Kobayashi, A., Sugawara, E., 1999. Flavor components of *shoyu* and miso Japanese fermented soybean seasonings. In: Shahidi, F., Ho, C.-T. (Eds.), Flavor Chemistry of Ethnic Foods. Springer, New York, pp. 5–14.
Kum, S.-J., Yang, S.-O., Lee, S.M., Chang, P.-S., Choi, Y.H., Lee, J.J., Hurh, B.S., Kim, Y.-S., 2015. Effects of *Aspergillus* species inoculation and their enzymatic activities on the formation of volatile components in fermented soybean paste (*doenjang*). J. Agric. Food Chem. 63, 1401–1418.
Lee, D.E., Lee, S.M., Jang, E.S., Shin, H.W., Moon, B.S., Lee, C.H., 2016. Metabolomic profiles of *Aspergillus oryzae* and *Bacillus amyloliquefaciens* during rice *koji* fermentation. Molecules 21, 773.
Mohd-Zaki, Z., Bastidas-Oyanedel, J.R., Lu, Y., Hoelzle, R., Pratt, S., Slater, F.R., Batstone, D.J., 2016. Influence of pH regulation mode in glucose fermentation on product selection and process stability. Microorganisms 4 (1), 2–13.
Nguyen, D., Nicolau, L., Dykes, S.I., Kilmartin, P.A., 2010. Influence of microoxygenation on reductive sulfur off-odors and color development in a Cabernet Sauvignon wine. Am. J. Enol. Vitic. 61, 457–464.
Park, M.K., Choi, H.-K., Kwon, D.-Y., Kim, Y.-S., 2007. Study of volatile organic acids in freeze-dried *Cheonggukjang* formed during fermentation using SPME and stable-isotope dilution assay (SIDA). Food Chem. 105, 1276–1280.
Ravasio, D., Wendland, J., Walther, A., 2014. Major contribution of the Ehrlich pathway for 2-phenylethanol/rose flavor production in *Ashbya gossypii*. FEMS Yeast Res. 14, 833–844.
Robin, X., Turck, N., Hainard, A., Tiberti, N., Lisacek, F., Sanchez, J.-C., Müller, M., 2011. pROC: an open-source package for R and S+ to analyze and compare ROC curves. BMC Bioinf. 12, 77.
Scherlach, K., Hertweck, C., 2009. Triggering cryptic natural product biosynthesis in microorganisms. Org. Biomol. Chem. 7, 1753–1760.
Schwab, W., Davidovich-Rikanati, R., Lewinsohn, E., 2008. Biosynthesis of plant-derived flavor compounds. Plant J. 54, 712–732.
Shin, H.M., Lim, J.W., Shin, C.G., Shin, C.S., 2017. Comparative characteristics of rice wine fermentations using *Monascus* koji and rice nuruk. Food Sci. Biotechnol. 26, 1349–1355.
Smit, G., Smit, B.A., Engels, W.J.M., 2005. Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products. FEMS Microbiol. Rev. 29, 591–610.
Son, E.Y., Lee, S.M., Kim, M.J., Seo, J.-A., Kim, Y.-S., 2018. Comparison of volatile and non-volatile metabolites in rice wine fermented by *Koji* inoculated with *Saccharomycopsis fibuligera* and *Aspergillus oryzae*. Food Res. Int. 109, 596–605.
Thomson, C.A., Delaquis, P.J., Mazza, G., 2006. Detection and measurement of microbial lipase activity: a review. Crit. Rev. Food Sci. Nutr. 39, 165–187.
Wu, S., YU, H., Liu, Z., You, C., 2019. Influence of *Monascus purpureus* BD-M-4 on the physiochemical properties, proteolysis and volatile compounds of surface mould-ripened cheese. Food Sci. Biotechnol. 28, 129–138.
Xie, C., Zeng, H., Wang, C., Xu, Z., Qin, L., 2018. Volatile flavour components, microbiota and their correlations in different sufu, a Chinese fermented soybean food. J. Appl. Microbiol. 125, 1761–1773.
Yoo, Y.J., Hong, J., Hatch, R.T., 1987. Comparison of α-amylase activities from different assay methods. Biotechnol. Bioeng. 30, 147–151.
Zapelena, M., Astiasarán, I., Bello, J., 1999. Dry fermented sausages made with a protease from *Aspergillus oryzae* and/or a starter culture. Meat Sci. 52, 403–409.
Zhao, G., Ding, L.-L., Pan, Z.-H., Kong, D.-H., Hadiatullah, H., Fan, Z.-C., 2018. Proteinase and glycoside hydrolase production is enhanced in solid-state fermentation by manipulating the carbon and nitrogen fluxes in *Aspergillus oryzae*. Food Chem. 271, 606–613.