國立臺灣大學生物資源暨農學院食品科技研究所

碩士論文

Graduate Institute of Food Science and Technology
College of Bioresources and Agriculture
National Taiwan University

Master Thesis

以台灣式製程製備豆腐乳時風味物質組成及抗致突變性之變化

Flavor Content and Antimutagenic Activity of Sufu Prepared with Taiwanese Manufacture Process

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中華民國 98 年 7 月

July, 2009

中文摘要

豆腐乳 (Sufu) 是軟質乾酪狀由黃豆凝結塊(豆腐)製成之東方發酵黃豆食品。台式豆腐乳之釀造過程包括先製備鹽漬豆腐塊，以 *Aspergillus oryzae* 為發酵之豆米麴，然後再將鹽漬豆腐塊浸漬於麴醪 (豆米麴加糖水) 中發酵而成。本研究主要在探討台式豆腐乳發酵過程中 (揮發性成分，醣類與有機酸組成分) 與抗致突變活性變化之情形。

結果顯示隨著發酵時間增加，大部分揮發性成分之含量亦隨之上升，尤其是醇類及酯類。豆腐乳樣品中共鑑定出 90 種揮發性成分，其中包括 22 種醇類，22 種酯類，21 種醛類，10 種脂肪酸，9 種酮類與 6 種其它成分。

未發酵之豆腐中主要之醣類為水蘇糖，棉子糖，蔗糖，葡萄糖與果糖。而豆米麴醪中除發現上述之醣類外還含有麥芽糖與半乳糖且有大量之棉子糖與葡萄糖。大致而言，水蘇糖、棉子糖及蔗糖含量隨發酵時間之增加而減少，但單醣含量則隨發酵時間增加而上升，發酵 46 天後，豆腐乳與豆米麴中以葡萄糖之含量最多。

在發酵 8 天後豆腐乳與豆米麴中檢測出四種有機酸，包括草酸，乳酸，醋酸及檸檬酸，其中以醋酸含量最高。大致而言，除檸檬酸外，豆腐乳中有機酸含量皆於發酵第 8 天達到最高。隨著發酵時間增加，豆米麴中之乳酸，醋酸及檸檬酸含量也隨發酵時間之延長而增加，但草酸則呈現下降之趨勢。此外，豆腐塊對 4-NQO (4-nitroquinoline N-oxide) 與 DMAB (3, 2-Dimethyl-4-amino-biphenyl hydrochloride) 所呈現之抗致突變活性隨著發酵時間之延長而增加。

關鍵字: 豆腐乳，豆米麴，揮發性成分，醣類，有機酸，抗致突變活性

Abstract

Sufu, the oriental fermented product of soy bean, is a soft cheese-like product made from cubes of soy bean curd (tofu). During the Taiwanese manufacturing process of sufu, the salted-tofu cubes and koji mash were first prepared. Fermentation was then proceeded by soaking the salted-tofu cubes in the prepared koji mash. Koji mash was prepared by mixing *Aspergillus oryzae* fermented rice-soybean koji with syrup containing 65% sucrose. In this study, changes in volatile, sugar, organic acid constituents and antimutagenicity of sufu during fermentation were investigated.

A total of 90 compounds of volatiles including 22 alcohols, 22 esters, 21 aldehydes, 10 fatty acids, 9 ketones and 6 other compounds from the sufu samples examined were identified. It was found that as the fermentation period was extended, contents of nearly all volatile fractions increased gradually, especially alcohols and ester.

The main sugars noted in the non-fermented tofu were stachyose, raffinose, sucrose, glucose and fructose. As the fermentation time extended, generally, content of stachyose, raffinose and sucrose decreased, while content of glucose and fructose increased. Glucose was the most abundant sugar detected in the 46-day-sufu.

Four organic acids including oxalic, lactic, acetic and citric acid were detected from the tofu and rice-soybean koji samples after fermentation for 8 days or longer. Acetic acid was the most predominant organic detected. It was generally found that content of these organic acids, except citric acid in the tofu cube increased to the highest point after 8 days of fermentation. The level of lactic, acetic and citric acid also increased in the collected koji as the fermentation time was extended.

Methanol extract of tofu showed antimutagenicity against 4-nitroquinoline-N-oxide (4-NQO) and 3, 2′-dimethyl-4-amino-biphenyl (DMAB). Fermentation was found to enhance the antimutagenicity of tofu extract. Antimutagenicity of tofu extract increased with the extension of fermentation time. Beside, sufu extract exerted a significantly higher ($p < 0.05$) antimutagenicity against DMAB than 4-NQO.

Keyword: sufu, koji, volatiles, sugar, organic acid, antimutagenic

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I. Introduction

Sufu, also known as fu-su, fu-ru, doufurn, tou-fu-ru, Chinese cheese or bean cake, is a Chinese fermented product of soybean. According to "Ben-Tsao Gong-Mu", an ancient Chinese-Botanical Encyclopedia written in the early 16th century, sufu was first invented by Liu An (179-122 B.C.) of the Han Dynasty (Lee, 1916). This fermented product, possessing a characteristic flavor and a relatively high protein content, is widely consumed by Chinese people as an appetizer.

Sufu can be notable according to the different manufacturing process. Taiwanese sufu is classified as the enzymatically ripened sufu. Different from the preparation of Chinese sufu and without the growth of fungus on the tofu cubes, salted tofu cubes and koji mash were first prepared during the manufacture of Taiwanese sufu. According to Huang (2008), koji is prepared by inoculating *Aspergillus oryzae* on steamed rice-soybean and not on the tofu cubes. Fermentation was then proceeded by soaking the salted tofu cubes in the prepared koji mash. Compare with Chinese sufu, fermentation period of Taiwanese sufu is rather short in addition to the difference in the microorganism involved. Additionally, Taiwanese sufu was packed in jar along with the koji-mash containing granules of rice and soybean koji for sale in the market. It generally has lower degree of saltiness with a relatively higher sweetness compared with the Chinese sufu.

As known, freshly molded soybean curd is bland in taste, and the characteristic flavor and aroma of the Chinese sufu gradually develops during the brining and ripening process as described by Wang and Hesseltine (1970). A few studies have been conducted to demonstrate the volatile compounds in various Chinese sufu samples (Chung, 1999; Hwan and Chou, 1999; Chung, 2000; Han *et al*., 2001b). Besides flavor, researchers have also

proved that fermented soybeans exhibit enhanced antimutagenic activity (Kim *et al*., 2000; Park *et al*., 2003). Additionally, Ren *et al*. (2006) reported that antimutagenic activity of sufu varied with locality, ingredients added and manufacturer.

Despite the abundant literature available on the Chinese sufu, no scientific assessment on the aroma, flavor and biochemical changes during the fermentation of Taiwanese sufu has been reported. This study was then conducted in an attempt to provide the detailed information on dynamic changes of flavor, sugar and organic acid content of Taiwanese sufu during fermentation. Additionally, the effect of fermentation on the antimutagenicity of the sufu product was also examined. This information obtained will be useful to optimize the production of Taiwanese sufu and its quality control.

II. Literature Review

1. Introduction

Sufu, the oriental fermented product of soy bean, is a soft cheese-type product made from cubes of soybean curd (tofu). Sufu is a form of processed, preserved fermented soybean food frequently used in Chinese's traditional cuisine (Hesseltine and Wang, 1967). This Chinese fermented-product of soybean is also known as fu-su, fu-ru, doufurn, tou-fu-ru, Chinese cheese or bean cake. According to "Ben-Tsao Gong-Mu" (Botanical Encyclopedia) or Chinese Materia Medica of 1596 by Li Shi-Chin, sufu was first invented by Liu An (179-122 B.C.) in Han Dynasty (Lee, 1916).

It has a pleasing special flavor and texture, and can be used as seasoning in an average person's daily life. Sufu is oftenly consumed as an appetizer or a side dish, e.g. with breakfast rice or steamed-bread. Sufu adds zest to the otherwise bland taste of a rice and flour meal. Sufu is highly nutritious, known to have rich in free amino acid and free fatty acid and protein content changing from 12% to 17%. (Wang and Hesseltine, 1970; Su, 1980). Sufu is a highly flavored, so it would be expected to be suitable for use in western countries as a healthy, non-cholesterol food from plant origin (Han *et al*., 2001b). The Chinese people consider it as a health food because it is made from soybeans and is an easily digested and nutritious protein food. Table 1 summarizes some major chemical constituents of Chinese sufu.

Table 1. Proximate composition of commercial Chinese sufu (Han *et al*., 2001b)

Component	Content[a]
Moisture (g)	58–70
Crude protein (g)	12–17
Crude lipid (g)	8–12
Crude fibre (g)	0.2–1.5
Carbohydrate (g)	6–12
Ash (g)	4–9
Calcium (mg)	100–230
Phosphorus (mg)	150–300
Iron (mg)	7–16
Thiamin (V_{B1}) (mg)	0.04–0.09
Riboflavin (V_{B2}) (mg)	0.13–0.36
Niacin (mg)	0.5–1.2
V_{B12} (μg)	1.7–22
Food energy (KJ)	460–750

2. Manufacturing process of sufu

Preparation of sufu can be divided into two category: Chinese manufacture process and Taiwanese manufacture process.

2.1 Chinese sufu manufacturing process

The Chinese manufacturing process of sufu can be divided into three steps (Fig. 1): (1) Preparation of soybean curd as the raw material, (2) Preparing pehtze (tofu freshly grown with the fungus) (3) Salting and aging (Wang and Hesseltine, 1970；Su, 1980；Nout and Aidoo, 2000).

To prepare tofu, selected soybeans are washed and ground with added water to milky slurry in a miller. The slurries are filtered and pressed to obtain soymilk which obtains soluble proteins, some lipids and other soluble constituents. The milk is then heated to boil and calcium sulfate as well as magnesium sulfate is added to initiate curdling for

precipitating the proteins. The precipitate is pressed to remove excess soy whey. A soft but firm tofu is formed which is then cut into desired sizes.

According to Han *et* al. (2001b), pehtze, the pre-fermented bean curd, is prepared by growing the fungus on tofu (Fig. 2). The spore suspension is inoculated on the surfaces of the tofu with manually operated sprayers. The inoculated tofu is placed, evenly spaced in wooden or plastic trays, the bottoms of which are made of bamboo or wooden strips. They are then kept at room temperature of 12 - 20°C. In spring or autumn, after 3-7 days depending on the temperature there would be growth of white mycelium of the fungus on the surface of the tofu cubes.

The pehtze was placed in an earthen jar in such a way that one layer pehtze was followed by one layer of salt. However, it can also be done by rubbing the surface of pehtze with salt. During the salting, the pehtze absorbs salt and this imparts a salty taste to the sufu. Additionally, the salts retard further growth of mold and the undesirable microbial (Han *et al*., 2001a). Wang and Hesseltine (1970) indicated that the fungal proteases were not extracellular and that they are loosely bound to the mycelium. The added salts enable the enzymes to diffuse into the tofu for substrate degradation as well as releasing the mycelia-bound proteases. During the fermentation, the pehtze was placed in various type of brine solutions according to the flavor desired and depending on the kind of sufu prepared. The enzymes produced by the mold act upon their respective substrates, and are likely that hydrolysis of protein and lipid provide the principal compounds of the mild, characteristic flavor of sufu (Chou *et al*, 1998a).

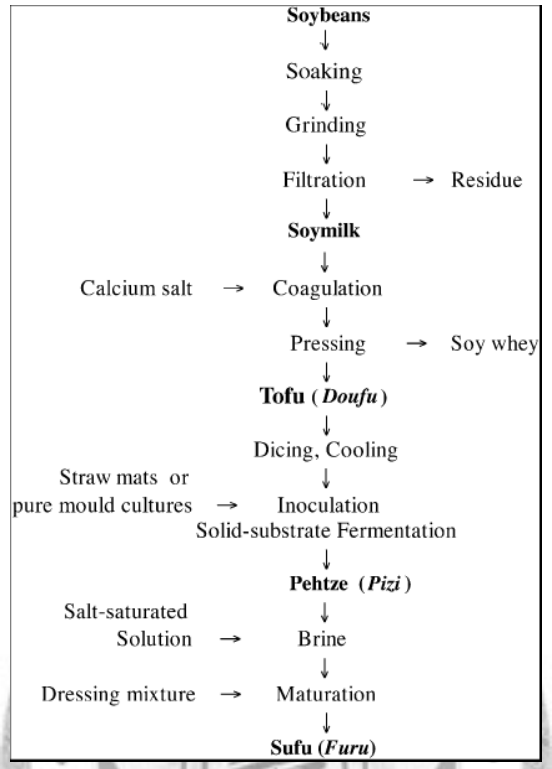

Fig. 1. The schematic diagram for production of Chinese sufu. (Nout and Aidoo, 2000)

Fig. 2 Pehtze (Han *et al*., 2001b)

The differences between various types of sufu are mainly caused by different dressing mixtures are added in salted pehtze during the ripening process. The ingredients of dressing mixture varied with social customs, climate, and locations. The most common dressing mixture used consists of angkak, alcoholic beverage, salt, sugar, flour (or soybean) paste, and some spices. Additional essence can be added into the dressing mixture to supply a special flavor. In Taiwan, *Actinomucor elegans* and *Actinomucor taiwanensis* are the best moulds that are used for pehtze production (Chou *et al*., 1988).

2.2 Taiwanese sufu manufacturing process

Different from the Chinese manufacturing process, three steps are normally involved in the making of Taiwanese sufu (Fig.3): [1] preparation of tofu, [2] preparation of koji mash, and [3] fermentation of tofu cube. Instead of growing fungus on the tofu cubes, salted tofu cubes and koji mash were first prepared (Huang, 2008). Koji is prepared by inoculating *Aspergillus oryzae* on steamed rice-soybean instead of on the tofu cubes. While koji mash was obtained by mixing the prepared koji with syrup that contain ca 65% sucrose. Fermentation of sufu was then proceeded by soaking the salted tofu cubes in the prepared koji mash. During the fermentation period of Taiwanese sufu, it is expected that the hydrolytic enzymes which leached out from the koji into the infusion and then penetrated into the tofu cubes. These enzymes may catalyze the enzymatic degradation of large molecules such as protein, lipid, oligosaccharide in tofu cubes. Compare with Chinese sufu fermentation period, Taiwanese sufu is relatively short.

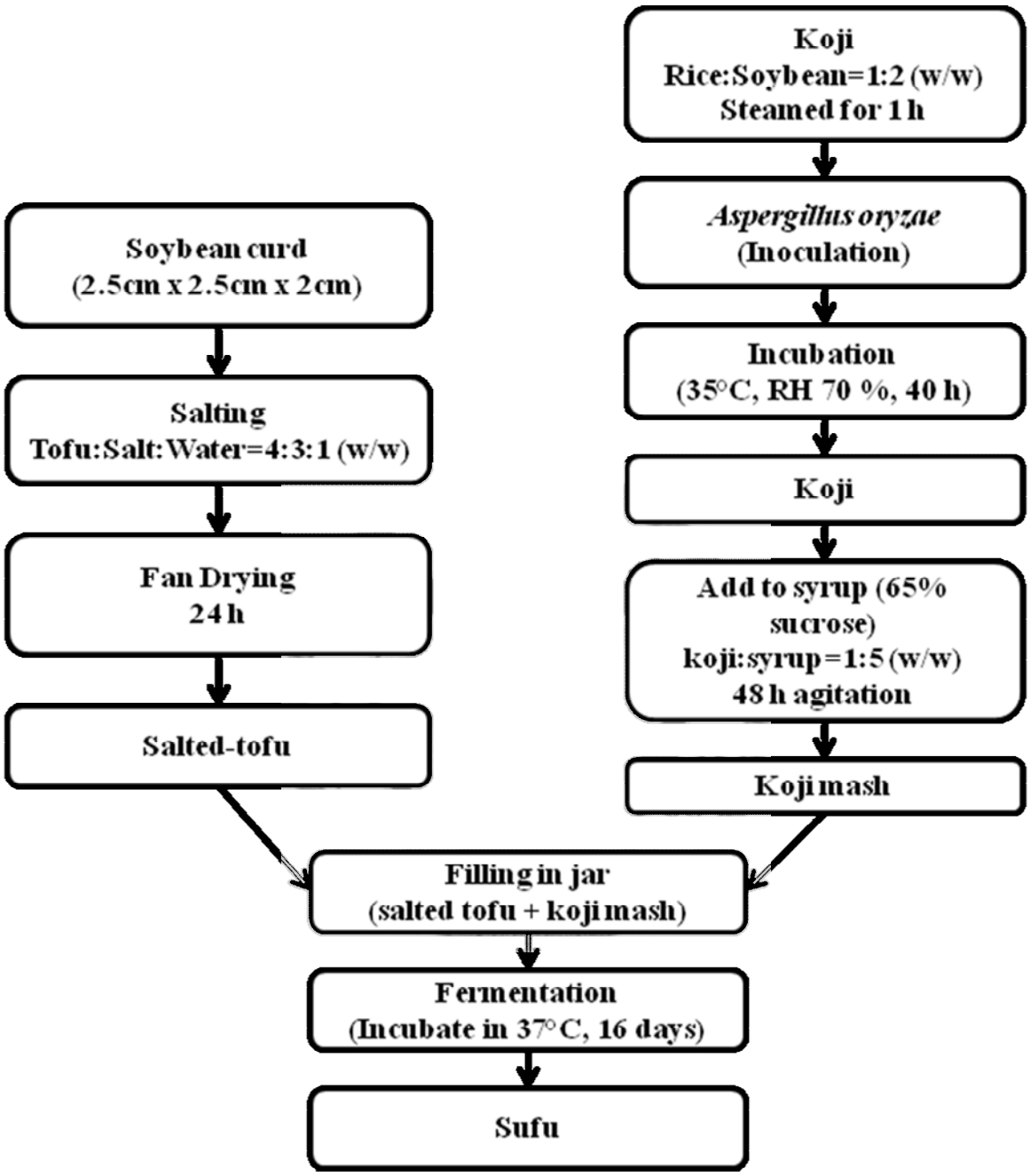

Fig. 3. The schematic diagram for production of Taiwanese sufu (Huang, 2008).

3. Microorganism involved in the manufacture of sufu.

Fermentation of sufu was once thought to be a natural phenomenon. However, presently it is understood that this category of product is result of the filamentous fungus fermentation (Wang and Hesseltine, 1970). In the Chinese manufacture process, the main mold strains, such as *Actinomucor, Mucor* and *Rhizopus.* (Wai, 1929; Su, 1980; Jong and Yuan, 1985) were used as the starter organism. It is suggested that these fungi should possess the ideal characteristics listed below (Han *et al*., 2001b):

- Ability to secrete high-activity of protease and lipase,
- Color of mycelium is either white or faint yellow to guarantee that the final sufu has an attractive appearance.
- Forms enclosure to maintain shape of tofu
- Prevent the formation of strange taste, toxin and also suppress other fungus growth.

On the other hand, *Asp. oryzae*, starter organism of soy sauce fermentation, was involved in the preparation of sufu with Taiwanese manufacture process.

4. *Aspergillus oryzae*

Asp. oryzae belongs to the *Asp. flavus* group (Raper and Fennell, 1965). The name was originally applied to strains used in China and Japan for the fermentation of rice and soy products (Wieklow, 1984).

For more than 2000 years, *Asp. oryzae* has been used in the Orient to produce koji, a complex enzyme preparation for the production of soy-sauce, miso and sake (Barbesgaard et al., 1992). In Europe, *Asp. oryzae* has been used since the beginning of last century in the

production of enzymes for baking and brewing (Barbesgaard *et al*., 1992). Both *Asp. oryzae* and its enzymes are accepted as constituents of food (FAO/WHO JECFA 1988). *Asp. oryzae* is generally regarded as a non-pathogenic fungus (Domsch *et al*. 1980). However, it has been isolated from clinical material in a few cases, but the number of case histories is extremely small (Barbesgaard *et al*., 1992). Hence, in the fermentation industry it is regularly checked that the levels of mycotoxins in the products comply with the requirements of the health authorities (FCC 1981; FAO 1990).

5. Biochemical changes during the fermentation of sufu

Enzymes produced by koji molds, are important for the production of the characteristic flavor, colors and textures of oriental traditional fermented products of soybeans, like soy sauce, miso and sufu (Whitaker, 1978). Enzymes such as proteinase, peptidase, lipase, glutaminase, α-galactosidase and amylase hydrolyze part of the protein, lipid and starch to simpler components during fermentation of sufu (Han *et al*., 2001b; Zhang *et al*., 2007). Li (2009) reported that protease and lipase activities reduced and the activities of amylase remained constant as the Taiwanese sufu fermentation was extended. Some physical properties of Chinese sufu have been investigated by Chou and Hwan (1994). They found that, in general, the hardness of sufu decreased as the fermentation period extended. This was similarly reported by Li (2009) with Taiwanese sufu. Additionally, Li (2009) also described that during fermentation, the color of tofu and infusion changed from ivory white to reddish brown.

Soybean contains oligosaccharides, raffinose and stachyose, which are non-digestible and commonly associated with stomach discomfort and flatulence (Rackis *et al*., 1970). Raffinose and stachyose are α-galactosides of sucrose (Fig. 4) comprised three and

four sugar moieties, respectively, and are indigestible due to the absence of α-galactosidase in the small intestinal mucosa of human beings. As a result, intact oligosaccharides pass directly into the lower intestine where they are metabolized by bacteria that possess the enzyme, resulting in the production of gases (Cristofaro *et al*., 1974). However, fermentation was reported to be more efficient in reducing the content oligosaccharides by inducing the formation of α-galactosidase (Mansour and Khalil, 1995). Additionally, Cruz *et al*. (1981) and Cruz and Park (1982) also reported that raffinose and stachyose, present in soy milk, were totally hydrolysed by α-galactosidase from *Asp. oryzae* secretion.

β-Fructosidase/invertase is another enzyme produced by *Asp. oryzae* that has gained considerable attention. This enzyme is able to catalyze the hydrolysis of glycosidic linkage in sucrose to liberate glucose and fructose (Delavega *et al*., 1991). Cameselle *et al*. (1998) indicated that sucrose, as the carbon source of *Aspergillus niger* in forming organic acids, was hydrolysed very quickly to glucose and fructose by its extracellular enzymes. After two days of fermentation, most of the available glucose was oxidised to gluconic acid which was then hydrolysed to oxalic acid. Marazza *et al*. (2009) also reported that glucose and fructose (products of sucrose hydrolysis) were scarcely detected, and suggests that they were consumed by the microorganism during growth in fermentation of soymilk to form other end products such as organic acids. In the preparation of Korean soy sauce, Lee and Kim (2008) observed that there was a rapid increased in reducing sugar content during the initial fermentation process and indicated that this may be attributed to the action of fungal amylase activity.

During fermentation, microorganisms produce organic acids by hydrolyzing simple carbohydrates with the secreted enzymes as shown in Fig. 5 (Cameselle *et al*, 1998). For example, citric acid produced by *Asp. niger* is carried out through the Krebs cycle with a series of compounds involved in the physiological oxidation of fats, proteins, and carbohydrates to carbon dioxide and water (Kubicek and Rohr, 1986). Acetic, lactic and l-pyroglutamic acid were detected in naturally fermented douche (*Aspergillus*-type Douchi, a Chinese traditional fermented product of soybean), douchi koji and unfermented black soybean (Zhang *et al*., 2007). It was found that the content of these organic acids is higher with douche than the unfermented black soybean. Zhang *et al*. (2007) stated that organic acids were mainly produced during secondary fermentation, rather than during primary fermentation in koji. In *Asp. oryzae*-fermented Korean soybean paste, citric, lactic, malic, acetic and oxalic acid were also identified. Among them, concentration of lactic acid was the highest (An *et al*., 1987).

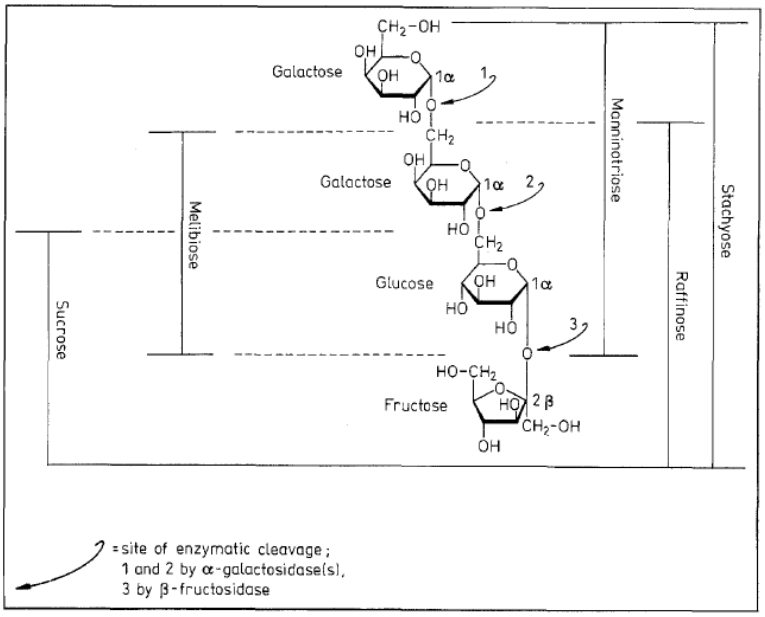

Fig. 4. Structural representation of oligosaccharides (raffinose stachyose and sucrose) (Rehms and Barz, 1995)

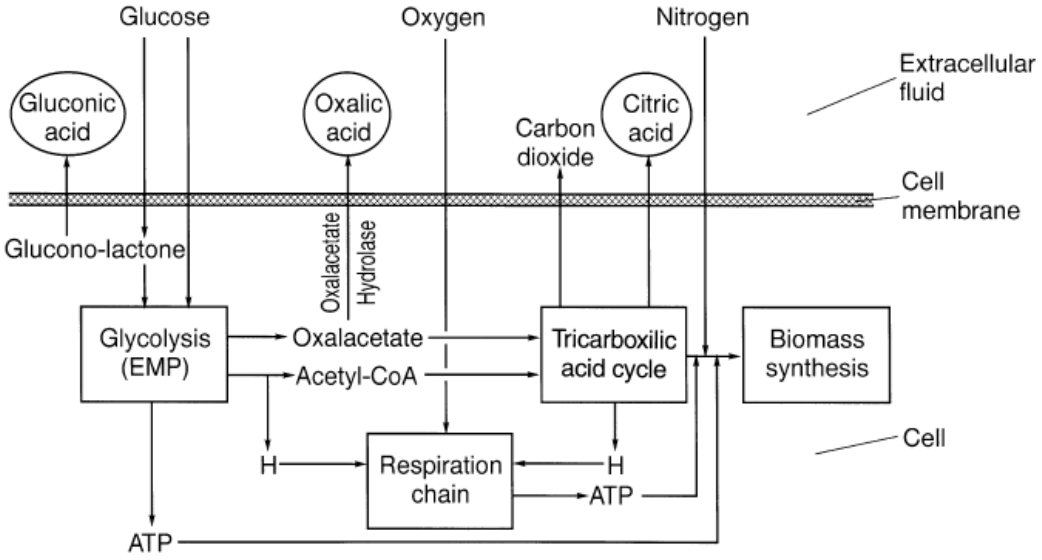

Fig. 5. Metabolic pathway for citric, gluconic and oxalic acid synthesis in *Asp. niger* (Cameselle *et al*, 1998)

6. Fermentation develops characteristic flavor in sufu

Basically, sufu has a salty and unami (sweet and meaty) taste, in addition to its distinctive pleasant fruit odor and alcoholic fragrance. Freshly molded soybean curd is bland in taste, and the characteristic flavor and aroma of sufu gradually develops during the brining and ripening process as described by Wang and Hesseltine (1970). During brining, the salt helps to release mycelia-bound protease and lipase from fungi, making them readily available to penetrate and to digest the protein in the matrix of a tofu (Wang, 1967; Wang and Hasseltine, 1970). Sufu has a peculiar palatable taste and aroma compared to ordinary soybean curd, tofu. Protease and lipases produced by the fungi during the fermentation periods of Chinese sufu generated large amounts of peptides and amino acids, as well as fatty acids (Steinkraus, 1983).

During the fermentation period of Chinese sufu, Wai (1968) and Chou and Hwan (1994) observed that soybean proteins were digested to peptides and amino acids by the catalytic action of proteases produced by the mold. It was also noted that during the Chinese sufu fermentation, contents of total nitrogen and amino nitrogen increased (Chou and Hwan, 1994). In addition, Li (2009) observed that as the Taiwanese sufu fermentation period extended, the content of free amino acid and amino nitrogen of the tofu also increased. Apparently, these are all the results of protein hydrolysis. On the other hand, Chou and Hwan (1994) observed that lipid content fluctuated during the fermentation process of Chinese sufu prepared with either *Act. taiwanensis* or *Act. elegans*, where the free fatty acid content increased and then decreased as the fermentation extended. However, Li (2009) reported that free fatty acid of the Taiwanese sufu increased gradually as the extension of fermentation period.

The fermentation process provides a pool of substrates for further biological and chemical reactions. The composition of the substrate in sufu fermentation is rather simple and it is likely that the hydrolytic product of protein and fat provides the principle flavor constituents of sufu. The flavor and aroma of sufu is further enhanced by the added wine, red rice, or fermented rice mash that contribute esters, organic acids, sugars, and alcohols. (Hesseltine and Wang, 1967). In fact, the cooked soybean can still retain a strong beany flavor, however fermented products contain much less of the undesirable flavor (Macrae *et al*., 1993).

The complex flavor of Chinese sufu was reported to contain esters, alcohols, ketones, aldehydes, pyrazines, phenols and other volatile compounds (Chung, 1999; Hwan and Chou 1999; Han *et al*., 2001b). The major volatile compounds detected in Chinese sufu

are ester and alcohol groups. A few researchers reported that when alcohol was added as a part of the dressing mixture in the fermentation period, it would react with the free fatty acid substrates to produce more desirable aroma in sufu (Wang and Hesseltine, 1970; Su, 1986). In addition, the presence of the ethanol is important in the qualitative and quantitative productions of volatile components during the aging period of Chinese sufu (Hwan and Chou, 1999). Hence, with the ample supply of ethanol in the final stage, most acids underwent esterification and formed ethyl esters (Wang and Hesseltine, 1970). Many of the volatile compounds identified posses the characteristic of the fruity odour such as ethyl propionate (rum-like), ethyl butyrate (pineapple-like), ethyl benzoate (floral-fruity), ethyl oleate (coconut-like, sweet), ethyl linoleate (sweet, creamy, mild), and ethyl 3-phenlpropionate (cool, prune-like) (Chung, 2000).

Alcohol is another large class of volatile compounds in sufu's flavor (Hwan and Chou, 1999; Chung, 1999; Han *et al*., 2001b). Some of the alcohols found in soybean or fermented soybean products are also observed in sufu. 1-propanol, 1-pentanol, 1-hexanol and 1-octen-3-ol noted in sufu were previously reported in soybean milk (Kobayashi *et al*., 1995). Besides, 2-methyl-1-propanol, 3-pentanol, 3-methyl-1-butanol, benzenemethanol, benzeenethaol, phenol, and 4-ethylphenol were also reported in soy sauce (Lee and Kwok, 1987). While 2-methoxy-4-vinylphenol and 4-vinylphenol exhibiting the cooked soybean flavor, were detected in all the fermented soybean products (Greuell, 1974). Most alcohols such as 1-propanol (alcoholic, sweet), 1-butanol (sweet, balsamic), 1-pentanol (sweet, balsamic), 1-hexanol (fruity, aromatic), 4-ethylphenol (woody, phenolic, sweet), 2-methoxy-4-vinylphenol (spicy, clove-like), 4-vinylphenol (vanilla-like), etc were detected in sufu that have characteristic aromas (Aldrich, 1998). A high concentration of alcohol

such as ethanol, 1-propanol, 2-methyl-1-propanol and 1-hexanol was noted in Chinese sufu (Chung, 1999).

Aldehydes, pyrazines, ketones, sulfur-containing compounds and furan were also detected in Chinese sufu. Aldehyde group includes pentanal, benzaldehyde and n-hexanal were found in sufu (Chung, 1999; Hwan and Chou, 1999; Chung, 2000). Pentanal and benzaldehyde were considered to be the desirable aromas (Aldrich, 1998), while n-hexanal contributed to undesirable green aroma to both the soybean milk and textured soy protein (Kobayashi *et al*., 1995). Since Chung (1999) reported that n-hexanal detected in sufu, it would be expected to give a greenish background flavor. Aldehydes compounds could be produced by lipid oxidation and degradation (Ames and Macleod, 1984). Both pyrazines and sulfur-containing compounds were significant to the aroma of food product. Pyrazines is generally described to have nutty aroma (Ho and Carlin, 1989). Pyrazines could be produced with the presence of fungal enzymes to disintegrate the tofu matrix and to release the necessary substrates for chemical reactions during the aging of sufu (Chung, 1999). Chinese sufu were found to contain two common sulfur-containing compounds including 2-(methylthio) ethanol and 3-(methylthio) propanol (Chung, 1999; Chung 2000). 3-(Methylthio) propanol was also reported in soy sauce (Nunomura *et al*., 1984; Lee and Kwok, 1987). Its aroma was described to have a powerful sweet soup or meat-like odor and flavor even in high dilution (Burdock, 1994). Ketones could be generated by fungal actions or by Maillard reaction (Fors, 1983). 3-Hydroxy-2-methyl-4*H*-pyran-4-one, having a malt, toasted flavor (Aldrich, 1998) was the most concentrated component within the ketone group and among all Chinese sufu studied by Chung (1999).

7. Changes in the constituents associated with functional properties during the fermentation of sufu

In recent years, some functional properties of sufu have also been investigated. Yin *et al*. (2004) reported that the levels of aglycones increased, while the corresponding levels of glucosides decreased during the fermentation Chinese sufu. A similar phenomenon was reported by Huang (2008) who observed that fermentation caused a marked increase in the content of aglycone (daidzein, glycitein and genistein), and a significant reduction in the content of malonylglucoside and β-glucoside isoflavone in Taiwanese sufu. Both studies revealed that the changes in the isoflavone composition were related to the activity of β-glucosidase during sufu fermentation (Yin *et al.*, 2004; Huang, 2008). Isoflavone, a biologically active substance in soybean and its products (Watanabe, 2001), was found to exhibit antimutagenic and antitumorial activities (Jing and Waxman, 1995). Researchers have also proved that fermented soybeans exhibit enhanced antimutagenic activity (Kim *et al*., 2000; Park *et al*., 2003; Lin and Chou, 2006).

Chinese sufu was also observed to possess antimutagenic and antioxidant activity. Generally, these activities of Chinese sufu differed with locality, ingredients added, and manufacturer (Ren *et al*., 2006). On the other hand, Huang (2008) also reported that DPPH free radical-scavenging activity, Fe^{2+}-chelating ability and reducing power of Taiwanese sufu enhanced as the time of fermentation increased.

III. Material and methods

i. Experimental Design

The objective of this study was to investigate the influence of fermentation on the chemical components of tofu such as volatiles, sugar and organic acid and also its antimutagenicity during the preparation of sufu with manufacture process as shown in Fig. 6.

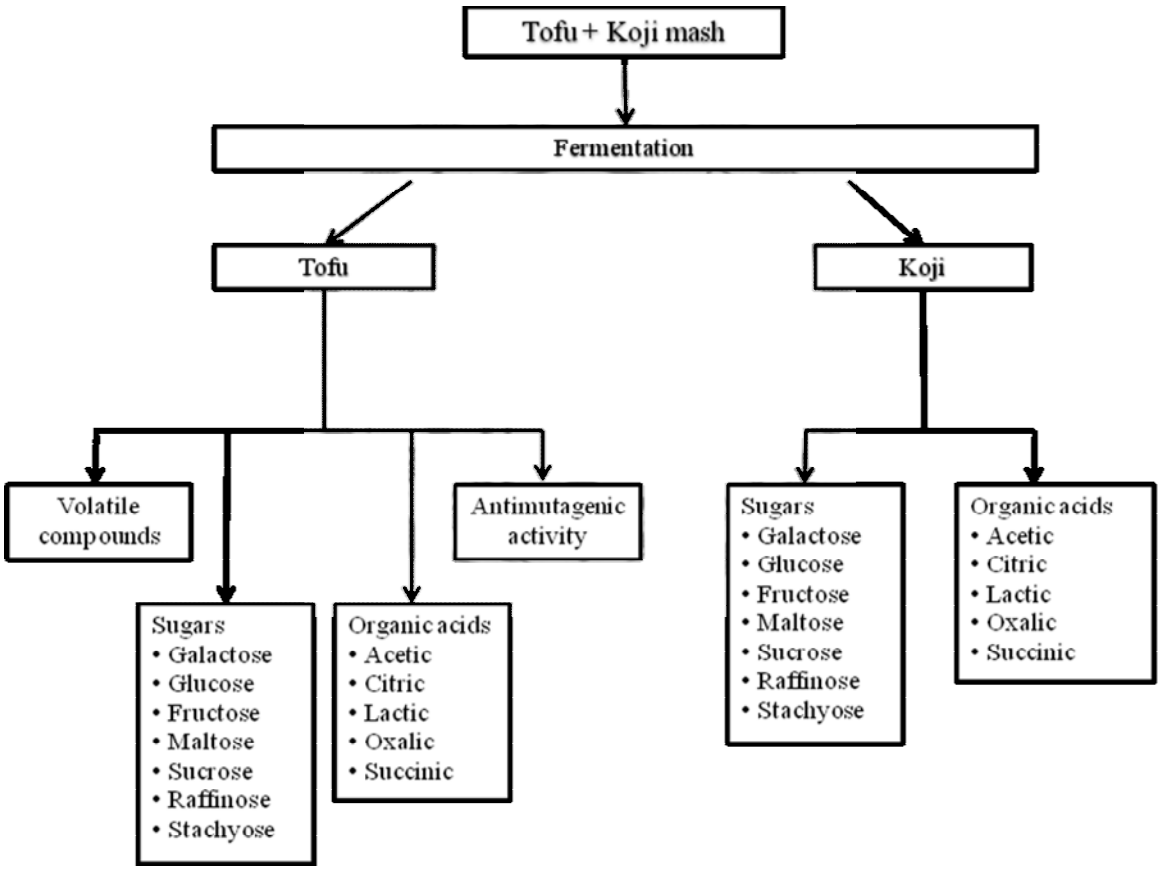

Fig. 6. The experimental design of this study

ii. Materials

1. Bacterial strain

The test strain of *Salmonella typhimurium* was TA100 BCRC 12378 which was obtained from Bioresources Collection and Research Center (BCRC), FIRDI, Hsinchu, Taiwan.

2. Chemicals

2.1 Taiwan Tobacco & Liquor Corporation (Tainan, Taiwan): alcohol

2.2 Acumedia (Michigan): Agar, Nutrient Broth

2.3 Oxoid Ltd. (Basingstoke Hampshire, UK): Oxoid Nutrient Broth No. 2

2.4 Merck (Darmstadt, Germany): Water (LC grade), Dichloromethane

2.5 Wako (Osaka, Japan)Potassium hydroxide (KOH)

2.6 Supelco Analytical (Pennsylvania, USA): Lactic acid, Citric acid, Acetic acid, Sucrose, Maltose

2.7 ABCR (Karlsruhe, Germany): Stachyose

2.8 Chem Service (Wester Chester, USA): D-Fructose, Oxalic acid, Succinic acid, D-Raffinose

2.9 Hayashi Pure Chemical Indusries LTD (Chuo-Ku, Japan): Perchloric acid

2.10 Ferak (Berlin, Germany): Sulfuric acid

2.11 J.T. Baker (New Jersey, USA): Dipotassium phosphate (K_2HPO_4), Di-sodium hydrogen phosphate (Na_2HPO_4), Potassium chloride (KCl)

2.12 Mallinkrodt Baker (New Jersey, USA): Sodium chloride (NaCl)

2.13 Sigma-Aldrich Co. (St. Louis, USA): D-Galactose, D-glucose, 3,2-Dimethyl-4-amino-biphenyl hydrochloride (DMAB), S9 from rat liver

(from male rats Sprague-Dawley), Methanol, Biotin, L-Histidine hydrochloride, Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), Citric acid monohydrate, Ammonium sodium hydrogen phosphate ($NaHNH_4PO_4.4H_2O$), Crystal violet, Ampicilin, Sodium dihydrogen phosphate (NaH_2PO_4), 4-nitroquinoline N-oxide (4-NQO), Dimethyl sulfoxide(DMSO), β-nicotinamide adenine dinucleotide phosphate (β-NADP), D-Glucose-6-phosphate disodium salt (G-6-P), Magnesium chloride ($MgCl_2.6H_2O$)

3. Equipment

3.1 Distillation unit (Buchi, Model 315, Flawil, Switzerland)

3.2 Incubator (Deng Yng, Model DBL.E600, Taipei, Taiwan)

3.3 Freeze Dry System (FreeZone® 4.5, Labconco, Missouri, USA)

3.4 Rotary evaporator (Rotavapor RE111, Büchi Co., Flawil, Switzerland)

3.5 Cooler (EYELA Cool Ace CA-1110, Tokyo Rikakikai Co., Tokyo, Japan)

3.6 Filter System, 0.22μm PVDF (Schleicher & Schuell GmbH, Dassel, Germany)

3.7 Water Deionized system (Millipore Co., Medford, Massachusetts, USA)

3.8 Micro-computer pH meter (Model F-21, Horiba Ltd., Kyoto, Japan)

3.9 Centrifuge (moldel RC5C, Dupont Co., Dover, USA)

3.10 Water bath (moldil E-200. Lauda Co., Darmetadt, Germany)

3.11 Gas chromatography- mass spectrometry, GC-MS:

A. HP-Agilent 5890 series II plus Gas Chromatography-5972 Mass spectrometry

B. Analytical column: DB-WAX (30 m x 0.25 mm i.d., film thickness 0.15 μm)

3.12 High performance liquid chromatography, HPLC:

A. Analytical column: Phenomenex RNM Carbohydrate column (Order no. 00H-0136-K0) (300 x 7.8 mm), Phenomenex Capcell Pak C18 UG-120A column (4.6 mm×250 mm, 5 mm)

B. Solvent delivery system:

Degassing system (Model DG-2410, Sanwa Tsusho Co., Tokyo, Japan)

HPLC pump (880-PU, Jasco Co., Tokyo, Japan)

C. Solvent mixing module (880-30, Jasco Co., Tokyo, Japan)

D. Autosampler (SFD S5200, Bad Honnef, Germany)

E. RI detector (model UV-970, Jasco Co., Tokyo, Japan)

F. Ultraviolet/Visible Detector (model UV-970, Jasco Co., Tokyo, Japan)

G. Data processor (EC2000 data system 1.0, Analab Corporation, Taipei, Taiwan)

iii. Preparation of *Salmonella typhimurium* TA100 inoculum

Freeze dried tester strain were incubated under 37°C in 0.5 % NaCl Nutrient broth for 3~4 days. After the incubation period, sterile wire loop was used to do streaking on the ampicillin plate and incubated for 48 h. A single colony was inoculated from ampicillin plate and grows in a flask of 50 mL Oxoid nutrient broth No. 2. The flask was covered with metal foil and incubated under 37°C and rotation 120 rpm for 16 h. To prepare frozen permanent copies of TA100, 1.0 mL of abovementioned fresh overnight cultures were added to 10 % (v/v) of dimethyl sulfoxide (DMSO) in sterile cryotubes. The mixtures were vortexed until the DMSO was dissolved and distributed evenly. Lastly, the cryotubes were placed upright and stored in -80°C freezer.

Prior to each toxicity, mutagenic and antimutagenic assay, one frozen permanent was used and the remaining culture will be disposed. The frozen cultures were thawed in room temperature. 0.5 mL culture was added to 50 mL Oxoid nutrient broth No. 2 and incubated under 37 °C, rotary 120 rpm and for 16 h prior to use.

iv. Sample preparation

Sufu prepared with Taiwanese manufacturing process. In the present study, Taiwanese sufu manufacturing process was adapted to prepare the sufu as shown in Fig. 3 (Huang, 2008).

1. Preparation of tofu

A soft, cake-like soy-tofu where cut into desired rectangular pieces, approximately 2.5 cm x 2.5 cm x 2 cm. The tofu is transferred to plastic containers, each layer of tofu being sprinkled with a layer of salt with the ratio of 4:1 (w/w) tofu and salt. By adding water,

where at the ratio 4:3:1 (w/w) of tofu: salt: water, thus the final concentration of salt, 25 % were obtained. After 24 h at room temperature, salted tofu cube were air-dried for 24 h.

2. Preparation of koji mash

Soybean and rice with ratio 2:1 (w/w) were soaked in a 60 °C~80 °C hot water for 1 h before steam-cook for 1 h. Koji was prepared by inoculating 22.5 g powder of seed koji of *Aspergillus oryzae*, obtained from 三益釀造化化學, Taichung, Taiwan, in every 50 kg steamed rice-soybean mix. The inoculated rice and soybean mix was placed evenly spaced in bamboo trays (thickness about 1 cm). The loaded trays are piled up in an incubation room, where temperature was controlled at 35 °C, with a relative humidity 70 % for 40 h. Koji mash was then prepared by mixing the prepared koji with syrup (65 % sucrose) at a ratio 1:5 (w/w), then proceed at room temperature for 48 h. Then the koji granules and syrup were separated from the koji mash.

3. Fermentation of sufu

To perform the fermentation of sufu, salted tofu cubes (195 g) were mixed with koji mash (175 g) where the ratio of koji granules: syrup was 1:4 (w/w) and added in a 400 mL glass jar and then incubated under 37°C for 16 days. Additionally, the sufu-containing jar was further placed at room temperature (Ca. 25°C) for another 30 days. This is the practice employed in some factory before the sufu products were subjected to sale in the market.

4. Sampling (Huang, 2008)

During the sufu fermentation period, bottled sufu samples withdrawed at specific interval. Contents of the bottle were separate into 3 parts: sufu (tofu cubes), koji granules and infusion. Tofu cubes were soaked in deionized water for 2 min. After rinsing, they were

dried and sealed in plastic bag. Koji granules separated from the koji mash were sealed in plastic bag. These samples were stored in -20°C before analyzed for volatiles, sugar, organic acids and antimutagenicity of the samples was performed.

v. Analysis methods

1. Assay for volatile compounds

1.1 Simultaneous steam distillation and extraction (SDE)

A Likens–Nickerson type (Likens and Nickerson, 1964) SDE apparatus was used to extract the volatile compounds. For the analysis, unfermented tofu or sufu samples (200 g each) were blended with 400 mL of distilled water and the aliquot was loaded in a 3 L roundbottom sample flask. 1 μL of internal standard (IS) [2, 4, 6-trimethylpyridine] was added to the sample before extraction. Each sample was extracted with 50 mL of dichloromethane. Each extraction was carried out for 2 h after the distilled water in the sample flask started to boil. The extract was concentrated to approximately 2 mL using a rota-vapor equipped with a water bath; the water bath temperature was 45 °C and the vacuum pressure 921 mbar. Triplicate extractions were carried out for each different fermentation period. The concentrated extract was stored at -20 °C until further analysis was performed.

1.2 Gas chromatography–mass spectrometry (GC–MS)

Method of Chung (1999) with minor modification was employed to determine the volatile compounds in the samples. One microlitre of each concentrated extract was analyzed on a HP-Agilent 5890 series II plus Gas Chromatography-5972 Mass spectrometry equipped with a DB-WAX capillary column (30 m x 0.25 mm i.d., film

thickness 0.15 μm). The detector was a mass spectrometer (HP 5973 Mass Selective Detector, Hewlett–Packard, Palo Alto, CA). GC oven conditions were initially at 40 °C and held for 3 min ,increased to 210 °C at 2 °C/min and held for 30 min. Injection port temperature is 250 °C and with split ratio 1:15. Helium purity 99.999% carrier gas flow was at constant pressure 2 psi. The total ion chromatograms acquired via GC–MS was used for peak area integration.

1.3 Volatile Compounds Identification and Quantification.

Tentative identifications of a component were based on matching mass spectra of unknown compounds with those in the Wiley library of mass spectral database (Hewlett-Packard Co., 1995). Quantification of compounds in each sample was determined by the standard curve method using the peak area of a specific fragment of a compound to that of the I.S., assuming a response factor of 1. Relative abundance of a tentatively identified compound was estimated by the ratio of the relative area of a specific fragment of the tentatively identified compound to the area of the internal standard (IS).

2. Assay for sugars and organic acids

2.1 Extraction procedures for sugars and organic acids

The extraction procedures were followed that described by Lefebvre *et al*. (2002). A 10 g of sample was homogenized with 90 mL distilled water in a blender (maximal speed, 30 sec). Five milliliters of 1 mol/L $HClO_4$ solution was added to a 10 mL aliquot of the homogenate. The mixture was centrifuged for 15 min at 4000 x g and at the temperature of 15°C, the supernatant was neutralized (pH 7.0 ± 0.1) with 2 mol/L KOH and the volume

was adjusted to 25 mL with distilled water. After 30 min precipitation on ice, the solution was filtered on 0.45 μm cellulose filter (Millipore).

2.2 Determination of sugars

The analytical method of Marazza *et al*. (2009) with minor modification was employed to determine the sugar content of the samples. The concentrations of stachyose, raffinose, glucose, fructose, galactose, maltose and sucrose in the samples were analyzed by High-performance liquid chromatography (HPLC). HPLC analyses were performed with a 20 μL automatic injection loop. Detection was performed with a refractive index detector. Chromatographic separation was performed using a Phenomenex RNM Carbohydrate column (Order no. 00H-0136-K0, dimension: 300 x 7.8 mm) under the following conditions: mobile phase HPLC grade Water, flow rate 0.35 mL/min, column temperature 75°C.

References of external standards (stachyose, raffinose, glucose, fructose, galactose, maltose and sucrose) were chromatographed to determine their retention times. Standards solutions of sugars at a concentration of 1 mg - 50 mg/mL were prepared by diluting specific amounts of sugar in deionized water. Standard curves were constructed and then least squares regression analysis was used to derive equations from the values obtained for each sugar.

2.3 Determination of organic acids

Method of Marazza *et al*. (2009) with minor modification was employed to determine the organic acid content in the samples. The HPLC equipped with a 20 mL automatic injection loop was used to determine the contents of organic acids (lactic acid, acetic acid, oxalic acid, citric acid and succinic acid) using Phenomenex Capcell Pak C18 UG-120A

column (4.6 mm×250 mm, 5 mm). Operational conditions were as follows: mobile phase: 5mM sulfuric acid (prepared using distilled deionized water); flow rate: 0.5 mL/min; column temperature: 30°C; UV 220 nm detector. References of external standards (lactic acid, acetic acid, oxalic acid, citric acid and succinic acid) were chromatographed to determine their retention times. Standards solutions of organic acids at a concentration of 0.001 M – 0.1 M of external standards were prepared by diluting specific amounts of organic acids in deionized water. Standard curves were constructed and then least squares regression analysis was used to derive equations from the values obtained for each organic acid.

3. Assay for antimutagenic activity

3.1 Preparation of methanol extracts (Hung *et al.*, 2007)

Sample of the unfermented salted-tofu and fermented sufu, were dried at 60°C for 24 h, then they were ground using a grinder. The sufu powders were extracted with methanol (1:10, w/v) by refluxing at Ca 25°C for 24 h with gentle (120 rpm) shaking. After filtering through Whatman No. 1 filter paper, the methanol extracts were vacuum concentrated and dried using a freeze-dryer.

3.2 Confirming the genotypes of the *Salmonella typhimurium* TA100

Tests of histidine requirement, *rfa* mutation, *uvr*B mutation and R-factor were carried out to confirm the genotypes of *Sal. typhimurium* TA100 (Maron and Ames, 1983).

3.21 Histidine requirement

A single streaking was made across the biotin control plate and histidine/biotin plate. The plates were incubated overnight at 37°C and examined for growth on the plates. Preparation of biotin control plate and histidine/biotin plate as below:

Biotin control plate

Ingredient	per liter
Agar	15 g
Distilled H_2O	924 mL
50 X VB salts	20 mL
40% Glucose (w/w)	50 mL
Sterile 0.5 mM biotin	6 mL

Vogel-Bonner medium E (50 X VB salts)

Ingredient	per liter
Warm distilled H_2O	670 mL
Magnesium sulfate ($MgSO_4.7\ H_2O$)	10 g
Citric acid monohydrate	100 g
Potassium phosphate, dibasic (K_2HPO_4)	500 g
Sodium ammonium phosphate ($NaHNH_4PO_4.4H_2O$)	175 g

Histidine-Biotin control plate

Ingredient	per liter
Agar	15 g
Distilled H_2O	914 mL
50 X VB salts	20 mL
40% Glucose (w/w)	50 mL
Sterile histidine . HCl . H_2O (2 g/400 mL H_2O)	10 mL
Sterile 0.5 mM biotin	6 mL

3.22 *rfa* mutation

0.1 mL of fresh overnight culture was added to a test tube containing 2 mL of molten top agar. The mixture was vortexed to mix well and poured on a nutrient agar plate. 10 µL of a 1 mg/mLsolution was pipeted to the centre of sterile filter paper disc (1/4 inch). Once the agar became firm, the disc was transferred to the seeded plate using sterile forceps. The plates were invert and incubated at 37°C for 12 h.

3.23 uvrB mutation

Fresh overnight cultures were streaked across a nutrient agar plate in parallel stripes. A piece of cardboard was placed over the uncovered plate so that half of each bacterial streak was covered. The plate was irradiated with 15 W UV lamp at a distance of 33 cm for 8 sec. The irradiated plates were incubated at 37°C for 12-24 h.

3.24 R-factor

Fresh overnight cultures were streaked across the surface of an ampicillin plate and incubated at 37°C for 12-24 h.

Ampicillin plate

Ingredient	per liter
Agar	15 g
Distilled H_2O	910 mL
50 X VB salts	20 mL
40% Glucose (w/w)	50 mL
Sterile histidine . HCl . H_2O (2 g/400 mL H_2O)	10 mL
Sterile 0.5mM biotin	6 mL
Sterile ampicillin solution (8 mg/mL 0.02 N NaOH)	3.15 mL

3.3 Toxicity Assay

To examine the toxic effects on *Sal. typhimurium* TA100, methanol extract (0.625 mg-5 mg) which dissolve in 0.1 mL DMSO were added to overnight-cultured *Sal. typhimurium* strains TA100 (0.1 mL) and S9 mix (0.5 mL) or 0.1 M phosphate buffer, pH 7 (0.5 mL), instead of S9 mix. S9 fractions were obtained from liver of Sprague-Dawley rats pretreated with a polychlorinated biphenyl mixture (Aroclor 1254). In control plate, methanol extracts were replaced with 0.1 mL of DMSO. The mixture was pre-incubated at 37 °C water bath with shaker 120 rpm for 20 min before it was diluted with sterile distilled water. The mixture was then poured with nutrient agar on petri dish. The plates were incubated at 37°C for 2 days, and the number of colonies was counted.

S9 mixture

Ingredient	per 50 mL
Rat liver S9 (Aroclor-1254-induced)	2mL
0.4 M $MgCl_2$-1.65 M KCl salt	1mL
1 M glucose-6-phosphate	0.25mL
0.1 M NADP	2mL
0.2 M phosphate buffer, pH 7.4	25mL
Sterile distilled H_2O	19.75mL

3.4 Mutagenic assay

The pre-incubation method of Maron and Ames (1983) with minor modification was employed to study the mutagenic effects of the methanol extracts. The mutagenic effects sufu extract were assayed according to the Ames test using *Sal. typhimurium* TA100 with and without metabolic activation. The methanol extracts were then tested for their mutagenic potency exclusively in the non-toxic concentration range. 0.1 mL of 16 h overnight culture of *Sal. typhimurium* TA100 was added to 0.1 mL DMSO containing 0 – 5.0 mg of an extract and 0.5 mL phosphate buffer (PBS), pH 7 or S9 mix. The entire mixture was incubated in 37°C water bath with shaker 120 rpm for 20 min, and after the incubation, 2.0 mL top agar was added and vortexed with the tube contents. The pre-incubated mixture was poured onto minimal glucose agar plate and His^+ revertant colonies were manually counted after incubation for 48 h at 37°C. A sample was considered to be mutagenic when the number of revertant colonies was at least twice the negative control

(without sample) yield and showed a significant difference ($p < 0.05$) in analysis of variance.

Minimal Glucose plate

Ingredient	per liter
Agar	15 g
Distilled H_2O	930 mL
50 X VB salts	20 mL
40% Glucose (w/w)	50 mL

3.5 Antimutagenic assay

The pre-incubation method of Maron and Ames (1983) with minor modification was employed to study the antimutagenic effects of the methanol extracts. The mutagens used as positive controls were 4-nitroquinoline N-oxide (4-NQO), which is a direct-acting mutagen, and 3, 2-Dimethyl-4-amino-biphenyl hydrochloride (DMAB) which is a indirect-acting mutagen and it required S9 mix for metabolic activation. 0.1 mL of 16 h overnight culture of *Sal. typhimurium* TA100 was added to 0.1 mL DMSO containing 0 – 5.0 mg of an extract, 0.1 mL mutagen and 0.5 mL phosphate buffer (PBS), pH 7 or S9 mix. The entire mixture was incubated in 37°C water bath with shaker 120 rpm for 20 min, and after the incubation, 2.0 mL top agar was added and vortexed with the tube contents. The pre-incubated mixture was poured onto a plate of minimal glucose agar and His^+ revertant colonies were manually counted after incubation for 48 h at 37 °C.

Antimutagenic activity was expressed as a percentage of mutagenic inhibition using the below formula:

Inhibition (%) = 1- [(A-C) / (B-D)] x 100

A: The numbers of mutagen-induced revertants in the presence of methanol extract

B: The numbers of mutagen-induced revertants in the absence of methanol extract

C: The number of spontaneous revertants with the presence of methanol extract

D: The number of spontaneous revertants with the absence of methanol extract.

5. Determination of moisture content

Firstly, aluminium dish were dried in 105 °C oven for overnight before weighing. Approximately 2 g of sample (S) were weighed and placed evenly on the aluminium dish (W1). The drying process was conducted in the 105 °C oven for 24 h. After the 24 h drying, the sample was removed from oven and transfer to desiccator for cooling. The dried sample was weighed again to calculate the moisture lost (AOAC, 2000).

$$\text{Moisture (g/100 g sufu)} = \frac{(W1-W2) \times 100}{S}$$

W1: The weight of aluminium dish + sample (g)

W2: The weight of aluminium dish + sample after 24 h drying (g)

S: The weight of sample (g)

6. Statistical analysis

All samples were analysed using triplicates. Each replication was prepared from single jar of sufu sample. The mean values and the standard deviation were calculated from the data obtained from three separate experiments. Means were compared using Duncan's multiple range test method in SAS (2001), version 8 (SAS Institute, Gary, NC, USA). The level of significance was chosen at $p < 0.05$ and the results are presented as mean ± s.e.m. (standard error of the mean).

IV. Results and Discussion

1. Changes in the content of the volatile components during sufu fermentation

During the manufacture of Taiwanese sufu, koji mash was first prepared by mixing the *Asp. oryzae*-fermented rice-soybean koji with syrup (65% sucrose). Fermentation was then proceeded by soaking tofu cubes in the koji mash. With this process, hydrolytic enzymes such as protease, lipases and amylase, released from the prepared rice-soybean koji into the syrup, may then penetrate into the tofu cube and catalyze the formation of volatile compounds. The volatiles in sufu samples were isolated using simultaneous steam distillation and extraction (SDE) and then analyzed by GC-MS. The gas chromatographic separation of the total volatile distillate is shown in Fig. 7, providing a comparison of the total volatile components identified in unfermented tofu cube and sufu with various fermentation periods. Table 2 and Fig. 8 show the results from the proximate analysis of volatiles in sufu samples with different fermentation period. Differences in both quantitative analysis and qualitative analysis were observed between the unfermented tofu cube and sufu with various fermentation periods. The identified components are grouped by functionality. A total of 90 compounds of volatiles including 22 alcohols, 22 esters, 21 aldehydes, 10 fatty acids, 9 ketones and 6 other compounds from the sufu samples examined were identified. Fifty percent of these compounds were previously reported in soybeans or soybean processed products (Wilkens and Lin, 1970; del Rosario *et al*., 1984; Hwan and Chou, 1999; Chung, 1999; Chung, 2000; Lee and Ahn, 2009). Twenty-two compounds were found in all the tofu samples examined regardless of fermentation while their quantitative levels varied.

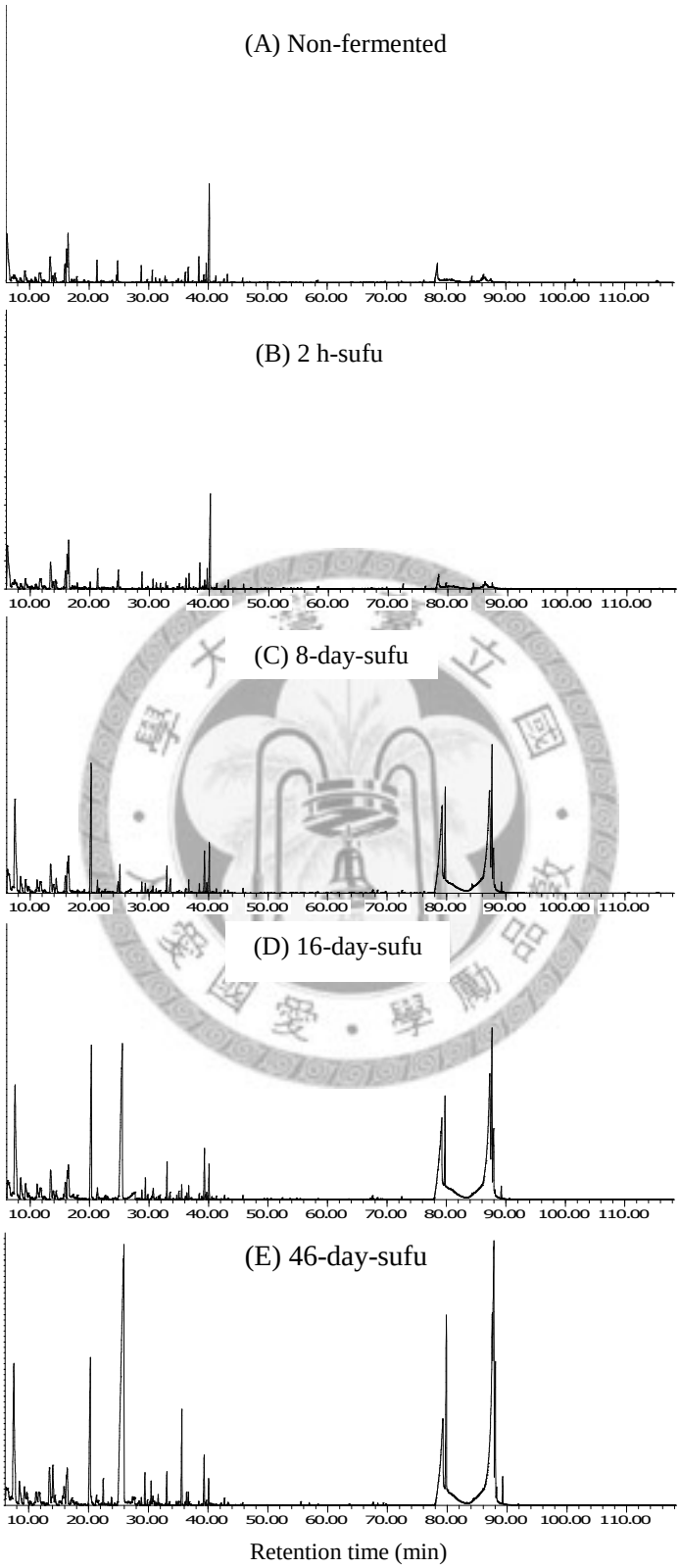

Fig. 7. Total ion chromatograms of volatile components in non-fermented tofu and sufu with various fermented time.

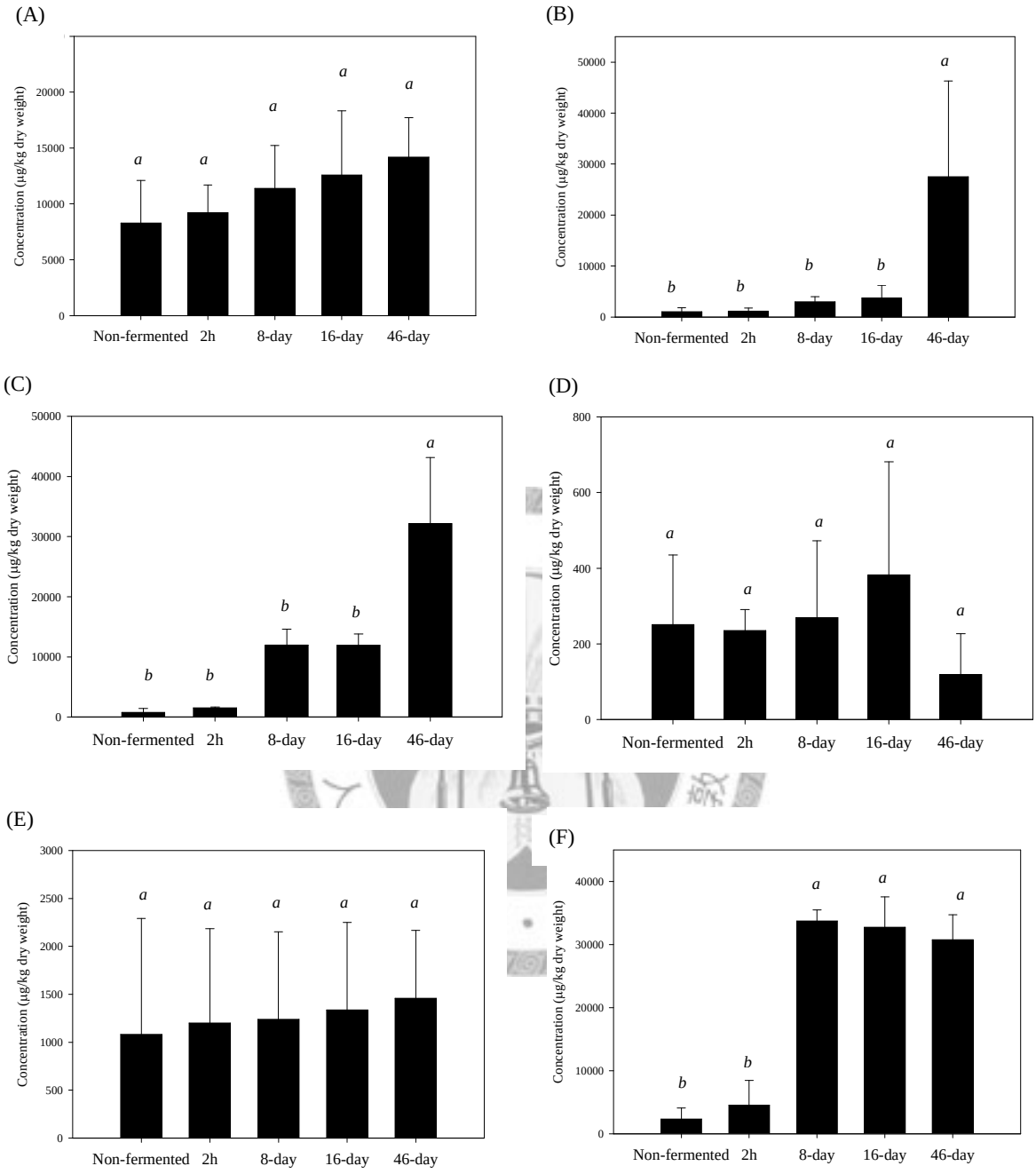

Fig. 8. Changes in concentration of various volatile fractions in sufu during fermentation process.

(A) Aldehydes (B) Alcohols (C) Esters (D) Ketones (E) Miscellaneous (F) Fatty acids

Table 2. Changes of various volatile compounds content during the fermentation period of Taiwanese sufu

[a]Components	[b]T_R (min)	[c]CAS Registry No.	[d]MW	[e]ref		[f]Sig	Non-fermented	2 h	8-day	16-day	46-day
				Chinese sufu	Other soybean product		[g]μg/kg	μg/kg	μg/kg	μg/kg	μg/kg
Aldehydes (21)											
N-Hexanal	6.21	66-25-1	100	1,2,3	4ab,5,6	*	2828 ± 1452*a*	286 ±1307*a*	882 ±26*b*	520 ±198*b*	310 ±118*b*
2-Furancarboxaldehyde	7.44	98-01-1	96	2,3	5	*	[h]nd	692 ±344*b*	4522 ±1935*ab*	5401 ±2992*a*	6924 ±3299*a*
2-Hexenal	8.44	505-57-7	98		6		134 ± 82	121 ±132	nd	nd	nd
Heptanal	10.91	111-71-7	114		4b,6	*	161 ± 86*a*	144 ±90*a*	39 ±55*b*	47 ±49*b*	nd
2-Heptenal	14.25	18829-55-5	112		6	*	323 ± 245a	nd	nd	8 ±14*b*	nd
5-Methyl-2-furancarboxaldehyde	14.38	620-02-0	110	2	5		nd	243 ±214	349 ±140	255 ±126	219 ±192
Benzaldehyde	14.39	100-52-7	106	1,2,3	4b,5,6		nd	nd	nd	104 ±180	170 ±166
2,4-Heptadienal	16.91	4313-03-5	110	2	6		126 ± 46	116 ±71	49 ±5	26 ±4	44 ±63
Octanal	17.46	124-13-0	128		4b		37 ± 33	10 ±35	38 ±13	14 ±13	nd
Benzeneacetaldehyde	20.07	122-78-1	120	3	5	*	55 ± 52*b*	188 ±30*b*	3544 ±1531*a*	4944 ±2254*a*	5377 ±79*a*
2-Octenal	21.32	2548-87-0	126	2	6	*	344 ± 145*a*	364 ±109*a*	143 ±26*b*	111 ±10*b*	209 ±112*ab*
Nonanal	24.79	124-19-6	142		4ab,6	*	309 ± 104*a*	255 ±137*ab*	171 ±173*abc*	53 ±48*bc*	25 ±26*c*
2-Phenylpropenal	28.17	4432-63-7	132				nd	nd	6 ±11	18 ±15	47 ±16
Nonenal	28.76	2277-19-2	140		6	*	214 ± 86*ab*	239 ±72*a*	106 ±20*b*	112 ±58*b*	106 ±50*b*
2,4-Nonadienal	32.75	5910-87-2	138		6	*	75 ± 25*ab*	92 ±16*a*	32 ±8*bc*	12 ±21*c*	nd
5-Hydroxymethylfuraldehyde (HMF)	33.60	67-47-0	126			*	nd	103±105*c*	449 ±100*a*	259 ±151*b*	157 ±78*bc*
3-Dodecen-1-al	35.41	68083-57-8	182				51±88	39±41	13 ±23	nd	nd
2-Decenal	36.16	3913-81-3	154		6		93±81	137±131	44 ±26	nd	nd
2-Phenyl-2-butenal	36.37	4411-89-6	146	3	5	*	nd	nd	nd	54 ±60*b*	136 ±77*a*
2,4 decadienal	40.19	2363-88-4	152	2	5,6	*	3528±1668 *a*	3552±980*a*	918 ±163*b*	416 ±26*b*	297 ±14*b*
9,17-Octadecadienal	88.18	56554-35-9	~				nd	nd	83 ±144	218 ±111	151 ±136

Table 2. Changes of various volatile compounds content during the fermentation period of Taiwanese sufu (continue)

[a]Components	[b]T_R (min)	[c]CAS Registry No.	[d]MW	[e]ref		[f]Sig	Non-fermented	2 h	8-day	16-day	46-day
				Chinese sufu	Other soybean product		[g]μg/kg	μg/kg	μg/kg	μg/kg	μg/kg
Alcohols (22)											
2-Furanmethanol	8.39	98-00-0	98	2		*	nd	nd	422±384*b*	660±302*a*	716±383*a*
1-Hexanol	9.17	111-27-3	102	2	5,6		309±245	293±255	487±133	356±43	537±230
2-Ethyl-1-butanol	12.34	97-95-0	102	2			90±84	104±93	90±87	54±94	nd
2-Methylpentanol	12.38	105-30-6	102				41±47	nd	nd	nd	863±1495
1-Heptanol	15.23	111-70-6	116	2			nd	nd	nd	19±17	25±22
1-Octen-3-ol	15.95	3391-86-4	128	2,3	4ab,5,6		234±406	229±396	218±378	222±385	278±482
3-Octanol	17.08	589-98-0	130	1,2	4ab,5,6		31±55	49±44	54±31	25±22	102±69
2-Butyl-1-octanol	17.36	3913-02-8	186				nd	46±44	nd	nd	nd
Phenol	21.62	108-95-2	94	2,3	5		nd	nd	27±23	16±28	30±33
2-Octen-1-ol	22.01	18409-17-1	128		6		26±28	25±22	14±13	14±13	nd
1-Octanol	22.34	111-87-5	130	2	6		31±13	41±16	31±11	23±10	31±16
2-Methoxy-phenol	23.11	90-05-1	124	2,3	5		nd	nd	nd	29±28	nd
Benzeneethanol	25.02	60-12-8	122	2,3		*	11±19*b*	31±14*b*	583±117*b*	1284±1182*b*	23267±15508*a*
Nonanol	29.74	28473-21-4	144		6		41±19	41±10	40±8	37±3	63±32
2-(2-Butoxyethoxy)ethanol	30.65	112-34-5	162				nd	nd	115±36	185±152	216±125
1-Methyl-4-isopropyl-1-cyclohexen-8-ol	31.16	98-55-5	154				62±61	17±30	16±16	30±15	19±33
4-Vinylphenol	32.98	000-00-0	120	2	5	*	nd	39±33*b*	202±232*b*	250±434*b*	495±174*a*
4-Vinyl-2-methoxy-phenol	39.24	7786-61-0	150	2,3	5	*	115±27*c*	153±10*c*	700±110*ab*	519±89*b*	873±260*a*
3-Isopropylphenol	39.71	618-45-1	136				nd	nd	nd	nd	49±45
2,4-Di-tert-butylphenol	52.42	96-76-4	206				10±18	101±104	16±18	18±5	nd
5-Tetradecen-1-ol	84.53	62936-14-5	212				17±15	7±13	nd	nd	nd
3-Tetradecen-1-ol	85.68	68900-86-7	212				15±26	9±16	nd	nd	nd

Table 2. Changes of various volatile compounds content during the fermentation period of Taiwanese sufu (continue)

[a]Components	[b]T_R (min)	[c]CAS Registry No.	[d]MW	[e]ref		[f]Sig	Non-fermented	2 h	8-day	16-day	46-day
				Chinese sufu	Other soybean product		μg/kg	μg/kg	μg/kg	μg/kg	μg/kg
Ester (22)											
1-Pentyl isobutyrate	13.43	2445-72-9	158				390±675	414±718	243±421	nd	nd
Isopentyl isobutanoate	13.50	2050-01-3	158				nd	456±462	nd	nd	nd
Ethyl hexanoate	17.22	123-66-0	144	1,2,3	5	*	9±16b	nd	nd	23±21*b*	194±46*a*
2-Octyl acetate	26.17	2051-50-5	172				nd	nd	27±24	nd	nd
Ethyl benzoate	29.37	93-89-0	150	1,3	5	*	nd	nd	141±31*c*	203±6*b*	594±13*a*
Diethyl butanedioate	30.46	123-25-1	174	3	5		nd	nd	nd	36±62	213±198
Ethyl octanoate	31.63	106-32-1	172	1,2,3	5	*	nd	nd	nd	31±34*b*	145±65*a*
Ethyl phenylacetate	34.66	101-97-3	164	2	5	*	nd	nd	6±10*c*	23±3*b*	62±7*a*
Phenethyl acetate	35.50	103-45-7	164	2,3	5	*	nd	nd	nd	81±140*b*	1761±956*a*
Isobutyl phthalate	72.49	84-69-5	278				55±28	48±35	35±8	40±13	nd
Methyl heneicosanoate	76.30	6064-90-0	341				nd	nd	17±15	nd	nd
Methyl palmitate	76.32	112-39-0	270				34±30	18±32	nd	nd	nd
Ethyl palmitate	79.66	628-97-7	284	1,3	5	*	nd	78±67*c*	3472±1029*ab*	2130±273*b*	4413±2200*a*
Methyl linolelaidate	84.27	2566-97-4	294				34±59	73±65	56±97	nd	nd
Ethyl linoleate	87.44	544-35-4	308	1,3		*	nd	322±186*b*	5028±567*ab*	6613±838*ab*	18514±8034*a*
Methyl-9,12,15-octadecatrienoate	87.86	301-00-8	292				nd	nd	nd	284±492	933±812
Ethyl oleate	87.92	111-62-6	311	1,3		*	nd	nd	2568±1839*b*	1906±1449*b*	4188±2350*a*
Ethyl stearate	89.24	111-61-5	313	1		*	nd	nd	219±67*b*	212±28*b*	429±138*a*
2-Ethylhexyl p-methoxycinnamate	95.84	5466-77-3	290				nd	nd	nd	30±28	nd
Dioctyl adipate	101.44	123-79-5	371				36±62	20±35	nd	nd	nd
Isooctyl phthalate	115.39	27554-26-3	391				52±50	nd	nd	nd	nd
Di-(2-ethylhexyl) phthalate	115.73	117-81-7	391			*	168±292*b*	89±104*b*	52±47*b*	286±319*ab*	704±268*a*

Table 2. Changes of various volatile compounds content during the fermentation period of Taiwanese sufu (continue)

[a]Components	[b]T_R (min)	[c]CAS Registry No.	[d]MW	[e]ref		[f]Sig	Non-fermented	2 h	8-day	16-day	46-day
				Chinese sufu	Other soybean product		[g]μg/kg	μg/kg	μg/kg	μg/kg	μg/kg
Ketones (9)											
Cyclohexanone	7.67	108-94-1	98	1			24±42	nd	nd	nd	nd
2-Heptanone	10.23	110-43-0	114	2,3	4b,5,6		74±73	42±71	nd	24±41	nd
2-Propylcyclopentanone	17.24	1122-98-1	126				78±33	38±34	35±34	19±33	nd
1-(2-Furanyl)-2-hydroxy-ethanone	22.69	17678-19-2	126				nd	nd	nd	10±17	24±21
2-Methyl-3-hydroxypyrone	24.62	118-71-8	126	3			74±76	57±83	118±182	76±132	nd
2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	27.38	000-00-0	272				nd	90±98	94±45	227±283	nd
2,4-Dimethyl-3-pentanone	28.69	565-80-0	114				nd	nd	nd	nd	44±47
Dihydro-5-propyl- 2(3H)-furanone	42.78	105-21-5	128				nd	nd	22±19	26±5	nd
5-Butyltetrahydro-2-furanone	42.79	104-50-7	142				nd	8±14	nd	nd	51±44
Fatty Acids (10)											
Heptanoic acid	17.00	111-14-8	130				nd	nd	29±25	nd	nd
Hexanoic acid	17.03	142-62-1	116		5,6	*	nd	nd	nd	71±63*a*	2±4*b*
2,2-Dimethyl-3-hydroxy-propanoic acid	26.67	000-00-0	~				nd	nd	22±19	nd	nd
Ethylhexoic acid	26.80	149-57-5	144			*	nd	nd	207±101*b*	498±481*ab*	841±251*a*
Octanoic acid	30.46	124-07-2	144				nd	nd	86±95	108±127	25±43
Decanoic acid	67.65	334-48-5	172				nd	nd	22±37	nd	29±25
Palmitic acid	78.52	57-10-3	256			*	1714±1070*b*	2941±2461*b*	16559±412*a*	13486±2186*a*	14845±2769*a*
Oleic acid	78.76	112-80-1	282				26±46	56±88	34±59	96±45	49±85
Linoleic acid	86.23	60-33-3	280			*	462±552*c*	1451±1286*c*	16782±2089*ab*	18496±2326*a*	14986±1573*b*
Stearic acid	87.95	57-11-4	284				107±185	72±125	nd	nd	nd
Miscellaneous (6)											
3 Methyl thio propanal	11.18	3268-49-3	104	3		*	nd	30±28*c*	379±145*b*	491±137*ab*	653±128*a*
4-Methyl-2,3-dihydrofuran	11.77	34314-83-5	84				102±176	39±50	nd	7±12	9±15
3-Methyl thiol propanol	15.63	505-10-2	106	2,3			nd	nd	nd	40±69	138±172
2-Pentylfuran	16.46	3777-69-3	138	2,3	4ab,5		898±1015	839±1047	405±702	344±597	411±712
2,3-Dihydrobenzofuran	32.99	496-16-2	120			*	18±31*b*	12±20*b*	401±86*a*	373±80*a*	211±366*ab*
1,3-Ditertiarybutylbenzene	35.00	1014-60-4	190				63±30	280±254	53±18	81±54	36±36

[a]Compounds in order of their elution sequences. [b]Retention time. [c]Chemical Abstracts Service Registry No. [d]Molecular weight. [e]Articles in which the compounds were reported: 1, Hwan and Chou, 1999; 2, Chung, 1999; 3, Chung, 2000; 4, del Rosario *et al.*, 1984a, raw soybean, 1984b, heated soybean; 5, Lee and Ahn, 2009; 6, Wilkens and Lin, 1970. [f] *,statistically significant difference at $p < 0.05$ in the same row. Values of the amount with different italic letters are significantly different (Duncan $p < 0.05$). [g]Mean concentration from three replicates on a dry weight basis. [h] nd, not detected.

Of the 90 compounds identified in the samples of Taiwanese sufu, a total of 37 common compounds were found in Chinese sufu (Hwan and Chou, 1999; Chung, 1999; Chung, 2000). Similar to the Chinese sufu, both esters and alcohols has the highest number of components (Hwan and Chou, 1999; Chung, 1999; Chung, 2000). These hydrolytic enzymes catalyze the degradation of larger molecular constituent of tofu with fermentation, a large amount of peptide, amino acids and fatty acids (Li, 2009) of which served as a pool of substrates for further biolytic and chemical reaction and results in the production of the volatile flavor components detected during the fermentation of Taiwanese sufu.

1.1 Aldehydes

A total of 21 aldehydes were identified from the Taiwanese sufu samples. Nine of these compounds (N-hexanal, 2-furancarboxaldehyde, 5-methyl-2-furancarbozaldehyde, benzaldehyde, 2, 4-heptadienal, benzeneacetaldehyde, 2-octenal, 2-phenyl-2-butenal and 2, 4-decadienal) were also found in Chinese sufu (Hwan and Chou, 1999; Chung, 1999; Chung, 2000). On the other hand, 13 aldehydes were also found in *Asp. oryzae*-fermented soybean paste (Lee and Ahn, 2009). It is reported that aromas of aldehydes mainly associated with sweet, fruity, nutty and caramel-like odors (Fors, 1983). The favorable odor of aldehyde compounds such as benzaldehyde (cherry or almond-like odor), benzeneacetaldehyde (rosy-like odor), 2, 4-decadienal (potato chip-like odor), and 2-phenyl-2-butenal (floral, prune-like odor) (Mookherjee *et al*., 1965; Chung, 2000) can be considered in the enhancement of flavor quality in sufu. The volatile compound, N-hexanal, contributed a green disagreeable aroma to the soybean milk and textured soy protein (Ames and Macleod, 1984). Content of N-hexanal in the 2h-sufu was higher compared to other sufu with a longer fermentation time. However, it is interesting to note that the content of

N-hexanal in tofu reduced as the fermentation period was extended. The high N-hexanal content of 2828 μg/kg noted in the unfermented tofu reduced to 310 μg/kg dried tofu after 46 days of fermentation. This demonstrated that fermentation may remove the undesirable bean odor from the soybean product. Similar phenomenon was also observed by Sugawara *et al*. (1985) on cooked soybean and natto. Benzeneacetaldehyde and 2-furancarboxaldehyde increased significantly ($p < 0.05$) during the fermentation process and were found to be the major aldehydes in Taiwanese sufu products. These aldehydes can be produced by lipid oxidation and degradation during fermentation (Ames and Macleod, 1984). On the other hand, hexanal, 2-heptanal, 2-octenal and 2, 4-decadienal can be formed from linoleic acid by catalytic action of lipoxygenase in soybean (Grosch *et al*., 1969). Beside, octanal, nonanal, 2-nonenal and 2-decenal can be arised from oleic acid while 2, 4-heptadienal can be arised from linolenic acid (Smouse and Chang, 1967).

1.2 Alcohols

Alcohol is another large class of volatiles found in the Taiwanese sufu samples. Among the 22 alcohols detected, twelve alcohols including 2-furanmethanol, 1-hexanol, 2-ethyl-1-butanol, 1-heptanol, 1-octen-3-ol, 3-octanol, phenol, 1-octanol, 2-methoxy-phenol, benzeneethanol, 4-vinylphenol and 4-vinyl-2-methoxy-phenol were also found in Chinese sufu (Hwan and Chou, 1999; Chung, 1999; Chung, 2000). Besides, benzeneethanol, 2-methoxy phenol, phenol, 4-vinyl-2-methoxy phenol and 1-octen-3-ol were reported to present in soy sauce (Lee *et al.*, 2006). 1-octen-3-ol and hexanol, the key compounds of green beany odor (Arai *et al*., 1966), remained even after 46 days of fermentation. A significant ($p < 0.05$) increased the content of 2-furanmethanol, benzeneethanol and 4-vinyl-2-methoxy-phenol was noted as the fermentation period was increased. 4-vinylphenol

and 4-vinyl-2-methoxy-phenol have the cooked soybean-like odor (Greuell, 1974), were detected in all the sufu samples, regardless of fermentation period. They were thought to be thermal degradation products of the lignin-related phenolic carboxylic acids (van den Ouweland and Schutte, 1978). Other favorable pleasant odor possessing alcohols, such as 3-octanol (nutty, herbaceous, melon, citrus-like odor), octanol (rosy-like odor), nonanol (rosy, citrus-like odor), 2-methoxy-4vinylphenol (spicy, clove-like odor) and 4-vinylphenol (vanilla-like odor) (Mookherjee *et al*., 1965; Chung, 1999) were detected in the Taiwanese sufu samples. The most abundant compound was benzeneethanol, which accounted for approximately 22% of the total volatiles detected in the 46-day-sufu. Results demonstrated that fermentation is a characteristic process that generates a pool of unique volatile compounds. The various alcohol compounds formed, may contribute to the flavor of sufu.

1.3 Esters

Ester was one of the largest class among the volatile compounds detected. In Taiwanese sufu, a total of 22 esters were detected. Ten of these compounds (ethyl hexanoate, ethyl benzoate, diethyl butanedioate, ethyl octanoate, ethyl phenylacetate, phenethyl acetate, ethyl palmitate, ethyl linoleate, ethyl oleate and ethyl stearate) were also found in Chinese sufu (Hwan and Chou, 1999; Chung, 1999; Chung, 2000). A number of low molecular weight fatty acid esters such as ethyl hexanoate, ethyl benzoate and ethyl octanoate detected in Taiwanese sufu samples have also been found in miso (Ku *et al*., 2000), soybean paste (Lee and Ahn, 2009) and soy sauce (Lee *et al*., 2006). On the other hand, a large number of high molecular weight fatty acid esters, such as ethyl palmitate, ethyl linoleate, ethyl oleate, ethyl stearate, methyl heneicosanoate and dioctyl adipate were detected. They were likely produced by the action of fungal lipases on the soybean lipid

(Chou and Hwan, 1994). Furthermore, a significant increase in the content of ethyl esters of linoleate, oleate and stearate, was noted in Taiwanese sufu as the fermentation period was extended. Total amount of these esters was found to be 27824 μg/kg dried sufu which contributes more than 50 % of the total ester detected in the 46-day-sufu. The presence of a large number of various esters suggested that most fatty acids probably underwent esterification with the formation of ethyl esters (Wang and Hesseltine, 1970) during the fermentation of Taiwanese sufu. Most esters have distinguished quality and distinctive fruit-like odor. The esters with more desirable aromas found in Taiwanese sufu are such as ethyl octanoate, ethyl phenylacetate, ethyl oleate and ethyl linoleate. These are all described to have fruity and floral odor with other pleasant aromas such as pineapple, coconut and honey (Bauer and Garbe, 1985; Chung, 2000).

1.4 Ketones

Nine ketones including four aliphatic ketones and five heterocyclic compounds were identified. Among them, three commons ones (cyclohexanone, 2-heptanone and 2-methyl-3-hydroxypyrone) were also found in Chinese sufu (Hwan and Chou, 1999; Chung, 1999; Chung, 2000; Lee and Ahn, 2009). Ketones can be generated by fungal enzymatic actions on lipids and/or amino acids, or by the Maillard reaction (Fors, 1983; Su, 1986). 2-heptanone which posses a fruity, spicy, cinnamon-like odor (Aldrich, 1998) were also found in soybean paste (Lee and Ahn, 2009), cooked soybean (del Rosario, 1984), soybean milk (Wilkens and Lin, 1970), chungkuk-jang and natto (Tanaka *et al*., 1998). Most heterocyclic compounds of the ketones class detected were not naturally occurring products but were thermally generated during steaming (Fors, 1983). Aliphatic ketone such as 2-heptanone could be the products of lipid oxidation or degradation assisted by the enzymes

(Ames and Macleod, 1984). Although the flavor notes of ketones are generally pleasant, their aroma contributions might be minimal since low concentration of these compounds were quantified in Taiwanese sufu.

1.5 Miscellaneous

Six miscellaneous volatile compounds including two sulfur containing components, three furans and 1, 3-Ditertiarybutylbenzene were identified from the sufu sample examined. Among them, two sulfur containing compounds which were also found in Chinese sufu. 3-methylthio propanal having a meaty, soy sauce-like odor and 3-methylthio propanol was previously detected in soy sauce (Lee and Kwok, 1987). Among the three furans detected, 2-pentylfuran which recognized as having grassy or green bean-like odor (Fors, 1983) was also found in Chinese sufu (Chung, 1999; Chung, 2000, Chung *et al*., 2005), soybean milk (del Rosario, 1984), soy bean paste (Lee and Ahn, 2009) and chungkuk-jang (Tanaka *et al*., 1998). Furans can be formed by sugar dehydration of fragmentation in the Maillard reaction (Fors, 1983). On the other hand, although Chinese sufu and other soybean products were reported to contain pyrazines (Sugawara *et al*., 1985; Hwan and Chou, 1999；Chung, 1999; Lee *et al*., 2006; Lee and Ahn, 2009), none was detected in Taiwanese sufu.

1.6 Fatty acids

Ten fatty acids including one short chain fatty acid (2,2-dimethyl-3-hydroxy-propanoic acid), five medium chain fatty acids (hexanoic acid, heptanoic acid, octanoic acid, ethylhexoic acid and decanoin acid) and four long chain fatty acids (palmitic acid, oleic acid, linolein acid and stearic acid) were identified from the sufu samples. Generally, total fatty acids content increased when the fermentation period extended. The major long chain fatty acids found in sufu samples were linoleic acid and palmitic acid. Both were the main fatty acid components in soybean (Hwan and Chou, 1999). Some of the above mentioned aldehydes and alcohols can be arised from linoleic acid or oleic acid (Smouse and Chang, 1967). Wu and Chen (1992) described soybean oil that was heated by deep frying generated volatile octanoic acid and decanoic acid. Besides, hexanoic acid which can be easily oxidized from hexanal, was also found in soybean milk (Wilkens and Lin, 1970) and soybean paste (Lee and Ahn, 2009). This compound was described as "sweat-like" odor (Arctander, 1969) as well as possessing a cheesy, fatty, sweaty, sour, rancid and pungent-like odor (Chung *et al*., 2005). Ethyl esters are derived from esterification of free fatty acids and ethanol. Hence, their corresponding fatty acids are expected to be present during the fermentation process. For example, the generation of ethyl linoleate from linoleic acid as well as ethyl oleate from oleic acid, this could be an indication of esterification catalyzed by fungal enzymes during the fermentation sufu.

Similar to Chinese sufu, Taiwanese sufu with different manufacturing processes also possess a distinctive pleasant fruit odor and alcohol fragrance in addition to its salty and umami (sweet and meaty) taste although other flavor ingredients such as hot pepper, sesame oil and rose essence etc maybe added to modify flavor. The development of flavor

and aroma of sufu during the fermentation process is further demonstrated with the data shown in Fig. 1. As the fermentation period extended, concentrations of nearly all the volatile fractions, particularly alcohol and ester, increased markedly. As shown in Table 2, 2 components (N-hexanal and 2, 4 decadienal) from aldehyde group and 2 components (palmitic acid and linoleic acid) from fatty acid group, has a volatile concentration of >1000μg/kg dried sufu in non-fermented soybean curd and/or 2h-sufu. When fermentation period extended to 16 days, more volatiles appeared in high concentration such as 2-furancarboxaldehyde, benzeneacetaldehyde, benzeneethanol, ethyl palmitate, ethyl linoleate and ethyl oleate. In the 46-day-sufu, the content of the major volatile components, benzeneethanol and ethyl linoleate, increased to >18000μg/kg dried sufu. As fermentation period extended, content of N-hexanal which were described to possess green beany-like odor decreased significantly ($p < 0.05$).

In conclusion, data collected from the present study provide qualitative and quantitative information concerning the development of volatile compounds during the fermentation process of Taiwanese sufu. The volatiles constituents found in the Taiwanese sufu samples were not exactly the same as those forms with Chinese sufu or other fermented soybean products. Difference in the microorganism involved and manufacture process may lead to this discrepancy. Besides, data obtained also demonstrated that the background volatile components of Taiwanese sufu were developed during the fermentation process. These volatile constituents were dominated by fruity and sweet flavor from a majority of the esters and alcohols.

2. Changes in the sugar content of tofu during sufu fermentation

During the fermentation of Taiwanese sufu, it is reasonable to expect that the hydrolytic enzymes which leached out from the koji into the syrup and those penetrated into the tofu cubes may catalyze the hydrolysis of sugar components of tofu substrate. Table 3 shows the changes in the content of various carbohydrates during the fermentation sufu. The main sugars noted in the non-fermented tofu, raw material used as the substrate for sufu fermentation, were stachyose, raffinose, sucrose, glucose and fructose. Among these sugars, fructose was the most abundant. Stachyose and raffinose, the main oligosaccharides in soy products, were the factor which causes flatulence in human after eating soybean food (LeBlanc *et al*., 2004). Comparing with the non-fermented tofu, the 2h-sufu sample which was taken immediately after soaking the tofu in the koji mash, exhibited a markedly high contents of raffinose. Apparently, the increased amount of this oligosaccharides found in the 2h-sufu was essentially came from the koji mash. Contents of these oligosaccharides decreased as the fermentation was extended. For example, a significantly ($p < 0.05$) reduced content of stachyose and raffinose, respectively, of 5.93 and 7.55 mg/g dried tofu was noted in the 46-day-sufu compared to 8.63 and 13.82 mg/g dried found in the non-fermented tofu. It was reported that *Asp. oryzae* was capable of producing α–galactosidase (Cruz and Park, 1982) in addition to β-glucosidase, protease, amylase and lipase (Wang and Murphy, 1994; Su *et al*., 2005; Li, 2009). α–galactosidase may catalyze the hydrolysis of α-1, 6 linked α-galactoside residue (Scalabrini *et al*., 1998). The catalytic action of α–galactosidase may thus lead to the reduced content of stachyose and raffinose in tofu substrate during the fermentation period. This results, in accordance with reports of Chumchuere and Robinson (1999) and Wang *et al*. (2003), demonstrated that fermentation

Table 3. Changes of various sugar contents in tofu during sufu fermentation

Content of sugars (mg/g dried tofu)	Non-fermented tofu	Time after fermentation			
		2h*[1]	8 days	16 days	46 days
Stachyose	8.63±0.73a*[2]	8.84±0.76a	8.81±1.74a	6.85±1.67ab	5.93±0.52b
Raffinose	13.82±1.16b	21.57±1.45a	13.09±0.56b	9.77±1.39c	7.55±0.67d
Sucrose	1.39±0.18b	28.50±4.47a	4.96±1.09b	2.93±0.39b	2.68±0.19b
Maltose	ND*[3]	ND	0.85±0.07c	1.37±0.12b	1.72±0.12a
Glucose	0.27±0.08d	98.49±12.27c	432.64±15.42a	358.35±12.69b	376.78±20.62b
Fructose	65.05±18.32b	256.23±30.36a	258.22±5.53a	257.56±21.91a	261.75±23.74a
Galactose	ND	ND	5.99±1.46b	8.60±1.23a	9.12±0.27a

*1. Tofu cubes were collected 2 h after mixing with koji mash.

*2. Values are presented as means ± SD (n = 3). Means in the same row with different letters were significantly different by Duncan's multiple range test ($p < 0.05$).

*3. Not detected.

may reduce the level of stachyose and raffinose, the flatulence factor, in soybean food. During fermentation, enzymes such as proteinase, amylase and peptidase hydrolyse part of the protein and starch to simpler components which served as substrates for further hydrolytic and chemical reaction.

As shown in Table 3, a markedly high content of sucrose was noted in the 2h–sufu when compared with that of the non-fermented tofu. This may be attributed to the penetration of sucrose from the koji mash into tofu cubes. Since the koji mash was prepared in syrup containing 65% sucrose. Similar to the changes of stachyose and raffinose and in accordance with that observed on Chinese sufu (Han *et al*., 2001b), sucrose content noted on the 2h-sufu reduced as the fermentation period was further extended. The significant ($p < 0.05$) reduction in the content of sucrose after 2h-sufu samples were probably due to enzymatic degradation of disaccharide to monosaccharide such as glucose and fructose.

Although maltose and galactose were not detectable in the non-fermented tofu and 2h-sufu samples, the presence of these sugars was noted in sufu after 8 days of fermentation. Along with the reduction in the contents of stachyose, raffinose and sucrose, an increase in the contents of maltose and monosaccharides such as glucose, fructose and galactose was noted during the fermentation of sufu. This phenomenon may be attributed to the hydrolysis of stachyose, raffinose and sucrose by the hydrolytic enzymes such as α–galactosidase and β-fructosidase/invertase (Rehms and Barz, 1995) which can be produced by *Asp. oryzae* extracellularly during the fermentation (Dhananjay and Mulimani, 2008). Invertase was capable to hydrolyse the bond (α-1, 2 glycosidic linkage) between fructose and glucose in the raffinose oligosaccharide family. After 46 days of fermentation, the sufu was found to contain 1.72, 376.78, 261.75 and 9.12 mg/g dried tofu, respectively, of maltose, glucose,

fructose and glucose. Contents of these sugars noted in the 46-day-sufu were all significantly higher ($p < 0.05$) than those noted with the non-fermented tofu. On the other hand, content of glucose was found decreased significantly ($p < 0.05$) after 16 days of fermentation which was probably caused by Browning reaction (Noguchi *et al*., 1982). A similar phenomenon was also previously reported on *Asp*. oryzae-Thai soy bean sauce (Lertsiri *et al*., 2003). The possibility of Mallard reaction occurred in latter aged Taiwanese sufu samples was also reported in Li (2009) when color measurement was performed. Soy products contain large amount of proteins and saccharides, which can be regarded as sources of browning reaction (Ibolya and Margit, 1986). Studies showed that about 50–60% of browning in soy sauce was developed during mash fermentation, and the remaining occurred during pasteurization (Lertsiri *et al*., 2001; Yokotsuka, 1986).

3. Changes in the sugar content of rice-soybean koji during sufu fermentation

Changes of various sugar contents of the rice-soybean koji and those collected from the koji mash during sufu fermentation is present in Table 4. Different from that observed on tofu cube, presence of maltose and galactose was found in the koji before mixing with syrup for sufu fermentation. This is probably due to the action of hydrolytic enzymes such as amylase and α-galactosidase produced by *Asp. oryzae* during the preparation of koji. Amylase released by *Asp. oryzae* degraded starch in rice-soybean koji to maltose in the preparation of koji and koji mash. The trend in the change of stachyose and raffinose contents of koji was found, generally, similar to that observed with the tofu substrate (Table 3). After 46 days of fermentation, the sufu sample was found to contain 1.04 mg/g dried tofu raffinose and non-detectable of stachyose. The 2 h collected rice-soybean koji after mixing with tofu showed a markedly increased content of sucrose. However, sucrose

Table 4. Changes of various sugar contents in rice-soybean koji during sufu fermentation

Content of sugars (mg/g dried koji)	Koji	Time after fermentation			
		2h*[1]	8 days	16 days	46 days
Stachyose	37.45±10.16a*[2]	3.34±0.11b	2.41±0.71b	1.17±0.42b	ND*[3]
Raffinose	50.57±6.55a	5.05±0.92b	2.67±0.33b	1.95±0.25b	1.04±0.21b
Sucrose	0.44±0.16c	23.07±1.49a	2.19±0.61b	1.61±0.42bc	1.58±0.19bc
Maltose	0.07±0.06e	0.52±0.11d	0.98±0.12c	1.15±0.04b	1.33±0.03a
Glucose	59.07±14.19c	399.70±24.80a	358.07±11.90b	348.26±15.75b	335.42±29.79b
Fructose	0.98±0.23d	145.34±16.06c	276.75±14.75a	261.27±51.14ab	212.34±36.43b
Galactose	13.26±0.94a	6.36±0.94b	6.02±1.23b	3.65±0.37c	3.66±0.46c

*1. Koji mash was prepared by mixing koji with syrup (65% sucrose) for 2 days before sufu fermentation was proceeded. 2h-koji referred to the koji which was collected 2 h after mixing koji mash with tofu.

*2. Values are presented as means ± SD (n = 3). Means in the same row with different letters were significantly different by Duncan's multiple range test ($p < 0.05$).

*3. Not detected

content of koji decreased when fermentation period was extended. The reduction in sucrose content may be caused by the action of invertase which was able to breakdown sucrose to fructose and glucose. Meanwhile, the koji collected from the koji mash showed a higher level of maltose, glucose and fructose when compared with that found in koji before the preparation of koji mash.

Summary, the incorporation of various kind of enzyme such as amylase, α-galactosidase, β-fructosidase/invertase and α-glucosidase, released by *Asp. oryzae* into tofu during the manufacturing of Taiwanese sufu, is expected to result in the changes of sugar contents observed.

4. Changes in the organic acid content of tofu during sufu fermentation

Acids often impart flavors in addition to the sour taste. The dominant flavor of organic acids is sourness, but they also contribute bitterness and astringency; the proportions of these sensations vary with different acids (Lawless *et al*., 1996). Additionally, antimicrobial activity of organic acid may also improved the keeping quality of a food product (Adams, 1988). As shown in Table 5, only small amount of oxalic and lactic acids was detectable in the non-fermented tofu samples. While four organic acids including oxalic lactic, acetic and citric acid were detected from the tofu samples fermented for 8 days or longer. It was generally found that content of these organic acids, except citric acid in the tofu cube increased to the highest point after 8 days of fermentation. Further extending the fermentation time did not change the contents of these organic acids significantly ($p > 0.05$). On the other hand, citric acid content of tofu was found to increase with the extension of fermentation time. The 46-day-sufu showed a citric acid content of 2.83mg/g dried, which is significantly ($p < 0.05$) higher than 0.15 and 0.91 mg/g dried tofu noted in the 8-day-sufu and 16-day-sufu respectively. Of the organic acids examined, acetic acid was found to be the predominant organic acid in the Taiwanese sufu. Although acetic acid was not detectable from the non-fermented tofu sample, the 8-day-sufu showed an acetic acid content of 18.01mg/g dried tofu which is not significantly different ($p > 0.05$) from those note with the 16-day-sufu and 46-day-sufu. The increased level of organic acid in the sufu samples observed is in agreement with that observed on douchi, an *Aspergillus*-fermented product of fresh soybean (Zhang *et al*., 2007) and might account for the reduced pH of sufu than the non-fermented tofu as reported by Li (2009). It is evident that that these organic acids was formed through the fermentation of sugar catalyzed by the enzyme produced by

Table 5. Changes of various organic acid contents in tofu during sufu fermentation

Content of organic acid (mg/g dried tofu)	Non-fermented tofu	Time after fermentation			
		2h*[1]	8 days	16 days	46 days
Oxalic	0.12±0.01b*[2]	0.17±0.03b	0.51±0.09a	0.58±0.07a	0.47±0.13a
Lactic	0.14±0.12b	ND*[3]	1.79±0.21a	2.35±0.26a	2.12±0.71a
Acetic	ND	1.13±0.22b	18.01±1.14a	17.18±1.09a	18.03±1.14a
Citric	ND	ND	0.15±0.13c	0.91±0.44b	2.83±0.34a
Succinic	ND	ND	ND	ND	ND

*1. Tofu cubes were collected 2 h after mixing with koji mash.

*2. Values are presented as means ± SD (n = 3). Means in the same row with different letters were significantly different by Duncan's multiple range test ($p < 0.05$).

*3. Not detected.

the Asp. oryzae. The hydrolytic enzymes released by *Asp. oryzae* which leached out from the koji into the infusion (syrup) and those penetrated into tofu cubes may catalyze the hydrolysis of complex sugars of tofu substrates. Hence, the results exhibited a significant yielding of various hydrolytic products as well as substantial amounts of organic acids in sufu by utilizing tofu substrates. The broken down sugars (glucose) went through glycolysis and down into pyruvates and then enter Kreb cycle which formed citric, oxalic and succinic acid as by products.

5. Changes in the organic acid content of rice-soybean koji during sufu fermentation

Table 6 shows the changes of organic acids in rice-soybean koji before and during the fermentation of sufu. Among the various organic acids examined, the prepared koji and the 2h-koji which was collected immediately after mixing with the tofu cubes was found to contain oxalic, lactic and citric acid, apparently they were formed through the catalytic action of enzymes produced by the *Asp. oryzae* during the preparation of koji. As the extension of sufu fermentation, content of oxalic acid in the koji collect from the tofu-koji mash mixture, decreased. While, the level of lactic, acetic and citric acid increased in the collected koji and showed a content of 4.80, 23.87 and 4.82 mg/g dried koji after 46 days of sufu fermentation. Zhang *et al*. (2008) indicated that lactic acid was formed through the reduction of pyruvic and the transformation of malic acid. It provides a sweeter acidic taste which is generally favorable to the palate (Tsangalis and Shah, 2004). As shown in Table 5 and 6, lactic acid content of tofu and koji increased as the time of fermentation was extended. Its content reached 2.21 mg/g dried tofu and 4.80 mg/g dried koji, respectively, at the end of the 46 days fermentation. In Chinese sufu fermented by *Actinomucor* Taiwanese, oxalic acid was the main organic acid (Hwang and Chou, 1994), while it was found to be

minor acid, quantitatively in Taiwanese sufu. Additionally, succinic acid was not detected in the sufu and koji samples examined, despite its presence in the *Act. Taiwanese*-fermented Chinese sufu (Hwang and Chou, 1994). This discrepancy may be attributed to the difference in the microorganism involved in the fermentation process. Different from that observed on tofu cubes, presence of acetic acids was found in rice-soybean koji before mixing with syrup for sufu fermentation. This may be due to the formation of acetic acid requires a saccharification step in addition to the alcohol fermentation and the oxidation of ethanol. Saccharification step in the process of vinegar fermentation was carried out from materials rich in sugar such as rice-starch in rice-koji (Hesseltine, 1965; Haruta *et al*., 2006).

Table 6. Changes of various organic acid contents in rice-soybean koji during sufu fermentation

Content of organic acid (mg/g dried koji)	Koji	Time after fermentation			
		2h*[1]	8 days	16 days	46 days
Oxalic	2.04±0.36a*[2]	0.66±0.07b	0.18±0.04c	0.25±0.04c	0.36±0.05bc
Lactic	1.35±0.39c	0.73±0.13c	3.90±0.42b	5.53±0.84a	4.80±0.09a
Acetic	3.36±0.72c	2.63±0.45c	20.10±0.53b	23.63±0.86a	23.87±0.99a
Citric	ND*[3]	ND	0.17±0.06c	3.25±0.45b	4.82±0.29a
Succinic	ND	ND	ND	ND	ND

*1. Koji mash was prepared by mixing koji with syrup (65% sucrose) for 2 days before sufu fermentation was proceeded. 2h-koji referred to the koji which was collected 2 h after mixing koji mash with tofu.

*2. Values are presented as means ± SD (n = 3). Means in the same row with different letters were significantly different by Duncan's multiple range test ($p < 0.05$).

*3. Not detected

7. Confirming the genotype of the *Salmonella typhimurium* TA100

A set of histidine-requiring strains was used for this antimutagenic assay. The *Sal. typhimurium* histidine reversion system is an assay that detects a mutation in the gene of a histidine-dependent bacterial strain. When the bacterial strain was exposed to a genotoxic agent, point mutations occurred. This mutation included that the bacterial were no longer required histidine for growth. The tester strain genotypes should be confirmed when a new set of frozen permenents or lyophilized cultures is prepared, when the number of spontaneous revertants per plate falls out of the normal range or when there is a loss of sensitivity to standard mutagens (Maron and Ames, 1983). The results for confirming the genotypes of TA100 were illustrated in Table 7.

6.1 Histidine requirement

The results exhibited that no growth of *Sal. typhimurium* TA100 on biotin plates and growth on histidine-biotin plates. It confirms the histidine dependent requirement for the growth of *Sal. typhimurium* TA100 strain.

6.2 *rfa* mutation

A clear zone of *Sal. typhimurium* TA100 inhibition produced around the crystal violet filter disc. Death of bacterial was due to *rfa* mutation that caused partial loss of lipopolysaccharide wall in the bacterial and it had increased permeability of the cell to large molecules such as crystal violet to enter

6.3 uvrB mutation

Sal. typhimurium TA100 exhibit sensitivity towards ultraviolet light and it only grow on the un-irradiated side of the plate. This uvrB mutation is a deletion of gene

coding for the DNA excision repair system which also increases the system's ability to detect mutagen.

6.4 R-factor

Sal. typhimurium TA100 exhibit resistance to ampicillin and it had growth along the streaks on ampicillin plates. Tester strain TA100 contains R-factor plasmid (pKM 101) which makes its resistant to ampicillin. The R-factor strains should be tested routinely for the presence of the ampicillin resistance factor as the plasmid is unstable and can be lost from the bacteria.

Table 7. Genotype test of *Sal. typhimurium* TA100

	Bio/His[*2]	Bio[*3]	uvrB[*4]	Rfa[*5]	R-factor[*6]
TA100	**+** *[1]	-	-	-	**+**

[*1] + : growth; - : no growth.

[*2] Medium contains biotin and histidine.

[*3] Medium contains biotin.

[*4] A deletion of a gene coding for the DNA excision repair system, resulting in greatly increased sensitivity in detecting many mutagens.

[*5] Lack of lipopolysaccharide on cell membrane leads to inhibition by crystal violet.

[*6] R-factor against ampicillin.

7. Toxic and mutagenic effect of tofu and sufu extract on *Sal. tyhimurium* TA 100

Toxicity of sample may lead to erroneous results. However extracts of sufu samples and non-fermented tofu exhibited non-toxicity on *Sal. typhimurium* TA100 at the four dose levels (0.625-5.0 mg extract/per plate) with or without S9 mix (Table 8). Since the survival of *Sal. typhimurium* TA100, in the presence of either tofu or sufu extract was close to those of the control. Besides toxicity, if the sample showed a mutagenic effect on *Sal. typhimurium* TA100, this may lead to improper interpretation of the antimutagenic assay. Thus mutagenicity of the tofu and sufu extracts were examined. As shown in Table 9, the revertants in presence of either the extract of sufu or the non-fermented tofu cubes for *Sal. typhimurium* TA100 both with and without the S9 mixture were close to those for the negative control (spontaneous revertants in absence of non-fermented tofu or sufu extracts) and were less than twice that of spontaneous revertants. The results demonstrated that extract of non-fermented tofu or sufu with various fermentation periods at the test concentrations (0.625-5.0 mg extract/per plate) had no mutagenic effect on *Sal. typhimurium* TA100.

8. Antimutagenicity of non-fermented tofu and sufu extracts against 4-NQO and DMAB in *Sal. tyhimurium* TA 100

In this study, suppression effect of methanol extracts of non-fermented tofu and sufu against mutagenesis induced by 4-NQO and DMAB were examined. DMAB is an experimental indirect colon carcinogen which need S9 rat liver to activate. Chinese sufu was reported to exert the antimutagenic effect against a few indirect-acting mutagens such as 3-amino-1-methyl-5H -pyrido [4,3-b] indole acetate (Trp-P-2), benzo(α)pyrene (BaP), 2-amino-3-methylimidazo[4,5-f] quinoline (IQ), and 2-amino-3,8-dimethylimidazo[4,5-f]

Table 8. Toxicity of non-fermented tofu and sufu extracts against *Sal. typhimurium* TA100

Extracts (mg/plate)	No. of colonies (log CFU/plate)				
	Non-fermented tofu	Time after sufu fermentation			
		2h*[1]	8 days	16 days	46 days
			Without S9		
None	7.98±0.10a*[2]	7.98±0.10a	7.98±0.10a	7.98±0.10a	7.98±0.11a
5.0	8.01±0.02a	8.09±0.06a	8.05±0.10a	7.94±0.13a	7.94±0.09a
2.5	8.06±0.17a	8.04±0.13a	7.97±0.06a	8.05±0.01a	8.02±0.14a
1.25	8.00±0.01a	7.96±0.19a	8.08±0.06a	8.03±0.02a	7.96±0.11a
0.625	7.97±0.25a	8.03±0.16a	8.10±0.16a	8.03±0.18a	7.97±0.11a
			With S9		
None	7.85±0.05a	7.85±0.05a	7.85±0.05a	7.85±0.05a	7.85±0.05a
5.0	7.85±0.04a	7.85±0.09a	7.82±0.04a	7.79±0.08a	7.78±0.10a
2.5	7.77±0.08a	7.85±0.08a	7.83±0.19a	7.75±0.08a	7.78±0.08a
1.25	7.77±0.13a	7.82±0.19a	7.87±0.03a	7.86±0.11a	7.79±0.09a
0.625	7.83±0.10a	7.89±0.04a	7.86±0.05a	7.86±0.03a	7.72±0.02a

*[1] Tofu cubes were collected 2 h after mixing with koji mash.

*[2]Results are presented as means ± SD from three separate experiments. Mean with the same letters in the same column are not significantly different ($p > 0.05$).

Table 9. Mutagenicity of non-fermented tofu and sufu extracts against *Sal.typhimurium* TA100

Extracts (mg/plate)	No. of colonies (log CFU/plate)									
	Non-fermented tofu		Time after sufu fermentation							
			2h*[1]		8 days		16 days		46 days	
	Revertants (CFU/plate)	Mutagenicity	Revertants (CFU/plate)	Mutagenicity	Revertants (CFU/plate)	Mutagenicity	Revertants (CFU/plate)	Mutagenicity	Revertants (CFU/plate)	Mutagenicity
					Without S9					
None	80±5	1.00	80±5	1.00	80±5	1.00	80±5	1.00	80±5	1.00
5.0	74±5	0.93	76±5	0.95	82±5	1.03	78±4	0.98	84±6	1.05
2.5	79±11	0.99	76±3	0.95	79±7	0.99	75±5	0.94	78±2	0.98
1.25	81±10	1.01	76±8	0.95	83±4	1.04	78±4	0.98	82±5	1.03
0.625	82±5	1.03	74±0	0.93	83±3	1.04	80±3	1.00	76±4	0.95
					With S9					
None	90±6	1.00	90±6	1.00	90±6	1.00	90±6	1.00	90±6	1.00
5.0	98±9	1.09	105±22	1.17	79±16	0.88	73±13	0.68	74±12	0.82
2.5	101±16	1.12	107±15	1.19	73±3	0.81	72±3	0.80	75±8	0.83
1.25	112±21	1.24	94±11	1.04	83±6	0.92	80±4	0.89	80±5	0.89
0.625	110±11	1.22	96±11	1.07	90±11	1.00	96±28	1.07	92±9	1.02

*[1] Tofu cubes were collected 2 h after mixing with koji mash.

*[2] Results are presented as means ± SD from three separate experiments.

quinoxaline (MeIQx) that are reported to be generated from overheating of protein rich food or found in smoked products (Ren *et al*., 2006). On the other hand, 4-NQO is a direct mutagen which exerts potent intracellular oxidative stress and its metabolic products binds to DNA predominantly at guanine residues. Fermentation with bacteria or fungi has been reported to enhanced the antimutagenicity of various bean products against 4-NQO (Park *et al*., 2003; Kanojia and Vaidya, 2006; Lin and Chou, 2006; Hsieh and Chou, 2006; Hung *et al*., 2007).

Table 10 shows the antimutagenic activities of the extracts of non-fermented tofu and sufu against 4-NQO and DMAB. It was found that extracts of the non-fermented tofu and sufu exerted antimutagenic activity against the mutagens examined. Additionally, it was also noted that methanol extracts of sufu exhibited a dose-dependent antimutagenic activity against the mutagens. Compounds such as vitamin E, saponin, phytic acid, linoleic acid, genistein and daidzein, commonly found in bean and bean products, have been reported to exhibit ntimutagenic activity (Tavan *et al*., 1997; Miyazawa *et al*., 1999; Park *et al*., 2003). All these compounds, may all contributed to the observed antimutagenicity of the extracts examined.

As shown in Table 10, antimutagenicity of sufu extract at the same dosage level increased as the fermentation period was extended. For example, extract of the non-fermented tofu at 5.0 mg/plate showed an antimutagenicity of 4.17 % and 18.97 % against 4-NQO and DMAB, respectively. While a higher suppression rate of 53.32% and 93.08 % was observed with extract of the 46-day-sufu extract at the same dosage level. This phenomenon observed is similar to the reports of Park *et al*. (2003), Hung *et al*. (2007) and Hsieh and Chou (2006). Edenharder and Tang (1997) reported that the flavonoid glycosides

Table 10. Effects of the non-fermented tofu and various fermentation period of sufu extracts against the mutagenic effects of 4-NQO and DMAB on *Sal.typhimurium* TA100

Mutagen	Extracts (mg/plate)	No. of colonies (log CFU/plate)									
		Non-fermented tofu		Time after sufu fermentation							
				2h*[1]		8 days		16 days		46 days	
		Revertants (CFU/plate)	Inhibition rate (%)*[2]	Revertants (CFU/plate)	Inhibition rate (%)	Revertants (CFU/plate)	Inhibition rate (%)	Revertants (CFU/plate)	Inhibition rate (%)	Revertants (CFU/plate)	Inhibition rate (%)
4-NQO						Without S9					
	None	679±70		679±70		679±70		679±70		679±70	
	5.0	A660±42a*[3]	A4.17c	C473±48b	A34.12b	C389±53c	A50.62a	C360±27c	A52.98a	C365±47c	A53.32a
	2.5	A639±20a	A5.69c	B550±36b	B22.75b	B496±30b	B30.41b	BC405±62c	AB45.21a	C375±55c	A50.73a
	1.25	A687±53a	A2.82c	B553±39bc	B22.86b	A587±45b	C16.61b	B475±40c	B34.35a	B579±43b	B15.93b
	0.625	A675±63a	A0.79a	A635±15a	C7.38a	A625±33a	C7.83a	A677±28a	C1.41a	A693±50a	B3.27a
DMAB						With S9					
	None	220±20		220±20		220±20		220±20		220±20	
	5.0	A203±13a	A18.97c	B193±6a	A32.56c	C120±27b	A68.21b	D88±11c	A88.72ab	D83±7c	A93.08a
	2.5	A215±10a	A12.31b	AB206±3a	AB23.85b	B158±19b	B34.87b	C123±12c	B60.26a	C111±6c	B72.05a
	1.25	A239±26a	A2.56c	A193±6b	B9.74c	AB182±3c	B23.85bc	B155±4d	B42.56ab	B132±13d	B60.26a
	0.625	A241±24a	A0.00b	A216±2b	B7.18b	A206±7b	B10.77b	A201±10b	C19.23ab	A172±3c	C38.72a

*[1] Tofu cubes were collected 2 h after mixing with koji mash.

*[2] Data were expressed as means±SD (CFU/plate) of three separate experiments. Value in the same row with different lower case letters (a, b, c, d) and column with upper case letters (A, B, C, D) are significantly different ($p < 0.05$).

*[3] Inhibition rate (%) = 1−number of induced revertants in the presence of extract /number of induced revertants in the absence of extract] ×100. Numbers of spontaneous revertants were 87±6 and 90±6, respectively, when studies on 4-NQO and DMAB were performed.

are less antimutagenic then the corresponding aglycones such as daidzein, glycitein and genistein. While Huang (2008) observed that contents of daidzein and genistein of Taiwanese sufu increased as fermentation period extended. In addition, incordance with report of Park *et al*. (2003), the content of linoleic acid, which possesses antimutagenicity, was also increased as sufu fermentation period extended (Table 2). The increased content of aglycone and linoleic acid may all contribute to the increased antimutagenicity observed with the extract of sufu with a longer period of fermentation. Additionally, formation of other bioactive compounds in tofu substrates following fermentation as reported by Park *et al*. (2003) may also contributed to the increased antimutagenicity of sufu.

In nearly all tested concentration examined, extracts of non-fermented tofu and sufu exhibited a lower inhibition rate against 4-NQO than DMAB. This indicates that the antimutagens present in the sufu might have acted differently with the direct-acting mutagen 4-NQO and the indirect-acting DMAB. A similar phenomenon was previously observed on the *Lactobacillus acidophilus* fermented-soymilk (Hsieh and Chou, 2006).

To further explore the effect of fermentation on the antimutagenicity of the sufu extracts, the efficient concentration of the test samples that inhibit 50% mutagenic activity (IC_{50}) was obtained by interpolation from regression analysis. Table 11 shows the IC_{50} for the antimutagenicity of the non-fermented tofu and sufu extract against 4-NQO and DMAB in *Sal. typhimurium* TA100. A significantly ($p < 0.05$) reduced IC_{50} or increased antimutagenicity was noted with the extract of sufu as fermentation time was extended. For example, the extract of non-fermented tofu showed an IC_{50} of 187.37 and 15.11 mg/plate against mutagenesis induced by 4-NQO and DMAB, respectively. While a significantly ($p < 0.05$) less IC_{50} of 4.06 and 0.90 mg/plate for 4-NQO and DMAB, respectively was noted

with the extract of the 46-day-sufu. This data further illustrated that antimutagenicity of the extract of sufu varied with fermentation periods and the mutagens.

Table 11. Half-inhibition (IC_{50}) of the antimutagenicity of sufu with various fermentation periods extract against 4-NQO and DMAB in *Sal. typhimurium* TA100

methanol extracts	IC_{50} (mg/plate)*[1] of tofu	
	4-NQO (-S9)	DMAB (+S9)
non-fermented tofu	187.37±73.84A*[2]	15.11±7.03A
2h-sufu*[3]	8.27±1.91B	8.24±3.54B
8-day-sufu	4.84±0.90C	3.75±0.93C
16-day-sufu	4.11±0.63C	2.13±0.41C
46-day-sufu	4.06±0.39C	0.90±0.19D

*[1] IC_{50} is the efficient concentration of the test samples that inhibit 50% mutagenic activity. IC_{50} was obtained by interpolation from linear regression analysis.

*[2] Values are given as mean ± SD from three separate experiments. Means with different letters in the same column are significantly different ($p < 0.05$).

*[3] Tofu cubes were collected 2 h after mixing with koji mash.

V. Conclusion

1. A total of 90 volatile compounds were identified from the non-fermented tofu and sufu sample examined. A large number of these volatile compounds belonged to aldehyde, alcohol and ester. As the aging period extended, concentrations of nearly all the volatile fractions, particularly alcohol and ester, increased ($p < 0.05$). Volatile components of Taiwanese sufu were dominated by fruity and sweet flavor from a majority of the esters and alcohols.

2. The content of raffinose, stachyose and sucrose decreased ($p < 0.05$) during fermentation while the content fructose and glucose increased ($p < 0.05$). Glucose was the most abundant sugar detected in the sufu and the koji collected from the koji mash.

3. Four organic acids including oxalic, lactic, acetic and citric acid were detected from the tofu and rice-soybean koji samples after 8 days of fermentation or longer. It was generally found that the content of these organic acids increased ($p < 0.05$) as the fermentation period was extended. Of the organic acids examined, acetic acid was found to be the predominant organic acid in the Taiwanese sufu and koji.

4. The antimutagenic activity of tofu extract toward 4-NQO and DMAB could be enhanced significantly ($p < 0.05$) through fermentation. Antimutagenicity of the extract of sufu increased as the fermentation time was extended. Sufu extract exerted a significantly higher ($p < 0.05$) antimutagenicity against DMAB then 4-NQO.

VI. References

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