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Full Length Article

Optimization of Enhanced Fermentation of Low-Salt Sufu Paste from Soybean and Evaluation of its Phenolic Acids

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Abstract

The optimum enhanced fermentation and phenolic acids profile of low-salt Sufu paste obtained from soybean [*Glycine max (Linn.) Merr.*] were investigated in this study. Under optimized condition, the total acid and amino acid nitrogen were 0.14 g/100 g and 1.35 g/100 g, which agreed closely with the predicted values. A total of 85 volatile flavor components (VFCs) were tentatively identified, in which esters were predominant, followed by alcohols. The total phenolic acids contents were ranked as Sufu paste (390.49 μg/g) > pehtze (259.33 μg/g) > soybean (154.25 μg/g) > tofu (107.37 μg/g). Among these compounds, cinnamic acid was the most prevailing in soybean and tofu, while gallic acid was the most predominant in pehtze and Sufu paste. Gallic acid increased from14.35 μg/g in tofu to 220.12 μg/g in Sufu paste, which might be related to the activity of esterase released by the complex microbiota during fermentation. Significantly, the total phenolic acids content in Sufu paste was approximately 2–4 times higher than that in the commercial Sufu paste samples, suggesting that the mixed fermentative starter and α-ketoglutarate are beneficial to increase the phenolic acids. Therefore, the preliminary results have extended to the knowledge about the changes of various phenolic acids in soybean, tofu, pheze and Sufu paste. This information would be useful for future improvements in the manufacturing process and quality of Sufu paste. © 2019 Friends Science Publishers

Keywords: Enhanced fermentation; Phenolic acid; Sufu paste; Soybean; Tofu

Introduction

Soybean, which was originated in Asia, is widely grown due to the nutritional benefits of amino acids and high-quality vegetal protein (about 40%) in a sufficient quantity and reasonable proportions. Especially, it contains abundant essential amino acids such as lysine and tryptophan, which lack in rice and wheat (Kim *et al.*, 2006). Hence, soybeans have been consumed as an important protein source to complement grain protein worldwide for a long time (Jin *et al.*, 2013). In addition, soybean is also an important source of secondary metabolites including isoflavones, anthocyanins, phenolic acids, saponins, phytic acids and oligosaccharides (Munro *et al.*, 2010; Thompson and Erdman, 2010; Zhang *et al.*, 2011; Park *et al.*, 2018). Due to its rich functional components, soybean consumption may be effective to reduce the risk of hypercholesterolemia, cardiovascular disease, atherosclerosis, osteoporosis and various cancers (Clarkson, 2002; Faraj and Vasanthan, 2004; Wei *et al.*, 2007; Rebholz *et al.*, 2013).

Phenolic compounds, including flavonoids, phenolic acids and hydroxycinnamic acid derivates, were reported to about 8000 types in various cereal crops, vegetables, fruits and so on (Coward *et al.*, 1998). Health-promoting effects of soybeans as well as their functional components, especially isoflavones, have received the most attention around the world (Xu and Chang, 2008a). In addition to isoflavones, phenolic acids, accounting for 28–72% over the total phenolic compounds content in soybean (Chung *et al.*, 2011), also have gained much attention due to the potential benefits on human health (Choi *et al.*, 2012). The phenolic acids are of about 3000 types, which are concerned with color, flavor, nutrition and functional activities (Scalbert and Williamson, 2000). According to the prior studies, the common phenolic acids in soybean were gallic, protocatechuic, vanillic, p-hydroxybenzoic, caffeic and p-coumaric acids (Xu and Chang, 2008a; Cho *et al.*, 2011). Phenolic acids in soybean seed and soybean products were influenced by soybean cultivars, cropping year, origin, analytical methods and soybean products processes such as heating and leaching (Lee *et al.*, 2008; Chung *et al.*, 2011). The dietary consumption of phenolic acids has been associated with the reduced risk of many chronic diseases such as cardiovascular disease and cancers in the epidemiological studies (Wu *et al.*, 2003).

As an ancient technology, fermentation remains one of

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the most practical methods for enhancing the nutritional and sensory qualities of fermented foods. It has been proved that fermentation process not only increased the protein quality and palatability, but also eliminates the antinutritional factors and flatulence component (Xie *et al.*, 2018a). Consumption of fermented soybean foods has potential health benefits against cardiovascular diseases, menopausal symptoms, osteoporosis and prostate cancers (Marã *et al.*, 2015). Various fermented soybean foods including soy sauce, douchi, Sufu, doenjang (soybean paste) and meju (soybean cake) have been developed (Chung *et al.*, 2011). Among them, Sufu, an oriental fermented food, is a highly flavored appetizer made from cubes of soybean curd. Nowadays, the post-ripening time is shorter than the traditional process, which took more than 6 months. However, the modern processes still take about 2–3 months. In addition, the traditional Sufu brine process usually leads to a high concentration of salt (10–30%), which could increase the dietary sodium intake and limit human consumption (Han *et al.*, 2001; Xie *et al.*, 2018b). Similar problems also existed in traditional Sufu paste, in which the pehtze fermentation and technical principle are exactly similar to Sufu. Therefore, Sufu paste with a low NaCl content may be beneficial to shorten the post-ripening period and preferable from the viewpoint of public nutritional health.

The aim of our work was to optimize the enhanced fermentation of low-salt Sufu paste and investigate the dynamic change of phenolic acids profile. In addition, the phenolic acids were compared with the commercial Sufu paste samples. The present study would be useful for future improvements in the manufacturing process and quality of Sufu paste.

Materials and Methods

Material and Chemicals

The raw soybean [*Glycine max* (Linn.) Merr.] classified as Yungui plateau summer soybean, was cultivated in Dafang County, Guizhou Province, China. It was sown on April 6th, 2017 and germinated after about five days. The flowering started on June 1, 2017 and lasted for 25–30 days. It was harvested on September 5th, 2017 and the yield was about 1600 kg/hm^2. The temperature and precipitation in producing area are listed in Table 1.

Actinomucor elegans (*A. elegans*, CICC 41043), *Pichia fermentans* (*P. fermentans*; CICC 33120), *Kodamaea ohmeri* (*K. ohmeri*; CICC 32993) and *Lactococcus lactis subsp. lactis* (*L. lactis subsp. lactis*; CICC 21030) were purchased from China Center of Industrial Culture Collection (CICC). α-Ketoglutarate (food grade) was purchased from Huao Co. Ltd, China. Internal standards (cyclohexanone, 2,4,6-trimethyl pyridine and methyl nonanoate) and eight phenolic acids, namely, gallic acid, protocatechuic acid, vanillic acid, p-hydroxybenzoic acid, caffeic acid, p-coumaric acid, ferulic acid and cinammic acid, were purchased from Sigma-Aldrich Co. Ltd, USA. Acetonitrile, methanol, acetic acid, and trifluoroacetic acid were of high-performance liquid chromatography (HPLC) grade. All other chemicals were of analytical grade.

Preparation of Sufu Paste

Preparation of pehtze: The schematic diagram for Sufu paste is shown in Fig. 1. Briefly, soybeans seeds were washed, ground, coagulated and pressed successively and the tofu was obtained. The tofu (moisture about 80%) was cut into rectangular pieces (2.0 cm × 2.0 cm × 1.5 cm) and inoculated with *A. elegans* by spraying inoculum suspension onto the surfaces. The inoculated tofu pieces were placed in an incubation room 28°C, relative humidity 90–95% and air circulation to ensure adequate aeration. Fresh pehtze was obtained until a slightly yellowish white color appeared.

Optimization of Sufu Paste Preparation

In order to optimize the preparation of Sufu paste, the single factor test (Table 2) and orthogonal test (Table 3) were performed. Briefly, the mixed fermentative starter, composed of *P. fermentans*, *K. ohmeri* and *L. lactis subsp. lactis*, was prepared just before use. The pehtze was micronized at 2800 rpm by colloid mill (JML-50, Shanghai Zhuheng Ltd., China). Then mixed starter, salt and α-ketoglutarate were added to the micronized pehtze and mixed well. About 150 g of fresh pehtze was placed in individual wide-mouthed glass bottles with a capacity of 280 mL. The post-ripening was performed and the sample SPSK (Sufu paste fermentation enhanced by mixed starter and α-ketoglutarate) was obtained. For SPS (Sufu paste enhanced by mixed starter), the Sufu paste was fermented without α-ketoglutarate, and all other conditions were consistent with SPSK. The SP was fermented without the mixed starter and α-ketoglutarate, and all other conditions were consistent with SPSK.

Analysis of Enzyme Activities

The dynamic changes of enzyme activities including protease, peptidase, branched-chain amino acid aminotransferase (BCAT), aromatic amino acid aminotransferase (ARAT), lipase and α-amylase were determined during different pehtze fermentation stages (0 h, 12 h, 24 h, 36 h, 48 h and 60 h). Briefly, 20 g of fresh pehtze was fully ground with 100 mL of saline solution. After extraction at 37°C for 1 h, the mixed solution was centrifuged at 10000 *g* for 20 min and the supernatant (namely crude exoenzyme extract) was collected. Subsequently, the precipitate was dissolved in a mixture of saline solution (80 mL) and 2% (V/V) cetyl trimethyl ammonium bromide (5 mL). After water bath at 37°C for 1 h, the endoenzyme was extracted by ultrasonic cell disrupter (ultrasonic time 3 s, interval time10 s, 10 times). The

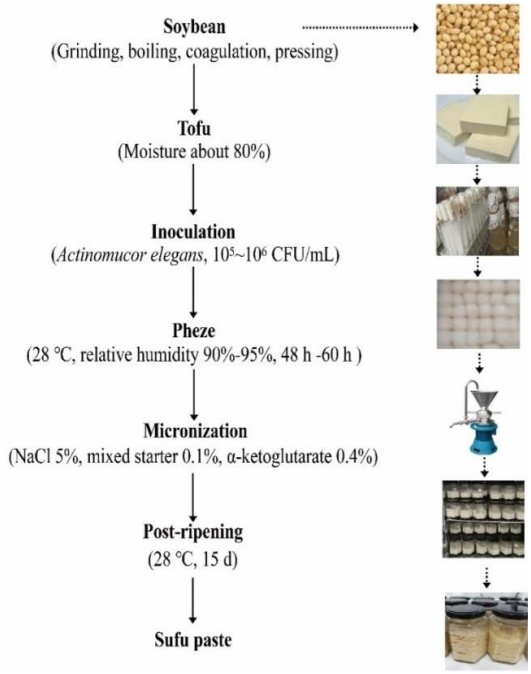

Fig. 1: The schematic diagram for Sufu paste

Table 1: The temperature and precipitation in producing area

Growth stage	Germination	Florescence	Physiological ripeness
Lowest temperature/℃	10	16	14
Highest temperature/℃	20	24	23
Average precipitation/mm	58	132	101

Table 2: Single factor test

Factors	Levels				
Fermentation time (d)	5	10	15	20	25
Temperature (℃)	24	26	28	30	32
Addition amount of salt (%)	1	3	5	7	9
Starter ratio (*P. fermentans*: *K. ohmeri*: *L. lactis subsp. lactis*)	1:1:1	2:1:1	2:2:1	3:2:1	3:3:1
Addition amount of starter (%)	0.03	0.07	0.1	0.13	0.16
Addition amount of α-ketoglutarate (%)	0.2	0.4	0.6	0.8	1.0

Table 3: Factors and levels for orthogonal test

Levels	Factors			
	A(Temperature)/℃	B(Starter)/%	C(α-Ketoglutarate)/%	D(Salt)/%
1	28	0.07	0.2	3
2	30	0.10	0.4	5
3	32	0.13	0.6	7

mixture was centrifuged at 10000 *g* for 20 min and the supernatant (namely crude endoenzyme extract) was collected. The exoenzyme and endoenzyme extract were mixed well and diluted with saline solution to 200 mL for the enzyme activities analysis. Protease activity was determined according to the method described by Leonard and Wildi (1970), using 2% casein as substrate. Lipase activity was assessed by potentiometric titration using polyvinyl alcohol and pure olive oil emulsion as substrate. α-Amylase activity was assayed as described by Inatsu *et al.* (2010). Peptidase activity was routinely determined using soybean peptide as substrate with some modifications (Boutrou *et al.,* 1998). Aromatic amino acid aminotransferase (ARAT) and branched-chain amino acid aminotransferase (BCAT) were traced according to the method as described by Yvon *et al.* (1997). Particularly, free amino groups were quantified with leucine as the standard. One unit of protease activity is defined as the amount of enzyme that liberates 1 μg of tyrosine per min. One unit of lipase activity is defined as the amount of enzyme that liberates 1 μmol of oleic acid per min. One unit of α-amylase activity is defined as the amount of enzyme that catalyzes the hydrolysis of 10 mg of soluble starch per 30 min. The units of ARAT and BCAT activities are defined as the amount of enzyme that catalyzes the hydrolysis of 1 μmol of phenylpyruvic acid and pyruvate per hour, respectively. Three replicates were analyzed.

Identification of Volatile Flavor Components by GC-MS

The volatile flavor components (VFCs) were extracted using headspace-solid phase microextraction (HS-SPME) and identified by gas chromatography-mass spectrometer (GC-MS). Two microlitre of internal standards solution of cyclohexanone (0.688 μg/μL), 2,4,6-trimethyl pyridine (0.588 μg/μL) and methyl nonanoate (0.452 μg/μL) dissolved in methanol, was added to samples (1 g each). Then the mixture was incubated in a 10 mL glass vial at 55°C for 40 min. Separations of the VFCs were performed on a HP-5 elastic quartz capillary column (60 m × 0.25 mm × 0.25 μm; Agilent Ltd, USA). All the other details of methods and instrument were the same as described by Xie *et al.* (2018b). All of the analyses were carried out in three replicates. Volatile and internal standard peak areas were used for qualification and quantification. Internal standards were used as follows: cyclohexanone for components with retention indices less than 950, 2,4,6-trimethyl pyridine for components with retention indices 950–1100, and methyl nonanoate for components with retention indices greater than 1100.

Analysis of Phenolic Acids by HPLC

Extraction of phenolic acids: Each sample was repeatedly freeze-dried in an IEC Lyoprep 3000 freeze drier (Lyoprep, Dunstable, UK) and then fully ground. Three grams of each powder were mixed with 40 mL of 80% ethanol. The mixture was sonicated (power 400 W) for 30 min at 40°C and then the volume was adjusted to 50 mL. Subsequently, the sample solution was filtered through a 0.22 μm P filter prior to HPLC injection.

HPLC analysis of phenolic acids: The HPLC system (Agilent 1260, Agilent Ltd, USA) equipped with a Phecda C18 column (250 × 4.6 mm, 5 μm) was used to measure the

phenolic acids according to the method of Kubola and Siriamornpun (Kubola and Siriamornpun, 2011) with some modifications. The mobile phase was composed of 0.1% formic acid in purified water (solvent A) and methanol (solvent B). The sample solution (20 μL) was injected at a flow-rate of 0.8 mL/min and the wavelength was 280 nm. The linear gradient solvent was performed as follows. Solvent B was increased from 15 to 20% in 5 min, then from 20 to 25% in 10 min, from 25 to 30% in 15 min, from 30 to 35% in 25 min, from 35 to 40% in 30 min, from 40 to 80% in 40 min, and kept at 85% of B for 10 min. The column was equilibrated for 15 min before injection of the next sample. Three replicates were performed.

Statistical Analysis

The statistical analysis was performed using Origin 8.0 software. The mean values and standard deviations were calculated from the data obtained from three separate experiments and data are expressed as the mean ± standard deviation (SD), where feasible. The orthogonal test was designed and analyzed by the Orthogonal design assistant software (Version 3.1).

Results

Dynamic Changes of Enzyme Activities during Pehtze Fermentation

As shown in Fig. 2, the enzyme activities increased along with the pehtze fermentation. The highest enzyme activities of BCAT (2581.57 U/g), ARAT (61.98 U/g) and peptidase (38.35 mmol/L) were detected at 48 h. The lipase activities reach a maximum (8.75 U/g) at 36 h, while the α-amylase (141.00 U/g) increased sharply from 24 h to 36 h and then slowed down to a constant value. The protease activity reached up to 2137.54 U/g at 60 h. Generally, the activities increased slowly within 24 h, due to the lag phase of *A. elegans*. Therefore, the optimal pehtze fermentation should be 48 h.

Optimization of Enhanced Fermentation of Low-salt Sufu Paste

The total acid and amino acid nitrogen of Sufu paste, affected by the fermentation time, temperature, starter ratio, addition amount of starter, salt and α-ketoglutarate, are shown in Fig. 3. The total acid decreased steadily with the increasing fermentation time and temperature, whilst amino acid nitrogen reached the peak values when the fermentation time and temperature were 15 d (1.25 g/100 g) and 28°C (0.96 g/100g), respectively (Fig. 3A and B). Both of the total acid and amino acid nitrogen increased with a change of starter ratio and reached the maximum values at the starter ratio of 2:1:1 (0.22 g/100 g) and 2:2:1 (0.91 g/100 g) respectively and then dropped (Fig. 3C). The amino acid

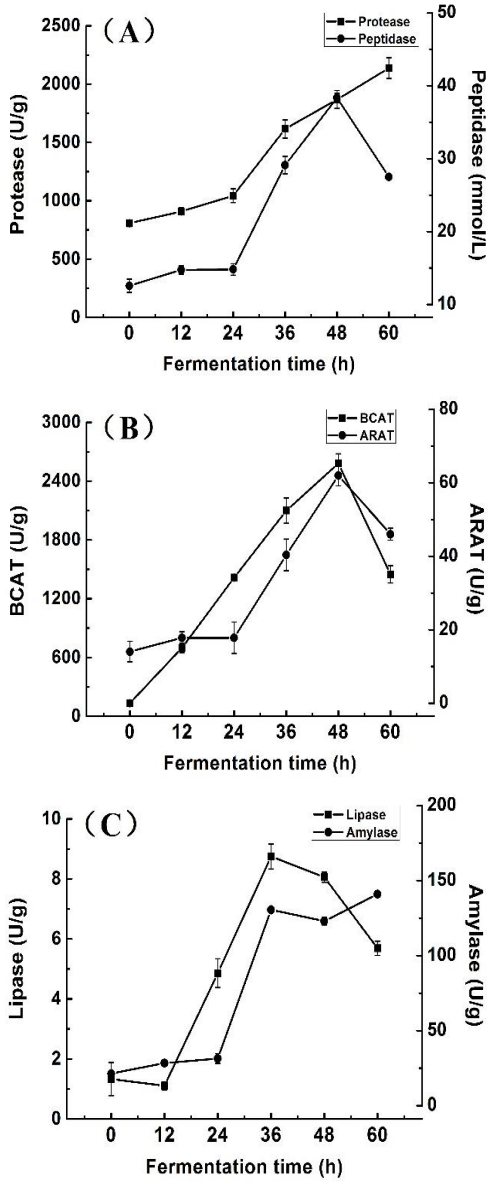

Fig. 2: Dynamic changes of enzyme activity during pehtze fermentation. (A) Protease and Peptidase; (B) Branched-chain amino acid aminotransferase (BCAT) and Aromatic amino acid aminotransferase (ARAT); (C) Lipase and α-Amylase

nitrogen contents reached critical values when the addition amounts of starter and α-ketoglutarate were 0.10 and 0.4%, and then increased in a mild slope (Fig. 3D, E and F). The total acid and amino acid nitrogen continuously decreased with the increasing of salt addition. Notably, the total acid decreased dramatically when the amount of salt was lower than 5%, While the amino acid nitrogen decreased quickly when the salt addition amount higher than 5%. So in this study, fermentation temperature (28–32°C), addition amount of starter (0.07–0.13%), salt (3–7%) and α-ketoglutarate (0.2–0.6%) were selected for further study in the orthogonal test design.

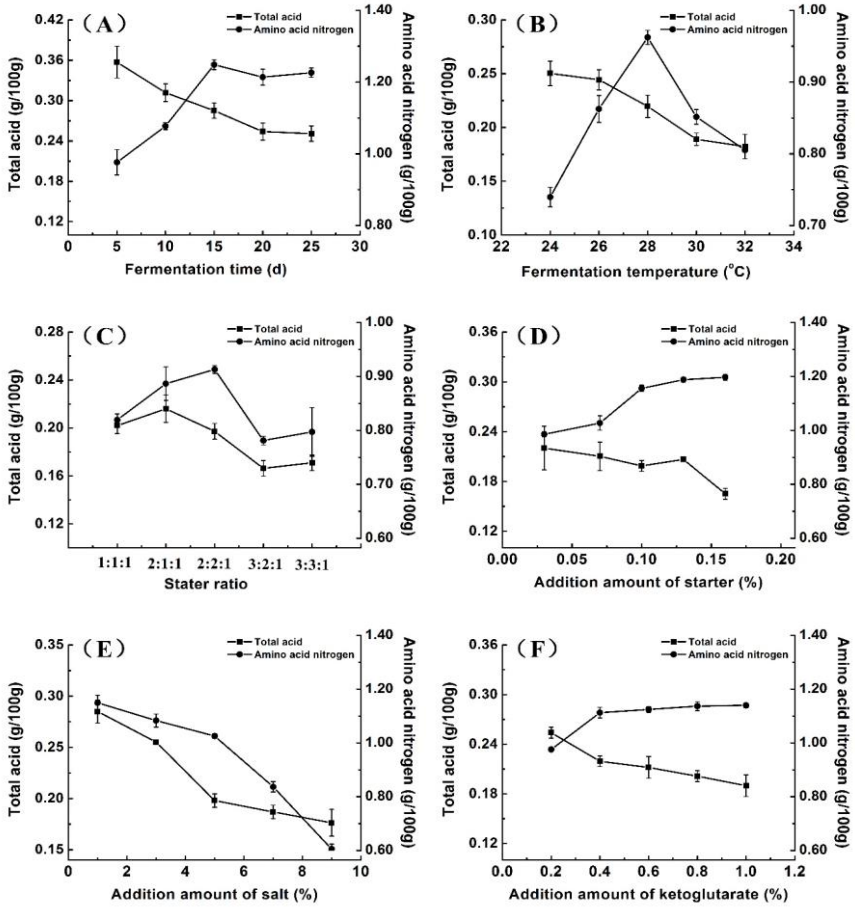

Fig. 3: Effects of fermentation time (A), fermentation temperature (B), starter ratio (C), addition amount of starter (D), addition amount of salt (E) and addition amount of α-ketoglutarate (F) on enhanced fermentation of low-salt Sufu paste

The analysis results of orthogonal test, performed by statistical software, are presented in Table 4. The lowest total acid was 0.12 g/100 g and the highest amino acid nitrogen was 1.30 g/100 g, corresponding to the condition $A_1B_2C_2D_2$. According to the R values, the influence by the parameters on the total acid decreased in the order: C (addition amount of α-ketoglutarate) > D (addition amount of salt) > B (addition amount of starter) > A (fermentation temperature). So the minimum total acid content was obtained in the optimum condition $A_2B_2C_2D_2$. The factors influence the amino acid nitrogen were listed in a decreasing order as follows: B (addition amount of starter) > C (addition amount of α-ketoglutarate) > D (addition amount of salt) >A (fermentation temperature). So the maximum amino acid nitrogen content was obtained in the optimum condition $A_1B_2C_2D_2$. Considering the above results, the optimum condition of Sufu paste enhanced fermentation was as follows: fermentation temperature 28°C, addition amount of starter 0.10%, addition amount of α-ketoglutarate 0.4% and addition amount of salt 5%. Under the optimized condition, the total acid and amino acid nitrogen were 0.14 g/100 g and 1.35 g/100 g in the confirmatory test.

Comparison of Varieties and Contents of Volatile Flavor Components

The volatile flavor components (VFCs) in Pehtze, SPSK, SPS and SP were identified by GC-MS (Fig. 4 and 5). Totals of 45, 85, 82 and 73 VFCs were tentatively identified in pehtze, SPSK, SPS and SP, respectively. They were composed of esters, hydrocarbons, alcohols, aldehydes, ketones, acids and miscellaneous components. Among them, esters were predominant, followed by alcohols and miscellaneous components. The esters were 8, 17, 18 and 13, while the alcohols were 9, 16, 15 and 14 in pehtze SPSK, SPS and SP, respectively (Fig. 5A). As shown in Fig. 5B, the contents of VFCs in SPSK were the highest, up to 142.48 μg/g, followed by SPS (121.35 μg/g) and SP (112.88 μg/g). The VFCs content in pehtze was the lowest, only 28.18 μg/g.

Phenolic Acids Changes during Different Stages of Sufu Paste Preparation

The phenolic acid contents of the soybean, tofu, pehtze,

Table 4: Analysis of $L_9(3)^4$ test results

No.		Factors				Total acid (g/100 g)	Amino acid nitrogen (g/100 g)
		A	B	C	D		
1		1	1	1	1	0.30±0.02	0.99±0.09
2		1	2	2	2	0.12±0.01	1.30±0.06
3		1	3	3	3	0.30±0.03	0.95±0.08
4		2	1	2	3	0.21±0.01	1.15±0.08
5		2	2	3	1	0.18±0.01	1.06±0.05
6		2	3	1	2	0.30±0.04	1.01±0.02
7		3	1	3	2	0.17±0.00	0.98±0.04
8		3	2	1	3	0.32±0.05	1.19±0.08
9		3	3	2	1	0.26±0.02	1.03±0.10
Total acid[#]	$k^{\#}_1$	0.24	0.23	0.31	0.25		
	$k^{\#}_2$	0.23	0.21	0.20	0.20		
	$k^{\#}_3$	0.25	0.29	0.22	0.28		
	$R^{\#}$	0.02	0.08	0.11	0.08		
Amino acid nitrogen[*]	K^{*}_1	1.08	1.04	1.06	1.03		
	K^{*}_2	1.07	1.18	1.16	1.10		
	K^{*}_3	1.07	1.00	1.00	1.10		
	R^{*}	0.01	0.19	0.16	0.07		

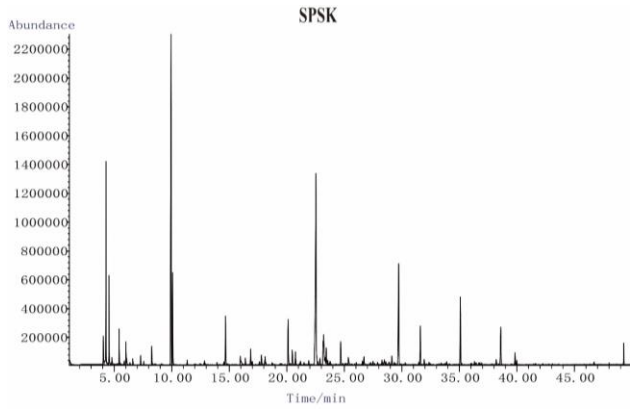

Fig. 4: Typical total ion chromatogram of Sufu paste (SPSK)

SPSK, SPS and SP are presented in Table 5. A total of three hydroxybenzoic acids (protocatechuic acid, gallicand and vanillic acid) and four hydroxycinnamic acids (caffeic, p-coumaric, ferullic and cinnamic acid) were detected in the test samples. Among these compounds, cinnamic acid was the most abundant in soybean and tofu, up to 81.29 μg/g and 69.30 μg/g, respectively. Gallic acid was the most predominant in pehtze and Sufu paste (SPSK, SPS and SP), followed by cinnamic acid. Gallic acid increased from 14.35 μg/g in tofu to 220.12 μg/gin SPSK. p-Hydroxybenzoic acid was not detectable in any of the test samples.

The total phenolic acids contents were ranked as SPSK > SPS > SP > pehtze > soybean > tofu. The total phenolic acids content in Sufu paste (390.49 μg/g) was about three times higher than in tofu (107.37 μg/g) and soybean (154.25 μg/g). Especially, the phenolic acid contents in SPSK were superior to SPS and SP. The total phenolic acids content in soybean was 154.25 μg/g and the content of SPSK was the highest, up to 390.49 μg/g. The hydroxycinnamic acids content in soybean was 120.66 μg/g, which was about four times as many as hydroxybenzoic

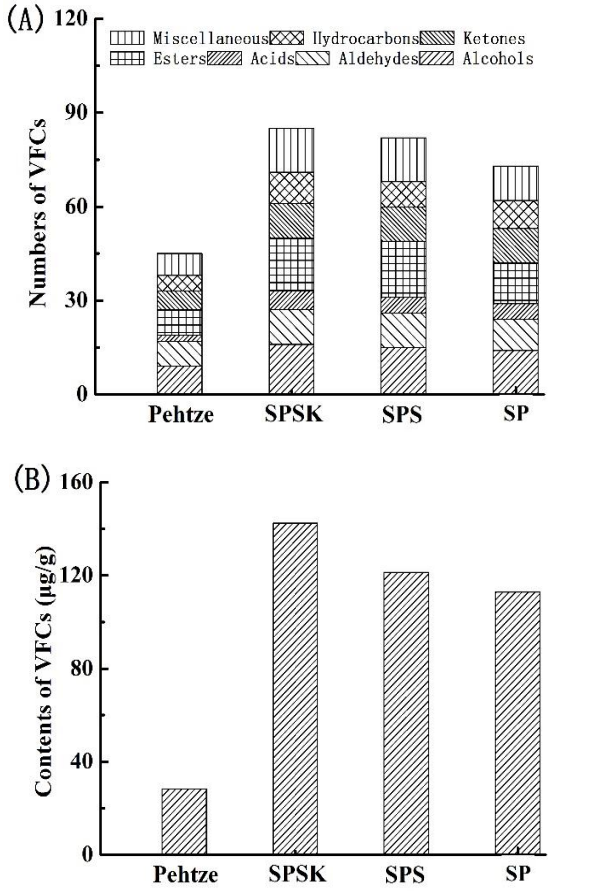

Fig. 5: Comparison of varieties and contents of volatile flavor components (VFCs) in pehtze, SPSK, SPS and SP

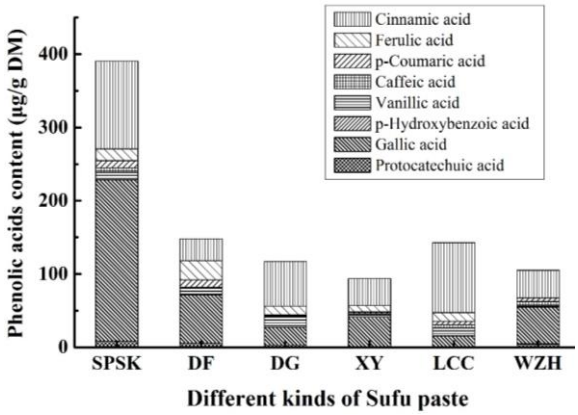

Fig. 6: A comparison of phenolic acids profile between SPSK and the commercial Sufu paste

acids (33.59 μg/g). In addition, the phenolic acids was more abundant in pheze and Sufu paste than in soybean and tofu.

As shown in Fig. 6, the total phenolic acids content among 5 commercial Sufu paste samples varied between 93.93 and 148.10 μg/g. The highest amount of total phenolic acids was identified in DF, while the lowest was

Table 5: Phenolic acids changes during different stages of Sufu paste preparation[a]

Phenolic acids	Soybean	Tofu	Pehtze	SPSK[b]	SPS[b]	SP[b]
			Hydroxybenzoic acids			
Protocatechuic acid	10.24±0.17	1.04±0.04	2.18±0.17	8.71±0.27	8.49±0.15	7.93±0.04
Gallic acid	18.08±0.41	14.35±0.16	127.16±1.08	220.12±6.36	212.18±3.88	166.93±5.33
p-Hydroxybenzoic acid	ND[c]	ND	ND	ND	ND	ND
Vanillic acid	5.27±1.01	3.24±0.02	4.22±0.31	5.78±0.17	4.15±0.27	3.26±0.02
Total	33.59±0.43	18.64±0.08	133.56±1.22	234.60±2.80	224.83±3.98	178.12±2.50
			Hydroxycinnamic acids			
Caffeic acid	14.63±0.12	11.63±0.52	11.78±0.36	10.40±0.35	10.41±0.34	10.35±0.09
p-Coumaric acid	5.36±0.17	3.08±0.03	7.35±0.05	9.84±0.04	8.80±0.08	7.26±0.02
Ferulic acid	19.38±0.22	4.73±0.03	11.77±0.06	15.83±0.03	13.82±0.04	11.10±0.01
Cinnamic acid	81.29±2.56	69.30±0.56	94.87±1.18	119.82±0.24	115.90±1.16	101.01±1.17
Total	120.66±5.18	88.74±0.60	125.77±0.66	155.88±0.85	148.94±0.58	129.73±1.16
Sum	154.25±5.61	107.37±0.51	259.33±1.88	390.49±2.95	373.77±3.39	307.85±3.66

Note: [a]All the data are expressed in the unit of μg/g. Each value is expressed as mean ± SD (n=3)
[b]SPSK, Sufu paste fermented with starter and α-ketoglutarate; SPS, Sufu paste fermented with starter but without α-ketoglutarate; SP, Sufu paste fermented without mixed starter or α-ketoglutarate;
[c]ND: not detected

determined in the XY. Gallic acid and cinnamic acid were the predominant phenolic acid in all the commercial Sufu paste samples assayed in the current study. p-Hydroxybenzoic acid, which was not detected in soybean, tofu, pheze and some Sufu paste samples, was present in very small amounts (0.28–0.90 μg/g) in DF, LCC and WZH. Ferulic acid ranged from 2.66–26.34 μg/g, with the exception of WZH. Significantly, the total phenolic acids in SPSK (390.49 μg/g) was approximately 2–4 times higher than that in the commercial Sufu paste samples.

Discussion

Enzymes, including protease, lipase, peptidase, α-amylase, aromatic amino acid aminotransferase (ARAT) and branched-chain amino acid aminotransferase (BCAT), are important for the characteristic flavor, color and texture of traditional fermented soybean food. The protease could cleave peptide bonds to produce peptide fragments and peptidase will hydrolyse peptides to amino acids deeply. BCAT and AAAT are two important enzymes in transamination reaction involving in amino acid conversion to aroma components (Yvon and Rijnen, 2001). Lipase catalyzes the hydrolysis and synthesis of esters formed from glycerol and long-chain fatty acids (Sharma *et al.*, 2001). α-Amylase could cleave α-1, 4-glucoside linkages at random sites in starch. Generally, the activities increased slowly within 24 h, due to the lag phase of *A. elegans*. The highest enzyme activities of BCAT, ARAT and peptidase were detected at 48 h, while the lipase and α-amylase activities reach a maximum at 36 h. In addition, the protease activity reached up to 2137.54 U/g at 60 h. Therefore, the optimal pehtze fermentation should be 48 h.

Both of total acid and amino acid nitrogen are important indices of Sufu quality, which indicate the degree of ripening during fermentation (Xia *et al.*, 2014). Therefore, the total acid and amino acid nitrogen were selected for the enhanced fermentation optimization of low-salt Sufu paste. The total acid decreased steadily with the increasing fermentation time and temperature, whilst amino acid nitrogen reached the peak values when the fermentation time and temperature were 15 d. The microorganism growth and microbial cells autolysis during the enhanced fermentation may have contributed to the phenomenon. Both of the total acid and amino acid nitrogen increased with the change of starter ratio and reached the maximum values at the starter ratio of 2:1:1 and 2:2:1, respectively. Yeasts were deduced as an indication of a beneficial effect on the lactic acid bacteria. Previous study has shown that carbon dioxide, pyruvate, propionate, acetate and succinate excreted by the yeast, could stimulate the growth of lactic acid bacteria (Leroi and Pidoux, 1993). In addition, some lactic acid bacteria release galactose into the medium as a by-product of lactose metabolism, which may be used by galactose-assimilating but lactose-negative yeasts (Gadaga *et al.*, 2001). The α-ketoglutarate also had effective influences on Sufu paste fermentation. Transamination is a major reaction of amino acid metabolism and limited by the a-keto acid (Yvon and Rijnen, 2001). The exogenous α-ketoglutarate has proved to be effective to intensify amino acid degradation and accelerate cheese curd ripening (Banks *et al.*, 2001). The amino acid nitrogen contents reached to the critical values when the addition amount of α-ketoglutarate was 0.4%. Salt had multiple impacts on the quality fermented food. It not only imparts a salty taste, but also influences biochemical changes and inhibits microorganism growth by controlling the enzyme activity at some high concentrations (Kang *et al.*, 2018). The total acid decreased dramatically when the salt addition amount lower than 5%, while the amino acid nitrogen decreased quickly when the salt addition amount higher than 5%. Therefore, fermentation temperature, addition amount of starter, salt and α-ketoglutarate were selected for further study in the orthogonal test design. According to the analysis results orthogonal test, the optimum condition of Sufu paste enhanced fermentation was determined as follows: fermentation temperature 28°C,

addition amount of starter 0.10%, addition amount of α-ketoglutarate 0.4% and addition amount of salt 5%. Under the optimized condition, the total acid and amino acid nitrogen were 0.14 and 1.35 g/100 g in the confirmatory test.

Volatile flavor components (VFCs) have important influences on the quality and acceptability of Sufu paste. Therefore, it is a key index for the palatability of human consumption of Sufu paste. Totals of 45, 85, 82 and 73 VFCs were tentatively identified in pehtze, SPSK, SPS and SP, respectively. Among them, esters were predominant, followed by alcohols. The esters were 8, 17, 18 and 13 in pehtze SPSK, SPS and SP, respectively. The results were in accordance with the report by Chung (2000), who found that both esters and alcohols had the highest number of components in red Sufu. Similar phenomena also occurred in white Sufu (Chung *et al.*, 2005) and enzyme-ripened Sufu (Moy *et al.*, 2012). The presence of a large number of various esters in Sufu paste suggests that most fatty acids probably underwent esterification with the formation of ethyl esters during the fermentation (Wang and Hesseltine, 1970). Most esters are described to have a general fruity and floral odor such as pineapple, coconut and honey (Moy *et al.*, 2012). In addition, the contents of VFCs in SPSK were the highest, up to 142.48 μg/g, followed by SPS (121.35 μg/g) and SP (112.88 μg/g). The VFCs content in pehtze was the lowest, only 28.18 μg/g. Both varieties and contents of VFCs in SPSK were the highest, indicating that fermentative starter and α-ketoglutarate were beneficial for the flavor formation during fermentation.

Phenolic acids were related to flavor and the particular savor (bitterness and astringency) as well as to the functional activity (Chung *et al.*, 2011). As the secondary plant products commonly found in plant-derived foods (Cho *et al.*, 2009), a total of three hydroxybenzoic acids and four hydroxycinnamic acids were detected in the test samples. Among these compounds, cinnamic acid was the most abundant in soybean and tofu, while gallic acid was the most predominant in pehtze and Sufu paste. Our results were consistent with the previous report that the content of gallic acid in the fermented cheonggukjang increased from 361 to 1012 μg/g by *Bacillus pumilus* HY1 and from 306.40 mg/kg to 1062.54 mg/kg by *B. subtilis* CS90 at the end of fermentation (60 h) (Cho *et al.*, 2009; Cho *et al.*, 2011). A significant increase in the gallic acid contents might be related to the activity of esterase released by the complex microbiota during fermentation, because esterase could catalyze epigallocatechin gallate into epigallocatechin (Cho *et al.*, 2011).

The total phenolic acids contents were ranked as SPSK > SPS > SP > pehtze > soybean > tofu. The low total phenolic acids content in tofu may be due to the heating, filtering and coagulation process during tofu making. For instance, the heating process had important influences on some thermal degradation or transformation of phenolic acids (Chung *et al.*, 2011). Especially, the phenolic acid contents in SPSK was superior to SPS and SP, indicating that the mixed fermentative starter, composed of *P. fermentans*, *K. ohmeri* and *L. lactis subsp. lactis*, was important for the increase of phenolic acids during the enhanced fermentation. These results are also consistent with the previous reports that fermentation of soybean with lactic acid bacteria and yeasts could produce an increase in total phenolic acids content (Fernandezorozco *et al.*, 2007; Marã *et al.*, 2015). According to the prior study, fermentation guarantees that every consumer, independently of their intestinal microbiota, could possibly derive health benefits resulting from the formation of phenolic acids (Marã *et al.*, 2015). The total phenolic acids content in soybean was 154.25 μg/g, higher than the content (87 μg/g) reported by (Kim *et al.*, 2006), due to the different soybean varieties, cropping year and origin. The phenolic acids content in soy paste aged for 3 days was higher than that in tofu (Chung *et al.*, 2011). This is similar to our present study, that the total phenolic acid in SPSK was the highest. The hydroxycinnamic acids content in soybean was about four times as many as hydroxybenzoic acids. Xu and Chang (2008a) also found that the hydroxycinnamic acids content in soybean samples was higher than hydroxybenzoic acids. In addition, the phenolic acids were more abundant in pheze and Sufu paste than in soybean and tofu. We presumed that the cell membranes and cell walls were disrupted and soluble phenolic acids were released from the insoluble ester bonds during the fermentation. Especially, the total phenolic acids in SPSK (390.49 μg/g) was approximately 2–4 times higher than that in the commercial Sufu paste samples, which may due to the enhanced fermentation by the fermentative starter. The differences may be also attributed to the differences in the sources of the materials (Xu and Chang, 2008b). Our results substantiate that fermentation is an appropriate and effective process for improvement nutritional and biological quality owing to the increasing of phenolic acids, which had good antioxidant potential (Dueñas *et al.*, 2005; Chang *et al.*, 2010).

Conclusion

This is the first scientific report concerning the optimum process and phenolic acids change of Sufu paste, which was enhanced by mixed starter and α-ketoglutarate during post-ripening. The total phenolic acids content in Sufu paste was about three times higher than in tofu and soybean, indicating that fermentation is an appropriate and effective process for improvement nutritional and biological quality owing to the increasing of phenolic acids. Significantly, the total phenolic acids in SPSK (390.49 μg/g) was approximately 2–4 times higher than that in the commercial Sufu paste samples, suggesting that the mixed fermentative starter and α-ketoglutarate are beneficial to increase the phenolic acids.

Our results would be useful for future improvements in the manufacturing process and quality of Sufu paste.

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