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Novel conditions for tofu and pehtze preparation to overcome bacterial contamination in pehtze

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Abstract

Sufu is one of the many soy food products. It is prepared when tofu is fermented with a strain of fungus, producing pehtze, which is then allowed to ripen with various dressing mixtures. Although pasteurization is applied to most commercial sufu, salt-tolerant pathogenic bacteria such as *Bacillus* spp. and *Staphylococcus aureus* are still detected in marketed sufu products. This study proposed new conditions for tofu and pehtze preparation to reduce bacterial contamination in pehtze, which would then be more suitable for using in further sufu production. At first, seven isolates of *Rhizopus* spp. were screened from fermented tofu obtained from a sufu manufacturer in Thailand. *Rhizopus* sp. KUPR4 strain was the only one out of strains containing protease activity at pH 4.0. The lipase activity of the *Rhizopus* sp. KUPR4 strain was as good as that of the reference *R. oligosporus* (TISTR 3001) at pH 4.0 (13.74 unit/ml). *Rhizopus* sp. KUPR4 strain was thus suggested for using in further acidic pehtze preparation. For protocol optimization of tofu production, different coagulants were tested, including: 1.0% w/v calcium sulfate (C-tofu); 0.5% w/v acetic acid (A-tofu); 1.0% w/v magnesium sulfate (M-tofu); and a combination of coagulum from 1.0% w/v calcium sulfate mixed with coagulum from 0.5% w/v acetic acid in a ratio of 1:1 w/w (AC-tofu). AC-tofu presented the most appropriate quality in terms of smoothness and hardness, and contained the lowest moisture content. Its pH was in a moderately acidic range (pH 5.0) in which *Rhizopus* sp. KUPR4 could grow very well at 33°C for 20 - 24 h or at 40°C for 16 - 18 h. Acidic AC-tofu was incubated with *Rhizopus* sp. KUPR4 during pehtze preparation under the above conditions, and then a microbiological contamination assay was performed. After 24 h incubation, pehtze prepared from AC-tofu (pH 5.09) exhibited <10 CFU/g of *B. cereus*; whereas $>10^5$ CFU/g of *B. cereus* was detected on 24-h-incubated pehtze prepared from C-tofu (pH 5.95), which is similar to the conditions for the preparation of pehtze used in typical sufu manufacturing. From the perspective of food safety, the use of AC-tofu with *Rhizopus* sp. KUPR4 in pehtze production is suggested to reduce the microbial contamination of sufu. The *Rhizopus* sp. KUPR4 strain was genetically analyzed and verified to be *Rhizopus microsporus* var. *microsporus*.

Introduction

Filamentous fungi are widely used in several fermented food industries. In Asia, sufu has long been the one of the most popular fermented soybean products. Originating in China, sufu is made by solid-state fungal fermentation of tofu (Steinkraus *et al*., 1996). However, several types of sufu can be distinguished according to the different processes, colors, and flavors (Han *et al*., 2001b). Among many kinds of sufu, red sufu is the most popular type due to its attractive color and characteristic flavor. In addition, red sufu (known in Thailand as *tao-huu-yi*) is widely used as a seasoning and food coloring, in dishes such as *yen-ta-fo* and *su-ki-ya-ki*, which are very popular in Thailand.

Basically, the production of sufu consists of four major steps: tofu preparation; pehtze-making by fungal solid-state fermentation of tofu; salting of pehtze; and aging in a dressing mixture (Han *et al*., 2001b; Hui *et al*., 2004; Yin *et al*., 2004). This article primarily focuses on the first two main steps.

Tofu, the essential raw material for pehtze preparation, can be produced using various kinds of coagulants and their selection is very important, according to the desired characteristics of the final tofu product. In this study, different coagulants were chosen to compare the characteristics of the resulting tofu products in order to select the appropriate coagulants for providing good tofu texture suitable for further sufu production. These included calcium sulfate, magnesium sulfate, acetic acid, and a combination of calcium sulfate and acetic acid. Each type of coagulant has advantages and disadvantages. Calcium sulfate is the most widely used tofu coagulant because of its ability to associate

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with water into the tofu structure (Liu, 1999). The main reason for choosing sulfate compounds is because these coagulants provide a higher yield of tofu with greater hardness and more elasticity than when using chloride compounds. Moreover, tofu made from calcium sulfate has a smoother texture than chloride compound-coagulated tofu, and hence it is more suitable for the production of sufu (Saio, 1979). Acetic acid was another coagulant chosen for acidic (low pH) tofu production but the texture, sour taste and strong odor of tofu make this much less attractive compared to other coagulants. The coagulation mechanisms of acetic acid and sulfate compounds are quite different (Liu, 1999). However, it is interesting to consider the application of a combination of curds from both calcium sulfate and acetic acid as this may contribute the advantages of both coagulants to produce the better tofu desired for sufu production.

In the second step of sufu production, pehtze is commercially made from tofu inoculated with the mycelium of molds such as *Actinomucor* spp., *Mucor* spp. or *Rhizopus* spp. The specific activities of the important enzymes produced by these fungi (such as protease and lipase) play a great role in influencing the quality of the finished product by developing the flavor of sufu during the ripening process. The protein and lipid hydrolytic products provide the principal constituents responsible for the mild flavor of sufu (Steinkraus *et al*., 1996). These fungi thrive under different growing conditions. *Actinomucor* spp. grows well at temperatures of 25 - 30°C (Han *et al*., 2001b). As a result, this leads to slower growth in the summertime when the temperature is higher. On the other hand, *Rhizopus* strains can grow in a temperature range of 14 - 44°C (Liu, 1999). *R. oligosporus*, for example, has been proposed as an efficient fungus for tofu fermentation during the summer because it grows well at temperatures up to 40°C (Han *et al.*, 2003) and provides pehtze of a quality equal to that produced using *A. elegans* (Han and Nout, 2000).

Although a pure culture has been used in pehtze preparation, sufu is typically made under non-sterile conditions, and normally contains 5 - 20% of NaCl for antimicrobial activity. However, various salt-tolerant pathogenic bacteria, such as *Bacillus* spp., can often be detected in sufu products in spite of the pasteurization process that is applied to most commercial sufu (Brewer, 2000; Han *et al*., 2001b). High levels of such bacteria can particularly be found after prolonged pehtze incubation and can produce endotoxins in sufu products (Ashraf *et al*., 1999). These contaminants are a potential hazard for the consumer. Consequently, the microbiological safety of tofu is of great concern in sufu manufacturing. Technical solutions have been suggested in some previous reports such as using fresh tofu immersed in an acid-saline solution and then subjected to pasteurization using dressing mixtures with low microbiological load to maximize the sanitation of the process (Ashraf *et al*., 1999; Liu, 1999; Han *et al*., 2001a). However, none of these proposals has been found to be practical in the sufu production process.

The aims of this paper are to propose better conditions for tofu and pehtze production in order to achieve a better quality of pehtze and to decrease the risk of bacterial contamination. Our experiment was divided into three main parts: 1) isolation and characterization of *Rhizopus* spp. – *Rhizopus* spp. with acidic protease was initially characterized, and the effect of pH on protease, lipase, and α-amylase enzyme activity of isolated *Rhizopus* spp. was explored; 2) optimization of the tofu preparation process – the tofu production process was optimized, and the effects of soy protein coagulants on properties of coagulated tofu were discussed; 3) determination of pehtze preparation – the effect of temperature on the growth of selected *Rhizopus* spp. was determined and finished pehtze prepared under these conditions was microbiologically investigated.

Materials and Methods

Isolation and characterization of Rhizopus spp.

Fungal strains

The strains of *Rhizopus* spp. were screened and selected from fermented tofu obtained from a sufu manufacturer in Thailand, using dilution plate method. The standard fungal culture, *R. oligosporus* (TISTR 3001), was obtained from the Thailand Institute of Scientific and Technological Research.

Morphological characterization of Rhizopus spp.

Rhizopus strains were isolated by enzymatic activity assay. The selected *Rhizopus* strains were stocked in agar slants at 4ºC for further use. Identification of *Rhizopus* spp. was carried out based on microscopic morphology and genetic study.

Genetic study of isolated Rhizopus spp.

To identify the species of new isolated *Rhizopus* spp., the polymerase chain reaction (PCR) method was performed with fungal genetic data (White *et al.*, 1990). After plating fungi on a potato dextrose agar (PDA) plate for 7 days at 30ºC, DNA was extracted using the hexadecyltrimethylammonium bromide (CTAB) method (Zhou *et al*., 1996). PCR was then carried out

using ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS4 (5'TCCTCCGCTTATTGATATGC3') primers, which are specific to ribosomal RNA nucleotide sequences and can distinguish fungal species belonging to the same genus (White *et al*., 1990). Automated DNA sequencing of purified PCR products was performed to clarify nucleotide sequences. The species of isolated fungi were identified by comparing DNA sequences with those in the fungal nucleotide database, NCBI (*www.ncbi.com*), and then matching those with 100% similarity.

Biochemical characterization of isolated Rhizopus spp.

Preparation of the crude enzyme sample

The crude enzyme preparation method was modified from Kanlayakrit (1987). Each *Rhizopus* strain was separately inoculated on autoclaved wheat bran medium (25 g wheat bran, 5 g rice hull, 0.5 g ammonium citrate, 25 ml tap water and 0.5 g tapioca starch, sterilized at 121°C for 30 min) in a 500 ml Erlenmeyer flask and cultivated for 4 days at 30°C. Crude enzyme was extracted by adding 150 ml of tap water to wheat bran medium; this was kept at 4°C for 4 h. The mixture was then centrifuged at 6,000 rpm for 20 min. The supernatant was stored at 4°C until protease, lipase, and amylase activity were assayed.

Assay of protease enzyme activity

The protease assay was modified from the method of Kanlayakrit (1987). One ml of enzyme solution was incubated with 5 ml of 1.2% casein in 0.05 M lactate buffer (pH 4.0), 0.05 M phosphate buffer (pH 7.0) for acid and neutral protease, and then pre-incubated at 37°C for 10 min. A 1 ml crude enzyme sample was added, well shaken, and incubated at 37°C for 10 min. The reaction was terminated by adding 5 ml of 0.44 M trichloroacetic acid (TCA), and then incubating at 37°C for 30 min. After filtered the mixture, 5 ml of 0.5 M Na_2CO_3 was added to 2 ml of the filtrate and was shaken and then 1 ml of Folin reagent was added and followed by incubation at 37°C for 30 min. The absorbance of the solution was measured at 660 nm. One unit of protease activity was defined as the amount of tyrosine in μg that was liberated per min at 37°C.

Assay of α-amylase enzyme activity

The α-amylase activity was assayed using a colorimetric method, as described in Kanlayakrit (1987).

Assay of lipase enzyme activity

Lipase activity was assayed using the method of Yamada *et al.* (1962) and Khayami-Horani (1996).

Tofu preparation and characterization

Preparation of soy milk

Soybeans purchased from a local market were washed and soaked in water for 6 - 8 h at 30 - 35°C. After again well washed the soaked beans were disintegrated with water in a grinder to obtain soybean milk in which the content of total soluble solids was controlled at about 8.0 - 9.0°Brix (Prutsirisombat, 1998). Soy milk was then separated and boiled at 90 - 95°C for 10 min with frequent stirring.

Preparation of tofu cubes with different coagulants

Soy milk was cooled down to 70 - 75°C and coagulant was then added choosing from either 0.5% w/v acetic acid, 1.0% w/v calcium sulfate, or 1.0% w/v magnesium sulfate. The mixture was vigorously stirred in order to obtain a smaller precipitate and then set aside for 10 - 15 min to complete coagulation. The coagulum was poured onto tofu trays (16 x 19 x 6 cm^3/tray) and then covered with semi-wet cheese cloth. The curd was pressed with a weight of 20 g/cm^2 on top for 20 min, followed by 30 g/cm^2 for 20 min, to eliminate excess water (soy whey). Prior to pehtze making and textural analysis, tofu was cut into cubes (2 x 2 x 2 cm). Tofu prepared from each type of coagulant was then physically and chemically characterized for appearance, textural properties and pH, moisture content, lipid and protein composition, respectively.

Measurement of tofu texture

Pieces of tofu were evaluated by a texture profile analysis using a TA plus texture analyzer (Lloyd Instruments, West Sussex, UK). A cylindrical plunger with 5 mm diameter was used. The test speed of the crosshead and the deformation distance were set at 60 mm/min and 50%, respectively. Textural parameters, including hardness and elasticity of tofu, were measured.

Measurement of tofu moisture

Moisture content was determined according to AOAC (1990).

Measurement of protein content

Protein content was determined according to AOAC (1990).

Measurement of lipid content

Total lipid content was determined according to AOAC (1990).

pH measurement

A 2 g tofu sample was homogenized with 18 ml of distilled water. The pH of tofu and pehtze were measured using a digital pH meter.

Statistical analysis of data

All measurements were performed in three replicates and reported as means ± SD. ANOVA was conducted using SPSS software. The significance was determined at $P \leq 0.05$ levels. Subsequently, Duncan's new multiple range test was applied for comparison among treatment means.

Pehtze preparation and characterization

Preparation of pehtze

Tofu cubes were inoculated with selected *Rhizopus* strain by dropping 0.3 ml of spore suspension (approximately 10^7 spores/ml). The inoculated tofu pieces were placed in plastic trays (Chou and Hwan, 1994; Lu *et al*., 1996) and cultivated in an incubator at controlled temperatures of 33 and 40°C with adequate aeration. Fresh pehtze, overgrown with *Rhizopus* mycelium, was obtained after incubation for 16 - 24 h. The pehtze was stored at 4°C for microbial analysis.

Microbiological analysis

Sampling and sample treatment for microbiological assay

A representative 100 g sample of prepared pehtze was aseptically weighed and then homogenized for further microbial analysis.

Analysis of Bacillus cereus

Blended pehtze was prepared and analyzed for enumeration of *B. cereus* as described in the Bacteriological Analytical Manual (BAM, 2001) chapter 14.

Coliform analysis

Coliform analysis in pehtze samples was performed using lauryl tryptose (LST) broth and brilliant green lactose bile (BGLB) broth, according to BAM (2001) chapter 4.

Analysis of yeast and mold

Determination of yeast and mold contamination in prepared pehtze was performed according to BAM (2001) chapter 18.

Results and Discussion

Isolation and characterization of Rhizopus spp.

Effect of pH on protease, lipase and α-amylase enzyme activity of isolated Rhizopus spp.

From colony characterization of *Rhizopus* spp. isolated from molded tofu samples obtained from a sufu factory in Thailand, seven isolates (KUPR1–KUPR7) were observed and determined for protease, lipase, and α-amylase enzyme activity. Normally, *Rhizopus* spp. produce neutral protease, which degrades soybean protein during fermentation and give the sufu smooth texture. However, during the fermentation process, the pH of the system gradually decreases, which could affect the function of this enzyme and might alter the desired sufu quality. Therefore, protease enzymes which work well in acidic conditions, will be beneficial during sufu fermentation without any effects from pH variation. To study the effect of pH focused on the protease and lipase enzyme activity of isolated *Rhizopus* spp. assays were performed under pH 4.0 and 7.0.

At pH 4.0, compared with the standard fungal species, *R. oligosporus* TISTR 3001, only KUPR4 isolate exhibited protease enzyme activity of 16.50 unit/ml. At pH 7.0, KUPR6 presented the highest protease activity of 3.93 unit/ml; next were KUPR5 and KUPR2, with protease activity of 3.74 and 3.36 unit/ml, respectively. However, at pH 7.0, *R. oligosporus* TISTR 3001 still contained the highest protease activity of 5.79 unit/ml. This could indicate that at pH 7.0, which is the normal condition for tofu manufacturing in the sufu industry and the TISTR 3001 strain would be the most suitable fungus to use. For lipase activity, KUPR6 showed the highest level (22.33 unit/ml) at pH 4.0 while both KUPR3 and KUPR4 showed the highest lipase activity (27.48 unit/ml) at pH 7.0. Nevertheless, our novel protocol of producing pehtze proposed using the KUPR4 strain; this is the only one that can produce acid protease, showing the highest protease activity at pH 4.0. Protease activity plays a major role in degrading soybean protein during sufu ripening, thus liberating free amino acids and bringing about the unique sufu flavor plus smooth texture. In particular, glutamic acid is associated with the highly appreciated palatability of sufu products (Han *et al*., 2003).

Together with preparing tofu under moderately acidic conditions, the risk of microbial contamination can be reduced during pehtze production. Although the lipase activity of KUPR4 was not the highest, it was equal to that of the standard fungus (*R. oligosporus* TISTR 3001). The implication is that

Table 1. Chemical and physical properties of tofu samples with various coagulants

Tofu type	Chemical properties (mean ± SD, in triplicate)				Physical properties (mean ± SD, in triplicate)	
	Moisture (%)	Protein (%dry basis)	Lipid (%dry basis)	pH	Hardness (N)	Elasticity (mm)
A-tofu	73.38^{a}± 0.60	53.71^{a}± 0.72	27.68^{a}± 0.20	4.65–4.66	3.62^{a}± 0.23	5.31^{b}± 1.77
AC-tofu	65.84^{b}± 0.56	50.11^{b}± 0.89	22.42^{d}± 0.53	5.06–5.09	2.73^{b}± 0.04	4.41^{b}± 0.29
C-tofu	72.21^{a}± 0.67	46.68^{c}± 0.15	23.11^{c}± 0.09	5.94–5.95	2.06^{c}± 0.11	5.62^{b}± 1.63
M-tofu	72.57^{a}± 0.63	51.26^{b}± 0.59	26.53^{b}± 0.39	5.93–5.95	1.32^{d}± 0.02	7.99^{a}± 2.24

Different superscript letters within the same column represent significantly different values at $p \leq 0.05$; numbers within the same column containing the same superscript letters represented no significant difference at $p > 0.05$. Physical and chemical properties were compared independently.

this level of lipase activity (13.74 unit/ml) should be sufficient during sufu ripening to hydrolyze crude lipids and release free fatty acids (FFA), di- and monoglycerides (Hwan and Chou, 1999; Moy *et al.*, 2012). The advantage of free fatty acids is that when they react chemically or enzymatically with alcohol added during sufu ripening. Fatty acid esters and other volatile compounds are what cause the unique flavor of sufu products (Steinkraus *et al.*, 1996; Moy *et al*., 2012).

To determine α-amylase enzyme activity, all 7 strains of *Rhizopus* spp. and the standard *R. oligosporus* TISTR 3001 were tested. The KUPR7 strain exhibited the highest α-amylase enzyme activity, 37.83 unit/ml, compared to the reference *R. oligosporus* TISTR 3001, which contained amylase activity of 38.58 unit/ml. This enzyme activity plays a major role in hydrolyzing the starch of soybeans into oligosaccharides, di- and tri-saccharides, which can then be hydrolyzed completely into glucose by glucoamylase (Whitaker, 1994). The salt-tolerant yeast grows and converts the sugars into alcohol and organic acids, while the bacteria also help produce organic acids, amines, and esters creating more sophisticated flavors (Steinkraus *et al.*, 1996). As a result, because of the highest protease activities at pH 4.0, *Rhizopus* sp. KUPR4 was chosen for use in the further preparation of pehtze to achieve hygienic pehtze.

In addition, species verification of *Rhizopus* sp. KUPR4 was performed using polymerase chain reaction (PCR) technique (White *et al*., 1990). Since the nucleotide sequences of nuclear and mitochondrial ribosomal RNA (rRNA) – especially in the internal transcribed spacer region (ITS) and the intergenic spacer of nuclear rRNA repeat units – are different among species in the same genus of organisms, specific primers for rRNA genes can be used to easily identify the species or sub-species of fungi by showing differences in the nucleotide level (White *et al*., 1990). In this study, by using ITS1 and ITS4 specific primers, *Rhizopus* sp. KUPR4 was identified as *Rhizopus microsporus* var. *microsporus.*

Optimization of tofu preparation process

Effect of soy protein coagulants on properties of tofu

Tofu is perhaps the most important raw material used in the production of pehtze and sufu. The quality of tofu largely depends on the types of coagulants, so this processing step is the most important step in tofu preparation (Liu, 1999). Various kinds of soy protein coagulants were used in the tofu-making process, including calcium sulfate, calcium chloride, and magnesium chloride. Each type of coagulant requires a different optimum concentration to produce the unique characteristics of tofu (Sun and Breene, 1991; Liu, 1999). In this study, the previously mentioned method of producing tofu was modified (Prutsirisombat, 1998) to produce four kinds of tofu by using different coagulants: C-tofu, A-tofu, M-tofu, and AC-tofu. A-tofu is tofu coagulated using 0.5% w/v acetic acid, C-tofu is tofu coagulated using 1.0% w/v calcium sulfate, and M-tofu is tofu coagulated using 1.0%w/v magnesium sulfate. Due to the different mechanism of gel formation in tofu, AC-tofu was separately prepared by using a combination of coagulum from 1.0% w/v calcium sulfate mixed with coagulum from 0.5% w/v acetic acid in a ratio of 1:1 w/w. Then, the tofu properties including pH, moisture content, lipid and protein composition, textural properties (hardness and elasticity) and appearance were investigated.

Table 1 presents the chemical and physical properties of all prepared tofu. Calcium sulfate-coagulated tofu had the best appearance. The texture was smooth, compact and firm (but not hard), characteristics, which provided the right amount of chewiness and an overall excellent sensory evaluation. In contrast, tofu made from acetic acid presented a rough texture and had a stronger, more sour flavor and distinct odor. Using magnesium sulfate as a coagulant resulted in tofu with a very smooth texture but it was too compact and springy. Similar results were previously reported on the texture of tofu coagulated with 13 different coagulants. It was found that using 0.4% w/w calcium sulfate provided a better yield

Table 2. Comparison of *Rhizopus* sp.KUPR4 growth at 33°C and 40°C on tofu samples with various coagulants

Temperature (°C)	Tofu type	Incubation period(h) [a]					
		0	14	16	18	20	24
33±1	A-tofu	-	-	-	-	-	-
	AC-tofu	-	-	+	++	+++	++++ S
	C-tofu	–	m	+m	++m	++m	++m S
	M-tofu	-	m	+m	++m	++m	++m S
40±1	A-tofu	-	-	-	-	-	-
	AC-tofu	-	+	+++	++++S	++++S	++++S
	C-tofu	-	-	++m	++m S	++m S	++m S
	M-tofu	-	m	++m	++m S	++m S	++m S

a = appearance of fungal growth on tofu
- = no growth of fungi
+= mycelium growth (number of + symbols indicates greater mycelium growth)
m= mucus characteristic
S = spore stage with black color on the tofu surface

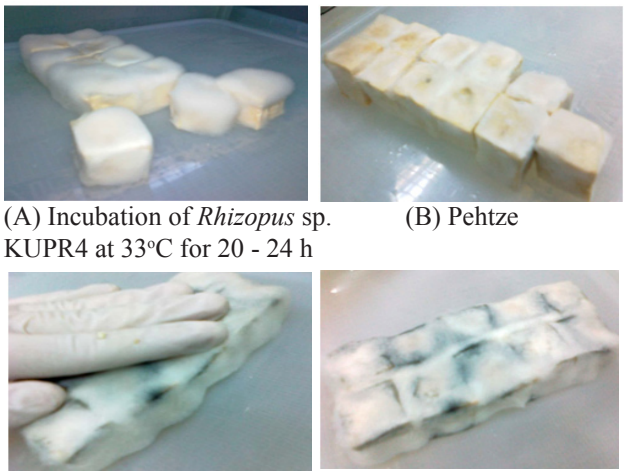
Table 3. Comparison of microbiological aspects of traditional and novel processed pehtze

Tofu selected for pehtze production	Incubation conditions with/without *Rhizopus* sp. KUPR4	Microbiological testing		
		Bacillus cereus (CFU/g)	Coliforms (MPN/g)	Yeast and mold (CFU/g)
C-tofu	0 h	<10	<3.0	<10
	24 h at 33°C without *Rhizopus*	4.8×10^6	N/A	N/A
	24 h at 33°C with *Rhizopus*	1.7×10^6	N/A	N/A
AC-tofu	0 h	<10	<3.0	<10
	24 h at 33°C without *Rhizopus*	<10	N/A	N/A
	24 h at 33°C with *Rhizopus*	<10	N/A	N/A

N/A = no analysis

than using other coagulants and resulted in a firm and smooth tofu (Prabhakaran *et al*., 2006). The texture of magnesium sulfate-coagulated tofu was far less hardness than of other types of tofu resulting from the different mechanisms of cross-linking between protein molecules and calcium ions or magnesium ions. This causes tofu to form a loose or compact network (Wang and Hesseltine, 1982). The pH of A-tofu and AC-tofu was not much different; about 4.66 and 5.09, respectively.

The texture of tofu is a primary concern. By vigorously stirring (180 rpm) at 70 - 75ºC, the smooth texture of AC-tofu was obtained. Because of its superior physical properties, taste, texture, appearance, and moderate acidity, AC-tofu was chosen for further production of hygienic pehtze made with *Rhizopus* sp. KUPR4. AC-tofu also presented the significantly lowest moisture content (65.84%), together with a smooth texture as shown in Table 1. As a result, there was no need for the oven-drying step before making pehtze. This presents an advantage over other kinds of tofu in reducing the number of steps involved in pehtze processing and the possibility of bacterial contamination. As bacteria can grow well in a moist environment, this level of moisture is still suitable for fungal growth in a solid-state fermentation system, which is normally confined to 65 - 75% (Krongtaew, 2004). Moreover, the advantage of the acidity of tofu is that it decreases the potential for contamination by pathogens, e.g. toxin-producing bacteria during the tofu preparation process, since normally these bacteria can grow well at pH 5.5 - 7.0 (Bell *et al*., 2005).



(A) Incubation of *Rhizopus* sp. KUPR4 at 33°C for 20 - 24 h (B) Pehtze

(C) Incubation of *Rhizopus* sp. KUPR4 at 40°C for 20 - 24 h (D) Pehtze with sporulation

Figure 1. The growth of *Rhizopus* sp. KUPR4 on cubes of tofu made from a mixture of coagulum from 1.0% w/v of calcium sulfate and 0.5% w/v of acetic acid (AC-tofu) at 33°C (A and B) and 40°C (C and D) for 20 - 24 h

Determination of pehtze preparation

Effect of temperature on the growth of Rhizopus sp. KUPR4

In the pehtze production step, *Rhizopus* sp. KUPR4 was inoculated onto tofu prepared from different coagulants and incubated at 33°C and 40°C to discover the optimal growing temperature. It was shown that this fungus could grow well on AC-tofu at 33°C for 20 - 24 h (Table 2). Alternatively, it could grow faster at 40°C for 16 - 18 h. Figure 1 shows the growth of *Rhizopus* sp. KUPR4 on AC-tofu after incubation at 33°C and 40°C for 20 - 24h.

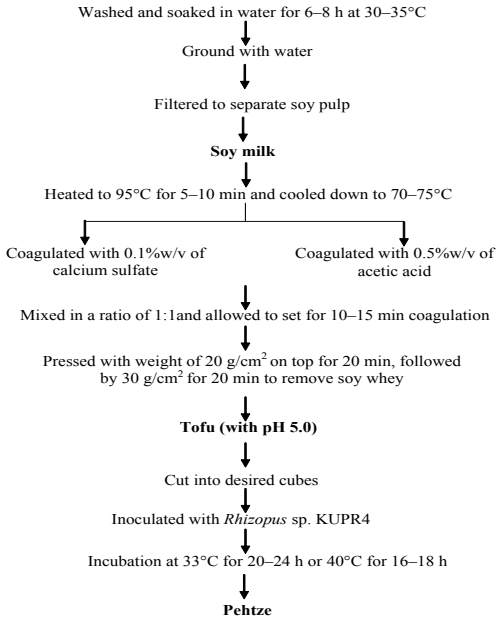


Figure 2. Flow chart of proposed novel conditions for tofu and pehtze preparation

These results indicate that *Rhizopus* spp. can be used as a potential pure culture for pehtze-making in tropical countries (Han *et al.*, 2003). Occasionally, a black appearance may be detected on pehtze due to the conidial stage of *Rhizopus* spp. (Han, 2003). Therefore, to avoid the black sporulation of *Rhizopus* spp., the fungus should be harvested before the mold starts to sporulate. In other words, the cultivation time and the culture conditions need to be carefully monitored.

Microbiological assay of prepared pehtze

The microbiological contamination of prepared pehtze using *Rhizopu*s sp. KUPR4 under moderately acidic tofu conditions was determined and compared between C-tofu with pH 5.95 and AC-tofu with pH 5.09 (Table 1). The results obtained after 24 h incubation at 33°C with and without *Rhizopus* sp. KUPR4 showed that pehtze prepared from a tofu sample with pH 5.09 exhibited <10 CFU/g of *Bacillus cereus* whereas >10^6 CFU/g of *Bacillus cereus* was detected on pehtze prepared from a tofu sample with pH 5.95 (Table 3). This implies that commercial tofu, with a pH generally in the range of 6.0 - 7.0, has a high risk of bacterial contamination. Although the pasteurization process is typically performed during sufu manufacturing to destroy live microbes and mold, some microbial spores, toxins, and mycotoxins might still be detected because they are not certain to be eliminated by the pasteurization process. Based on microbiological assays of most commercial sufu in China and The Netherlands, the level of *B. cereus* contamination is a potential hazard for consumers; poisoning by enterotoxins from *B. cereus* could be harmful (Han *et al*., 2001a). Therefore, from a food safety point of view, it is better to ensure that all steps of the sufu production process are safe, especially during pehtze and sufu incubation periods. The present results confirm that these novel conditions for tofu and pehtze preparation, using *Rhizopus* sp. KUPR4 or *Rhizopus microsporus* var. *microsporus* (as summarized in Figure 2), could help reduce the risk of microbial contamination during pehtze production.

Conclusion

To address food safety concerns, the proposed process can serve as an alternative method of pehtze production on an industrial scale in order to prevent bacterial contamination of pehtze and sufu products, an all-too-common occurrence that has an adverse impact on both the psychology and health of the consumer. More importantly, the entire preparation process should be conducted under conditions of good hygiene and sanitation. Tofu and pehtze produced using these novel techniques will be safer and have less microbial contamination.

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