Characterization and Product Innovation of Sufu

A Chinese Fermented Soybean Food

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Contents

Abstract

Over the centuries, Chinese people have consumed soybeans in various forms of traditional fermented soybean foods. Sufu (*Furu*), a cheese-like product originating in China, is one of the most popular fermented soybean foods in China, and is becoming popular in Chinese shops all over the world. It is made by fungal solid-state fermentation of tofu followed by salting and ripening in dressing mixture containing various ingredients. Several types of sufu can be distinguished according to processing method or colour and flavour.

High levels of surviving but inactive bacterial endospores and concomitant high numbers of culturable mesophilic aerobic bacteria were found in all process stages of sufu production as well as in commercial sufu. Most samples contained only low levels ($< 10^3$ cfu/g) of *Bacillus cereus*, whereas no Enterobacteriaceae were detectable in any of the commercial and experimental sufu products. From a microbiological safety point of view, sufu products are stable and safe when they are produced under the conventional conditions.

Phylogenetic relations based on sequencing of genomic DNA-its-1-4 regions of collected fungal starters and of relevant control strains indicate that the genera *Mucor*, *Actinomucor* and *Rhizopus* form distinct and homogeneous clusters. Most *Mucor* and *Actinomucor* spp., especially *Actinomucor elegans* (the most frequently used starter) cannot grow well over 30°C. *Rhizopus oligosporus* has similar growth and enzyme production abilities as *A. elegans* and could thus offer an alternative for the latter during the hot summer season.

NaCl strongly affects the changes of microflora, textural properties and the hydrolysis of protein and lipid in sufu. Low-salt sufu (7-8% salt content) is qualified to be termed “finished product” after only 45 d of ripening, which takes more than 3 months for conventional red sufu with over 11% salt content. However, sufu will spoil during the ripening stage at salt contents of 5% or lower.

SDS-PAGE profiles showed that after 60 d of ripening, all protein subunits had disappeared in sufu with 8% salt content, which indicates that most proteins were degraded into peptides and amino acids. Consequently, a large amount of free amino acids, notably glutamic acid, were found in matured sufu.

Foreword

This thesis contains the main results that were obtained during the past three and a half years of my research. My original purpose was to obtain a PhD degree rather than to produce this book. As I registered as a PhD student at Wageningen University, producing this thesis turned out to be a prerequisite to reach my goal! Of course, it would have been impossible for me to complete my study and to produce this booklet without the help from many people in the Netherlands and in China. It is with great pleasure that I am able to take the opportunity to thank them.

I would like to thank Dr. Cor van den Berg for introducing me to the Laboratory of Food Microbiology when I studied first time in Wageningen in 1997. Then I had the chance to apply for the PhD program with the help of Dr. Fre Pepping.

I am deeply indebted to my promoter Prof. Frans Rombouts, and my co-promotor Dr. Rob Nout. They, together, provided me the PhD position and the strong supervision. I could always get in-depth and clear comments and suggestions from Prof. Rombouts when we worked on the program. Talking with him about science and life was equally comfortable and attractive to me.

Discussions with Rob (actually, I always call him Mr. Nout) were never enough because it is so easy and pleasant for me to talk with him. His open and bright charisma provided me with encouragement and a constant source of inspiration. His daily supervision offered me a solid basis to carry out this study. Most of the chapters in the thesis have been published or submitted as separate paper. The efficient and critical reading and correcting of the manuscripts has contributed substantially to this timely completion. Special thanks go to his wife (Mrs. Nout) for her support and understanding when Rob was again reading and correcting my papers at home at weekends. Nout's family not only regarded me as one student but they also approached me as a friend, enjoying many activities together during my stay in the Netherlands.

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Chapter 1

General Introduction

Fermented foods

Fermented foods are those foods which have been subjected to the action of microorganisms or enzymes that cause desirable biochemical changes and significant modification of the food (Campbell-Platt, 1994). Fermented foods have been with us since humans arrived on Earth. They will be with us far into the future as they are the source of alcoholic foods/beverages, vinegar, pickled vegetables, sausages, cheeses, yogurts, leavened and sour-dough breads, vegetable protein amino acid/peptide sauces and pastes with meat-like flavours, etc. (Steinkraus, 1997). Fermented foods and beverages are of great significance because they provide and preserve vast quantities of nutritious foods in a wide diversity of flavours, aromas and textures that enrich the human diet. They globally provide about 20-40% of our food supply (Campbell-Platt, 1994).

Food fermentation represents one of the oldest known uses of biotechnology and the main advantages of food fermentation can be categorized as follows (Campbell-Platt, 1994; Steinkraus, 1994; 1997):

Food fermentation develops a diversity of appealing exteriors, textures, aromas and flavours in food substrates. The increasing popularity of different types of e.g., fermented milks has as much to do with different textures that are created during the fermentation, as with significant flavour changes. The formation of diacetyl by heterofermentative lactic acid bacteria in fermentations of yoghurt and butter is not only important as a major flavour component, but it may also help inhibit less desirable microorganisms.

Food fermentations create the utility of volume reduction and result in decreased cooking times and fuel requirements. Many fermented foods are ready-to-eat foods, such as cheese, sufu, wine, pickled vegetables, vinegar etc.

Food could get a longer shelf-life through lactic acid, alcoholic, acetic, and alkaline fermentations. Lactic acid fermented foods include yoghurts, cheeses, sauerkraut, and sour-dough bread. Alcoholic fermented beverages include beers, wines, Chinese liquor, and Japanese sake. Alcohol has antimicrobial effects and contributes to the stability and preservation of alcoholic beverages.

Food fermentations not only enrich the food substrates with protein, essential amino acids and fatty acids, but also with vitamins. Biosynthesis of B vitamins in food fermentations has been recognized to be of major nutritional significance, especially in the area where high-carbohydrate diets, particularly maize and sorghum diets can be deficient in essential B vitamins.

Food fermentations also have functions in digestibility, bio-availability, and

detoxification. For instance, the practice of soaking and fermenting peeled bitter cassava tubers allows the endogenous linamarase to hydrolyse the linamarin thus rendering the cassava tubers safe to eat as fermented products, such as West-African *gari*.

Fermented foods generally have a very good safety record even in the developing world where foods are often manufactured by people without formal training, and under conditions of poor hygiene.

Fermented soybean foods and their benefits

During the past several decades, soybeans have become an increasingly important agricultural commodity, with a steady increase in annual production. Major groups of soybean foods include traditional soybean foods, soy oil, soy protein products (e.g. used in bakery, breakfast cereals and infant formulas), new-generation soybean foods (e.g. meat alternatives like soy burgers), soy-enriched foods (e.g. soy snacks) and functional soy ingredients/dietary supplements (e.g. phytochemicals like lecithin, isoflavones, tocopherols and sterols) (Liu, 2000).

Traditional soybean foods have been consumed in Asia for many centuries, and they remain popular and can be classified into two categories: non-fermented and fermented. Non-fermented soybean foods include soymilk, tofu, soy-sprouts and others, whereas fermented soybean foods include among others, soy sauce, sufu, miso, tempe and natto (Kiers, 2001).

Primary benefits of soybean fermentation are improvement of sensory quality and nutritional value, rather than preservation. First of all, development of flavours and aromas through fermentation is a major characteristic of fermented soybean foods (Steinkraus, 1996). Texture dramatically changes during fungal fermentation leading to a cake-like product with a meat-like taste. Secondly, raw soybeans contain significant levels of anti-nutritional factors (ANFs), such as phytates. It has been reported however, that some of these bioactive molecules have potential beneficial health effects as well. The majority of the ANFs are removed or destroyed during soaking, cooking and fermentation of the soybeans (Nout & Rombouts, 1990; Tawali et al., 1998). Thirdly, soybean fermentation has shown to improve the bioavailability of dietary zinc and iron (Hirabayashi et al., 1998; Kasaoka et al., 1997), and can result in increased levels of vitamins (Denter et al., 1998; Sarkar et al., 1998).

Chinese fermented soybean foods

The soybean (*Glycine max* (L.) Merrill, family Leguminosae, subfamily Papilionoideae) originated in China. The Chinese use 58% of the soybean production for food (Liu, 2000), so soybean foods serve a large population in the world. The remaining 42% are processed into industrial products and animal feed.

Historically, most traditional soybean foods originated in China and were introduced later to other parts of East and Southeast Asia. Only during the recent 20-30 years they have made a significant inroad into western cultures and diets (Golbitz, 1995).

The major Chinese fermented soybean foods comprise soy sauce, sufu, bean paste, and *dou-chi*. The largest quantity of fermented soybean foods produced in China is represented by soy sauce, followed by sufu with an annual production of about 300,000 metric tons. With the development of fermentation technology, soy sauce (especially the Japanese product) has become a condiment of worldwide popularity. In contrast, most Chinese fermented soybean foods are still consumed domestically.

Sufu is a cheese-type product that can be used in the same way as cheese. It is made by fungal solid-state fermentation of tofu. The resulting "pehtze" is salted, followed by ripening in dressing mixture containing various ingredients. The merits of sufu are not yet appreciated or even known in western countries. Considering the increasing interest in non-meat protein foods, sufu may also become a world-commodity, especially when its organoleptic properties can be adjusted in agreement with regional food preferences.

Aim and outline of the thesis

This thesis describes the characterization and deals with aspects of product innovation in sufu. The main objective is to understand the microbiological and biochemical characteristics of sufu and the sufu production process, and to create a basis for improvement of processing technology to obtain high quality products.

Chapter 2 is a review about the product sufu and its manufacture, based on scientific data published in Chinese and international sources. For the first time, Sufu is classified in English language into several types, according to the processing method employed, or according to colour and flavour of the final product. Chemical composition and nutritional quality of sufu are also discussed.

In **Chapter 3** the microbiological composition and safety of commercial sufu from different regions of China are investigated. Microbiological analyses are reported, and a microbiological guideline for safe commercial sufu is proposed based on the analyses results.

In **Chapter 4** mould starters used in commercial pehtze fermentation are identified based on macroscopic and microscopic morphology as well as on relevant physiological distinctive features. Phylogenetic relations are discussed based on sequencing of genomic DNA.

Chapter 5 reports microbial changes during sufu production. The quantitative evolution of microflora of sufu was studied throughout the production, with special reference to the effect of different salt contents during the ripening.

Chapter 6 deals with the effects of temperature and relative humidity on

growth and enzyme production by the fungi *Actinomucor elegans* and *Rhizopus oligosporus* during sufu pehtze preparation. It is shown that *R. oligosporus* is a potential alternative to *A. elegans* as sufu pehtze starter during hot seasons.

Chapter 7 focuses on the effect of NaCl on textural changes and protein and lipid degradation during the ripening stage of sufu. Lower salt content in sufu resulted in reduced hardness and elasticity, and higher extent of degradation of protein and lipid, which enables a shorter ripening time of sufu.

In **Chapter 8** amino acid profiles of sufu are discussed. Amino acids are of relevance because of their taste-enhancing properties. Profiles of total and free amino acids were measured during consecutive stages of sufu manufacture, i.e. tofu, pehzte, salted pehtze and in white, red and gray sufu ripening in dressing mixtures of different salt content.

In **Chapter 9** the findings of this thesis in relation to the microbiological and chemical characterization of sufu are integrated and discussed.

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Chapter 2

Review: Sufu -- A Chinese Fermented Soybean Food

Abstract

Sufu or furu is a fermented soybean product originating in China. It is a cheese-like product with a spreadable creamy consistency and a pronounced flavour. Sufu is a popular side dish consumed mainly with breakfast rice or steamed bread. It has a long history and written records date back to the Wei Dynasty (220-265 AD). Sufu is made by fungal solid state fermentation of tofu (soybean curd) followed by aging in brine containing salt and alcohol. The present review is based on scientific data published in Chinese and international sources. Several types of sufu can be distinguished, according to processing method or according to colour and flavour. Choice of processing can result in mould fermented sufu, naturally fermented sufu, bacterial fermented sufu, or enzymatically ripened sufu. Depending on the choice of dressing mixture, red, white or grey sufu may be obtained. The stages of the process are discussed and include the preparation of tofu, the preparation of pehtze, salting and ripening. Fungal starters include *Actinomucor* spp., *Mucor* spp. and *Rhizopus* spp. The chemical composition is discussed with particular reference to the proximate composition, the amino acid content and profile, as well as the volatile flavour components of various types of sufu.

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INTRODUCTION

Over the centuries, the Chinese have used microorganisms to convert agricultural commodities into fermented food (Chen, 1989). These fermented foods have improved the quality of the diets of Chinese people and also enriched dishes all over the world. Traditional Chinese fermented foods cover a wide range of products, such as soy sauce, sufu, vinegar, distilled spirits, rice wine, fermented vegetable and meat products.

Sufu, *Fu-ru* written in hieroglyphics, is a fermented soybean curd and a highly flavoured, soft creamy cheese-type product which can be used in the same way as cheese (Su, 1986).

Sufu is the name for the product that first appeared in the literature (Wang & Hesseltine, 1970). Literally, sufu (*fu-ru*) means “moulded milk” and tosufu (*dou-fu-ru*) means "moulded soymilk". Because of the numerous dialects used in China and the difficulties of phonetic translation from Chinese into English, sufu has appeared in literature under many different names. The following synonyms for sufu in the literature have been found: *sufu, tosufu, fu-ru, dou-fu-ru, tou-fu-ru, toe-fu-ru, jiang-dou-fu, fu-yu,* and *foo-yue*. Sufu is also known as *tofuyo, nyu-fu* or *fu-nyu* in Japan (Yasuda & Kobayashi, 1989), *chao* in Vietnam, *ta-huri* in The Philippines, *taokaoan* in Indonesia and *tao-hu-yi* in Thailand (Beuchat, 1995). These names confuse Western people as well as the Chinese. Officially, sufu should be named *Furu* (or *Doufuru*) in Chinese.

Manufacture of tofu (soybean curd) began during the era of the Han Dynasty. The “*Ben-Cao-Gang-Mu*” (Chinese Materia Medica), compiled by Li Shi-zhen in 1597, indicated that tofu was invented by Liu An (179 BC to 122 BC), King of Weinan (Shi & Ren, 1993; Steinkraus, 1996). However, it is not known when sufu production began. Due to the long history and incomplete written records, no attempt was made to search for its origin. The first historical record mentioned that the sufu process was carried out in the Wei Dynasty (220~265 AD) (Wang & Du, 1998; Hong, 1985). It became popular in the Ming Dynasty (1368~1644), and there are many books describing sufu processing technologies (Zhang & Shi, 1993).

Sufu products are manufactured both commercially and domestically, and the annual production is estimated over 300,000 metric tons in China. Sufu is consumed as an appetizer and a side dish, e.g. with breakfast rice or steamed-bread. Sufu adds zest to the bland taste of the rice and flour diet. Since it is made from soybeans and is an easily digested and nutritious protein food, Chinese people consider it a health food. The food encyclopedia written by Wang Shi-Xun (1861) in the Qing Dynasty described the food as follows: “Hardened tofu is difficult to digest and not healthy for children, elderly persons or ill persons. Sufu fermented from tofu is better because it is matured and very good for patients.” Sufu is a highly flavoured, creamy cheese-like product, so it would be expected to be

suitable for use in western countries as a healthy, non-cholesterol food from plant origin. Table 2.1 summarizes some major chemical constituents of sufu.

Table 2.1 Proximate composition of commercial sufu

Component		**Content***
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

Sources: Wang and Du (1998) and Su (1986).

* per 100 g sufu fresh weight

THE CLASSIFICATION OF SUFU

There are many different types of sufu, which are produced by various processes in different localities in China (Li, 1997; Wang & Du, 1998).

Four types of sufu can be distinguished according to the processing technologies. The base for all form types is tofu, a curd from soybean milk by adding calcium salts.

Mould-fermented sufu

Four steps are normally involved in making this type of sufu; (1) Preparing tofu, (2) Preparing pehtze (pizi) with a pure culture mould fermentation, (3) Salting, (4) Ripening. (see: Innovated commercial process).

Naturally fermented sufu

Four steps are also normally involved in making this type of sufu; (1) Preparing tofu, (2) Preparing pehtze (pizi) with natural fermentation, (3) Salting, (4) Ripening. (see: Traditional process with natural fermentation).

Bacteria-fermented sufu

Five steps are normally involved in making this type of sufu; (1) Preparing tofu, (2) Pre-salting, (3) Preparing pehtze (pizi) with a pure culture bacterial fermentation, (4) Salting, (5) Ripening.

During the pre-salting, the tofu adsorbs the salt till the salt content of tofu reaches about 6.5%, which takes about 2 days. Pehtze is prepared by pure cultured *Bacillus* spp. or *Micrococcus* spp. at 30-38°C for about one week. In order to keep the shape of the final product, pehtze is dried at 50-60°C for 12 hours before salting. The ripening time normally takes less than 3 months. This sufu is made in some places, such as Kedong (Heilongjiang) & Wuhan (Hubei).

Enzymatically ripened sufu

Three steps are normally involved in making this type sufu; (1) Preparing tofu, (2) Salting, and (3) Ripening. Because there is no fermentation before ripening, some koji is added in the dressing mixture for enzymatic ripening. The ripening time takes 6-10 months. This product of sufu is produced only in a few areas of China, such as Taiyuan (Shanxi) and Shaoxin (Zhejiang).

According to the colour and flavour, sufu can be classified into four types, which are mainly based on the different ingredients of dressing mixtures in the ripening stage

Red sufu (see Fig. 2.1)

The dressing mixture of red sufu mainly consists of salt, angkak (red kojic rice), alcoholic beverage, sugar, flour (or soybean) paste and some spices. The outside colour of the sufu is from red to purple, and the interior colour is from light yellow to orange. Because red sufu

Fig. 2.1 Red sufu

possesses an attractive colour and strong flavour, it is the most popular product all over China.

Angkak (Anka, Red kojic rice or Red Qu) is a product produced by solid-substrate fermentation of cooked rice with various strains of *Monascus* spp., such as *M. purpureus*. It has a specific aroma and purple red colour and has been used as a natural colorant in red sufu and some other traditional food.

White sufu

White sufu has similar ingredients as red sufu in the dressing mixture but without angkak. It has an even light yellow colour inside and outside. White sufu is a popular product in the south of China because it is less salty than red sufu.

Grey sufu

The dressing mixture of grey sufu contains the soy whey left over from making tofu, salt and some spices. Grey sufu is ripened with a special dressing mixture, which could be dominated by both bacteria and mould enzymes and results in a product with a strong, offensive odour. The preparation of this type of sufu is a top secret in the industry and is slowly becoming a lost art (Wang & Fang, 1986)

Other types

Other sufu types are made by adding various ingredients to the dressing, including vegetables, rice, bacon, and even higher concentrations of alcohol. For instance, dressing mixture containing high levels of ethanol results in a product having a marked alcoholic bouquet. This product is called Zui-Fang (or tsui-fang), which means drunk sufu.

Sufu can also be classified according to size, such as Big Sufu and Small Sufu, and shape, Square Sufu and Chess (Round) Sufu (Huang, 1991)

MANUFACTURING PROCESS

Most sufu products are produced by a similar principle, which involves four main steps:

(1) Preparation of tofu; (2) Preparation of pehtze; (3) Salting; and (4) Ripening. The schematic diagram for production of sufu is shown in Fig. 2.2 (Nout & Aidoo, 2002).

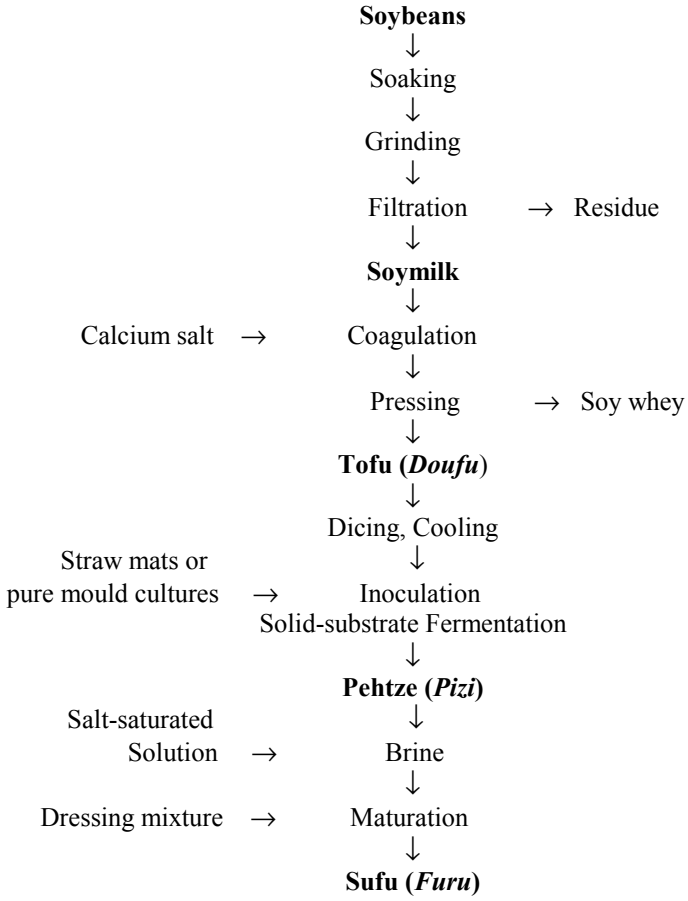

Fig. 2.2 The schematic diagram for production of sufu.

Traditional process with natural fermentation

Traditionally, the preparation of sufu is a centuries-old household activity involving natural fermentation. The process is still used in some places, such as Jiangsu and Zhejiang (Wang & Du, 1998).

Preparation of tofu

Soybeans are washed and soaked overnight in water, and then ground in a stone mill into a slurry. The slurry is diluted and pressed to obtain soymilk. Coagulation is achieved by acid or by addition of salts, such as calcium sulphate and magnesium sulphate. The precipitate is pressed to remove excess water (soy whey)

with cheesecloth bags using stones or wooden planks. Finally, a soft but firm cake-like tofu results, which can then be cut into cubes of desired sizes.

Fig. 2.3 Pehtze

Preparation of pehtze (pizi)

Pehtze (see Figure 2.3), fresh bean curd overgrown with mould mycelia, is prepared by means of natural fermentation. Cubes of tofu are placed in wooden trays, the bottom of which is made of bamboo strips loosely woven together. The loaded trays are piled up and surrounded with straw for natural inoculation and fermentation. The temperature is 15-20°C, which is not favourable for bacteria, yeasts and other moulds except for *Mucor* spp. This step takes 5-15 days and varies depending on locality and season.

The pehtze must have white or light yellow-white mycelium to ensure that the final sufu has an attractive appearance. Before the pehtze is moved to the salting treatment, the mycelial mat of mould should be flattened by hand so that a firm film will be formed over the surface of the sufu to keep its shape.

Salting

The pretreated pehtze is transferred into a big earthen jar and salt is spread between layers of pehtze as they pile up in the jar. During this period, the pehtze adsorbs the salt until salt content of pehtze reaches about 16%, which takes 6-12 days. The salted pehtze is removed from the jar, washed with water, and then transferred to another jar for further processing.

Ripening

The differences between the various types of sufu are mainly caused during the ripening process since different dressing mixtures are added in salted pehtze. The ingredients of dressing mixture vary with social customs, climate, locations and so on. The most common dressing mixture used consists of angkak, alcoholic beverage, salt, sugar, bean paste, and spices. Additional essence can be added into the dressing mixture to supply a special flavour.

For the ripening, alternate layers of pehtze and dressing mixture are packed into jars, and the ratio is about 2:1 between salted pehtze and dressing mixture. The mouth of the jar is wrapped with sheath leaves of bamboo and sealed with clay. The sealed jars are aged for 6 months for further maturation.

Innovated commercial process

Nowadays, sufu is manufactured at an industrial scale, following the same four main steps as the traditional process.

Preparation of tofu

The production of tofu is highly mechanised. The yield and quality of commercial tofu are affected by soybean composition (Murphy et al., 1997), soymilk characteristics (Lim et al., 1990), coagulant, and other factors (Moizuddin et al., 1999). Preparation of tofu used for sufu mainly follows the process technologies for commercial tofu except for slight differences in some steps. First, soybeans of selected quality are washed and ground with added water to a milky slurry in a steel mill. The slurry is then heated and filtered through cloth to separate soymilk. Coagulation is done at 70-80°C by addition of calcium sulphate and magnesium sulphate. Generally, 20% more coagulant is used to produce tofu for sufu than for regular tofu production. Coagulant added is 2.5-3.5% of the dry starting weight of soybean. Moreover, after the calcium salts are mixed with soymilk, the mixture needs to be agitated vigorously in order to get a homogenous coagulum, and then it is set aside for 10-15 min to complete the coagulation. The precipitate is pressed to remove excess water (soy whey) with cheese-cloth in a mechanized press. Finally, a soft, cake-like tofu results which can then be cut into desired sizes (normally rectangular pieces, approximately 3.2*3.2*1.6 cm). Moisture and pH of tofu are 70-79% and 6-7, respectively, varying with the type of sufu to be produced (Wang & Du, 1998; Li, 1998).

Preparation of pehtze (pizi)

Pehtze (or pizi), fresh bean curd overgrown with mycelium of moulds is produced by means of solid-substrate fermentation after inoculation with pure culture moulds. The process of making pehtze was considered a natural phenomenon until 1920, when a microorganism believed to be responsible for sufu fermentation was isolated and identified (Su, 1986; Wei, 1928) studied the microorganisms found in sufu and isolated a pure culture strain of *Mucor* from the fermenting pehtze samples.

---- Microorganisms

The fungal genera involved (*Actinomucor, Mucor* and *Rhizopus*) all belong to the Mucoraceae. The mould used in fermentation of sufu is crucial and has to possess certain characteristics. First of all, the mould must have enzyme systems with high proteolytic and lipolytic activities since it grows on tofu which is a protein and lipid-rich and carbohydrate-poor medium. Secondly, the mould must have white or, at most, slightly yellowish white mycelium to ensure that the sufu has an attractive appearance. Thirdly, the texture of the mycelial mat should be dense and tenacious so that a film formed on the surface of the pehtze will act as an over-casing to protect the final product of sufu from deformation. Finally, the mould growth should not develop any off-odor, astringent taste, or mycotoxins and the mould should resist undesired bacterial contamination during the fermentation.

Some *Mucor* spp., *Actinomucor* spp., and *Rhizopus* spp. fulfill all of these criteria and could be used for making high-quality sufu. Among them, *Actinomucor elegans* and *A. taiwanesis* seem to be the best moulds that are used for pehtze production commercially in Beijing and Taiwan, respectively. However, also other moulds such as *Mucor sufu* and *Mucor wutongqiao* have been mentioned as popular starter cultures. Nevertheless, most of *Actinomucor* spp. and *Mucor* spp. only grow well at 20-30°C, so it is hard to produce sufu during the hot summer (Hu & Zhao, 1998a). Hu and Zhao (1998b) and Deng et al.(1996) screened mutants, made by using conventional mutagens and isolated strains of *Mucor* sp. M_{263} and *Mucor* sp. H_4, which could grow at 30-40°C and ensure sufu production all year round. The authors did not provide information about the stability and safety of these mutants.

---- Inoculation

A pure culture inoculum of mould can be prepared, starting from agar slant culture, by liquid or solid substrate culture in roux bottles. The medium used for solid-substrate culture consists of bran and water (1 : 1.2~1.4) and that for liquid-substrate culture contains soy whey with added maltose (2-3%) and peptone (1.5-2.0%). The spore suspension (~10^5 CFU/ml) is harvested and inoculated on the surfaces of the tofu with manually operated sprayers, comparable to those used for spraying plants.

---- Incubation

The inoculated tofu is placed, evenly spaced in wooden or plastic trays, the bottoms of which are made of bamboo or wooden strips. The loaded trays are piled up in an incubation room, where a controlled temperature (about 25°C), a relative humidity (about 97%) and good aeration are needed for optimum growth of the

mycelia. The thin white mycelia are developed in 8-12 h and a thick mycelial mat is formed after 36-40 h of incubation. Then, the room temperature is decreased by aeration to prevent over-growth of mould, until a slightly yellowish white colour appears, at which point formation of fresh pehtze is complete. The total cultivation time is about 48 h, which is much less than in the traditional way (5-15 days).

Chou et al (1988) reported that the incubation temperature, humidity, and cultivation time greatly affected the growth of and enzyme production by *Actinomucor taiwanensis* on tofu. Optimum conditions observed for growth of *A. taiwanensis* were 25-30°C at 97% relative humidity when tofu of 65% moisture content was inoculated. Under these conditions, a maximum production of protease, lipase, α-amylase and α-galactosidase was achieved.

Before pehtze is transferred to the salt treatment, the mould mycelial mat should be flattened by hand, in the same way as done in the traditional way.

Salting

Freshly prepared pehtze has a bland taste. The flavour and aroma of sufu develop during the salting and ripening process. During the salting period, the pehtze absorbs salt and loses water until the salt content reaches a certain equilibrium level. The absorbed salt imparts a salty taste to the sufu, and it retards further mould growth and the growth of undesirable microbial contaminants. The added salt also plays another important role in releasing the mycelia-bound proteases. Wang and Hesseltine (1970) mentioned that the fungal proteases were not extracellular and that they are loosely bound to the mycelium, possibly by ionic linkage. On the other hand, the mould grows only on the surfaces of the tofu pieces, and the mycelium hardly penetrates into the tofu. The salting could enable the enzymes to diffuse into the tofu for substrate degradation.

Pehtze can be salted in many ways, e.g.:

- The pehtze is transferred to containers, each having a volume of 10-20 liters and each layer of pehtze being sprinkled with a layer of salt in accordance with a traditional method. This method not only takes longer, but also the pieces of pehtze have widely varying salt concentration.
- The pehtze is transferred to vessels containing a saturated salt solution. After 4-5 days at room temperature, the salt content of the pehtze can reach over 12% and the moisture content decreased by 10-15%. Final levels of moisture content may vary in the range 50-65% (w/w).
- Pehtze is immersed in an alcoholic saline solution consisting of 12% NaCl and 10% ethanol (distilled liquor or rice wine is used). Pehtze immersed in this solution can be sold as such. Actually, this method combines the salting and the ripening together in one step.

Ripening

The flavour and aroma of sufu develop during the ripening step. During this period, the enzymes produced by the mould act upon their respective substrates, and it is likely that hydrolysis of protein and lipid provide the principal compounds of the mild, characteristic flavour of sufu. The soybean proteins are hydrolysed by the proteinases into peptides and amino acids. Free amino acids, such as glutamic acid, aspartic acid, leucine/isoleucine and alanine, are predominant in sufu (Table 2.2).

Table 2.2 Amino acid content of sufu samples

Amino Acid	Red Sufu [A] (g/100 g sufu)	Grey Sufu [A] (g/100 g sufu)	Sufu [B] (g/100 g protein)	White Sufu [C] (Molar ratio %)
Alanine	0.32	0.70	10.0	7.0
Arginine	0.38	0.27	2.1	2.5
Aspartic acid	1.00	0.66	5.1	13.7
Cystine	0.59	0.20	0.4	
Glutamic acid	2.15	2.08	0.6	22.0
Glycine	0.54	0.42	4.4	7.0
Histidine	0.20	0.18	1.4	1.9
Isoleucine	0.88	0.58	4.8	4.5
Leucine	0.81	0.95	8.8	7.6
Lysine	0.59	0.29	7.0	7.3
Methionine	0.51	0.14	0.7	
Phenylalanine	0.59	0.59	4.6	2.6
Proline	0.38	0.29	2.4	7.7
Serine	0.34	0.27	2.3	5.2
Threonine	0.45	0.23	2.0	4.1
Tryptophan	0.09	0.05	0.6	
Tyrosine	0.54	0.25	2.2	1.0
Valine	0.16	0.58	5.3	5.2

A -Wang, (1995) and Wang and Du, (1998). B - Su, (1986): Commercial sample non-specified. C - Liu and Chou, (1994).

The pleasant and palatable taste is considered to be related to the content of free amino acids, mainly glutamic acid, in oriental fermented food (Chou et al, 1993). Purification and some properties of glutaminase (catalyses hydrolysis of glutamine to glutamate) from *Actinomucor taiwanensis* were studied by Lu et al. (1996). Glutaminase was stable at a temperature up to 35°C and at pH values of 6.0~8.0. In the presence of 10% NaCl, the enzyme activity was inhibited 50%.

Soybean lipids are also hydrolysed to some extent into fatty acids. The added alcohol reacts with the fatty acids chemically or enzymatically to form esters, providing the pleasant odor of the product. The alcohol also retards the degradation of soybean proteins (Chou & Hwan, 1994). A higher protein solubility and a higher content of peptides and amino acids were observed in the sufu ripened in a brine solution without ethanol.

Salted pehtze is ripened in various jars or bottles, ranging in size from approx. 0.25 to 10 litres, containing a dressing mixture that varies with the type of sufu. The dressing mixture that is most commonly used for red sufu includes salt (final salt content 10~12%), angkak (red kojic rice) 2%, flour (or soybean) paste 3~5%, alcohol content 8~12%, sugar 5~10% and some spices. Additional essence is also added to the dressing mixture to supply a special flavour. For instance, rose essence is added to the dressing mixture for Rose Sufu preparation. The addition of hot pepper to a dressing mixture would make Hot Sufu. Therefore, the flavour and aroma of sufu, in addition to its own characteristics can be easily improved or modified by the ingredients of the dressing mixture.

Ripening requires much time and space. Although nowadays the ripening time is shorter than the 6 months that the traditional process took, modern processes still take about 2-3 months. Reduction of ripening times can be achieved by using smaller cubes of tofu, lowering the salt content from ~14% to ~10%, lowering alcohol content from ~10% to ~6%, keeping the ripening temperature at a higher and more constant level, and using smaller jars. The high concentrations of salt are considered to retard the hydrolysis of protein and lipid. In addition, human consumption of sufu is also limited because of its saltiness. Unfortunately, lowering salt content could cause other problems, such as shorter shelf-life. In an attempt to overcome the problem, a coating of whole blocks of pehtze with paraffin (m.p. 60°C) was studied (Wai, 1964). The pehtze was first mixed with salt (7% of pehtze weight) and then coated with a layer of melted paraffin. Upon solidification of the paraffin, the product was stored in a glass container. After one month at room temperature, the paraffin layer was removed and the contents were subjected to sensory evaluation. The resulting sufu was found to be satisfactory. In order to accelerate the ripening of sufu, stem bromelain was used as a coagulant of soymilk and a hydrolytic enzyme (Fuke & Matsuoka, 1984). Increases in ripening rate and enhanced flavour were obtained by the addition of stem bromelain.

From the microbiological point of view, it may be expected that yeasts and bacteria play a role in the flavour and texture of sufu. Little literature is available

on these issues, except a study on the halophilic flora of sufu. Pao (1995) reported that high levels of non-halophiles and moderate halophiles were found in 60% of sufu brands tested. Predominant halophiles were identified as *Tetragenococcus halophila* (previously *Pediococcus halophilus*).

CHEMICAL COMPOSITION AND NUTRITIONAL QUALITY OF SUFU

From a nutritional point of view, sufu has a higher content of protein-nitrogen than other oriental soybean foods, such as miso and natto (Su, 1986). Nutritionally, soybean milk, tofu and sufu have the same importance to people of Asia as cows' milk and cheese do to the people of the Western Hemisphere. Asians prefer the salt-coagulated bean curd, not only because it has the desired texture, but also because it serves as an important source of calcium (Wang & Hesseltine, 1970; Zhao, 1997).

The chemical and nutritional compositions of sufu are shown in Table 2.1 (Li, 1998; Wang & Du, 1998; Su, 1986). In spite of their differences in colour and flavour, most types of sufu have a similar proximate composition.

The amino acid content of sufu is presented in Table 2.2 (Wang, 1995; Wang & Du, 1998; Liu & Chou, 1994; Su, 1986). Glutamic acid and aspartic acid were the most abundant amino acids found in red sufu and grey sufu. The ratio of (glutamic acid + aspartic acid) : (total amino acid content) was around 30%, which provides sufu with a delicious taste. The cystine and methionine contents of grey sufu may be lower than those of red sufu because of their degradation or conversion to other sulfur compounds during maturation, which may contribute to the offensive odor of grey sufu.

Yen (1986) reported that the average amine contents in 15 samples of commercial sufu from Taiwan, China were: cadaverine (0.039 mg/g), histamine (0.088 mg/g), beta-phenylethylamine (0.063 mg/g), putrescine (0.473 mg/g), tryptamine (0.150 mg/g), and tyramine (0.485 mg/g). Tyramine and putrescine were the major amines found, and these might have a potential harmful effect on human beings if levels are very high.

The complex flavour of sufu was reported to contain 22 esters, 18 alcohols, 7 ketones, 3 aldehydes, 2 pyrazines, 2 phenols and other volatile compounds by Hwan & Chou (1999). Maturation in the presence of ethanol resulted in higher levels of volatiles. Ho et al. (1989) compared the volatile flavour compounds of red sufu and white sufu. Red sufu contains much larger amounts of alcohols, esters, and acids, which may be due to the fermentation of angkak by *Monascus* spp. The esters give red sufu its characteristic fruity aroma. White sufu contains a large quantity of anethole, which seems to be the major contributor of its flavour. The volatile compounds detected in red/white types of sufu are shown in the Table 2.3.

Table 2.3 Volatile compounds detected in red/ white types of sufu

Alcohols	Ethanol,	2-Butanol,	Propanol,
	2-Methylpropanol,	Butanol,	3-Methylbutanol,
	Hexanol,	3-Octanol,	2-Ethylhexanol,
	Benzyl alcohol,	Phenylethyl alcohol	
Esters	Ethyl butyrate,	Ethyl 2-methylbutyrate,	Ethyl hexanoate,
	Ethyl heptanoate,	Ethyl octanoate,	Ethyl benzoate,
	Ethyl dodecanoate,	Phenylethyl propanoate,	Ethyl tetradecanoate,
	Ethyl palmitate,	Ethyl stearate,	Ethyl oleate,
	Ethyl linoleate		
Miscellaneous	Acetic acid,	Phenol,	2-Nonanone,
	2,6-Dimethylpyrazine,		2-Ethyl-5-methylpyrazine

Sources: Ho, et al., (1989) and Hwan and Chou, (1999).

CONCLUSION

To provide the increasing global population with a source of protein other than meat is a worldwide challenge. Alternative sources are legumes and cereals. But plant protein by itself often lacks desirable flavours. To overcome this deficiency, fermentation can either add desirable flavour or destroy unpleasant flavour. This is especially true in soybean products made by fermentation.

In China, sufu is one of the most important traditional fermented soybean foods. It has been widely consumed as a relish by Chinese people for more than 1,000 years, and further, it can be used in the same way as cheese. Throughout history, the Chinese seemed to follow their own ways in developing the product without foreign influences. Although a pure culture method for preparing sufu has been developed, further studies are still needed to guarantee uniform high quality products. If innovations in taste, flavour and product quality are made, sufu may become more widely popular all over the world.

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Chapter 3

Microbiological Safety and Quality of Commercial Sufu - A Chinese Fermented Soybean Food

Abstract

In this study, the microbiological safety and quality of commercial sufu were investigated. Twenty-three samples of three different types of sufu were obtained, mainly in China and some in the Netherlands. Chemical parameters analyzed included moisture, pH, free amino N, NaCl, ethanol, sucrose, glucose, and fructose. Concentrations of NaCl, ethanol, glucose and fructose varied from 6.2%, 0.5%, 0% and 0% to 14.8%, 6.3%, 6.2% and 4.8%, respectively. Microbiological analyses were done for total count of mesophilic aerobic bacteria (TMAB), bacterial endospores, total count of halotolerant bacteria at 10% (THB10) and at 17.5% NaCl (THB17.5), lactic acid bacteria (LAB), fungi, Enterobacteriaceae, and the following pathogens: *Bacillus cereus, Clostridium perfringens, Staphylococcus aureus* and *Listeria monocytogenes*. High levels (> 10^5 cfu/g) of TMAB and bacterial endospores were found in most samples, and 85% of TMAB was identified as Gram-positive. Considerable levels (10^5 and 10^7 cfu/g) of LAB were detected in two samples of white sufu, and isolates of LAB were identified as most probably *Lb. casei*. One third of the samples contained less then 10^3 cfu/g *B. cereus*, but 3 samples had over 10^5 cfu/g indicating potential hazard to consumers. All samples had less than 10^3 cfu/g *C. perfringens,* except sample R11 (~10^5 cfu/g). *S. aureus* could not be detected in any of the samples tested since the competitive microflora (usually bacilli) disturbed typical features on the selective medium used; however *Staphylococcus aureus* enterotoxin A was detected in some of the white and grey sufu samples. Fungi, Enterobacteriaceae, and *L. monocytogenes* were not detected in any of the samples. Based on these results, a microbiological guideline for safe commercial sufu is proposed.

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INTRODUCTION

Sufu or furu is a traditional Chinese fermented soybean curd resembling a soft creamy cheese-type product, which can be used in a similar way as cheese (Steinkraus, 1996). It has been one of the most popular highly-flavoured side dishes consumed in China for many centuries, and is becoming popular in Chinese shops all over the world.

Sufu is produced by various processes in different localities of China (Wang & Du, 1998). Four steps are usually involved in making sufu: (1) preparing tofu by salt precipitation from boiled soymilk, (2) preparing pehtze (*pizi*) by spray-inoculation of diced tofu with a pure culture fermentation starter, (3) salting, and (4) ripening in a dressing mixture. The sufu process and variations in recipes and compositions were described by Wang and Hesseltine (1970), Su (1986), and Han et al. (2001). The pure culture starters mainly consist of moulds -- Mucoraceae (*Actinomucor, Mucor* and *Rhizopus*) or bacteria – *Micrococcus* and *Bacillus* spp. Although a pure culture is used in the pehtze fermentation, the process of sufu manufacture itself is carried out under non-sterile conditions, and by consequence, microbial contamination will occur.

Whereas most sufu contains considerable levels of the antimicrobial NaCl (5-15 %) and ethanol (1-7 %) that could prevent the survival or growth of pathogens, it is also known that the endospore-forming rods such as *Bacillus* spp. and *Clostridium* spp. vary greatly in their salt tolerance (Brewer, 2000). The fact that several bacteria have a remarkable ability to survive different environmental stress conditions makes it very difficult for the food industry to exclude them from their products (Andersson et al., 1995).

Earlier investigations of fermented soybean foods, eg. tempe and kinema, revealed the presence of considerable levels of foodborne pathogenic bacteria such as *Bacillus cereus*, *Staphylococcus aureus* and Enterobactericeae (Samson et al., 1987; Nout et al., 1998). Tofu is a major precursor of sufu. Foodborne pathogenic bacteria, such as Enterobacteriaceae, *B. cereus* and *S. aureus* were found in commercial tofu (Van Kooij & De Boer, 1985; Rehberger et al., 1984; Ashraf et al., 1999). But to date, there is only scant information about the microbiological safety and quality of sufu (Pao, 1989).

From the microbiological public health point of view, it is of interest to investigate the microorganisms present in commercial products, with special reference to food-borne pathogenic bacteria. The data obtained from this study can be useful in developing and proposing microbiological guidelines for the commercial production of high quality sufu products.

MATERIALS AND METHODS

Collection of samples

Twenty-three samples of sufu were obtained from different markets and factories in China and the Netherlands. According to their colour and flavour, they were classified into three types (red, white and grey sufu) which are mainly based on the different ingredients of dressing mixture during the ripening phase (Han et al., 2001), which can be regarded as representative of the major types of sufu in China. All samples were stored at room temperature as is usual in sufu retail shops.

Chemical analysis

Moisture

Moisture content was determined by conventional oven-drying (80°C, 48 h) of 10 g sufu.

pH and free amino nitrogen

A 2-g sample of sufu was homogenised with 18 ml demineralized water. The pH was measured in this suspension using a digital pH meter (WTW, type 525, Weilheim, Germany). While stirring continuously, 0.1 M NaOH was added to the suspension to a constant pH 8.5, followed by addition of 5 ml formaldehyde (37%; Code no.1.04003.1000, Merck, Germany). After 2 min the suspension was titrated by semi-automated burette (Schott-Gerate, type T81; Hofheim a. Ts. Germany) with 0.100 M NaOH to constant pH 8.5 again (pH should be constant for 30 sec.). Free amino nitrogen (mM/g) was calculated as $0.1*V/m$; where V is the volume of 0.100 M NaOH used, and m is the sample fresh weight.

Salt concentration

The ten-fold diluted sufu was titrated with 0.1 M $AgNO_3$, and 10% w/v K_2CrO_4 solution was used as an indicator. The following equation was used: % Salt as % (w/w) NaCl = $V*M*0.0584*100/m$; where V and M are the volume and molarity of $AgNO_3$ used, and m is the mass of a fresh sample.

Ethanol, sucrose, glucose and fructose concentration

The determination of ethanol, sucrose, glucose and fructose content was performed on a HPLC isocratic RI analyser, column: Aminex HPX-87H (300*7.8 mm); sample: 20 µl; eluant: 5 mM H_2SO_4; flow rate: 0.6 ml/min; temperature: 40°C.

Sufu samples were deproteinized using Carrez reagents (a mixture of $ZnSO_4 \cdot 7H_2O$ and $K_4Fe(CN)_6 \cdot 3H_2O$) before injection into the analyser.

Subsampling and sample treatment for microbiological analysis

Representative 20 g portions were aseptically weighed, mixed with 180 ml peptone saline (0.1% neutral peptone, 2.5% NaCl) with higher than physiological salt concentration to minimise osmotic shock, and homogenized by blending for 30 sec at high speed in a Laboratory Blender (Stomacher 400, England). Subsequent decimal dilutions were prepared with the same diluent, and in all cases, duplicate counting plates were prepared of appropriate dilutions.

Microbiological analysis

Total count of Mesophilic Aerobic Bacteria (TMAB)

TMAB was enumerated in pour-plates of Plate Count Agar (PCA, CM325, Oxoid, England) to which 25 g NaCl was added per litre, after incubation at 30°C for 2-3 d.

Total count of Halotolerant Bacteria (THB10) with 10% and (THB17.5) with 17.5% salt concentration

THB10 and THB17.5 were enumerated in pour-plates of PCA to which 100 g and 175 g NaCl were added per litre, after incubation at 30°C for 7 d and 14 d, respectively.

Bacterial endospores

Sufu samples were pasteurised (80°C, 10 min) and bacterial endospores. were enumerated in pour-plates of PCA to which 25 g NaCl was added per litre, after incubation at 30°C for 2-3 d.

Lactic acid bacteria

Lactic acid bacteria (LAB) were enumerated in pour-plates of de Man, Rogosa and Sharpe medium (MRS, Cat. No. 1.10661, Merck, Germany) to which 12 g agar, 25 g NaCl and 2 g natamycin ("Delvocid", Gist-brocades, Delft, The Netherlands) were added per litre, after incubation at 30°C for 3-4 d. Initial confirmation was based on Gram stain, catalase and oxidase tests. Carbohydrate metabolism of isolates of LAB was determined using API 50 CH strips and API 50 CHL medium (bioMérieux, Lyon, France) according to the manufacturer's instructions. Tentative

identification based on phenotypical properties was done with IBIS software (Intelligent Bacteria Identification System, the Netherlands) (Wijtzes et al., 1997).

Yeasts and Moulds (Fungi)

Fungi were enumerated in pour-plates of standard and salt-enriched Oxytetracycline Glucose-Yeast Extract agar (OGYE, CM545, Oxoid, England) to which 0 g and 25 g NaCl was added per litre, respectively, after incubation at 30°C for 5 d.

Bacillus cereus

Selective enumeration was carried out on spread-plates of Mannitol Egg Yolk Polymyxin (MYP) (Cereus selective agar, Cat. No. 1.05267, Merck, Germany) to which 25 g NaCl was added per litre. The inoculated plates were incubated at 30°C for 24 h and examined for typical colonies. Presumptive colonies (pink colonies surrounded by a zone of precipitation) were counted and subsequently transferred to a Brain Heart Infusion (BHI, No. 2337500, DIFCO, France) agar with added fresh sheep blood (50-70 ml/l BHI) (Sheepblood Defibrinated, bio TRADING Benelux B.V., The Netherlands) for confirmation, as specified in ISO method (Anonymous, 1993). Carbohydrate metabolism by isolates of bacilli was determined using API 50 CH strips and API 50 CHB medium (bioMérieux, Lyon, France) according to the manufacturer's instructions. The resulting biochemical profiles were interpreted using APILAB software (Version 3.3.3, 1990, bioMérieux, Lyon, France).

Clostridium perfringens

Selective enumeration was carried out in pour-plates of Tryptose Sulfite Cycloserine Agar (TSCA, Cat. No. 1.11972, Merck, Germany) to which 25 g NaCl was added per litre. After incubation at 37°C for 18-24 h under anaerobic conditions, black colonies were counted and subsequently confirmed according to Eisgruber et al. (2000).

Enterobacteriaceae

Selective enumeration was carried out in pour-plates of Violet Red Bile Glucose agar (VRBG, CM485, Oxoid, England) with overlay to which 0 g and 25 g NaCl was added, after incubation at 30°C for 24-36 h.

Staphylococcus aureus

Selective enumeration was carried out in pour-plates of Baird Parker agar + Rabbit Plasma Fibrinogen (BP+RPF, No. 44003, bioMérieux, France). After incubation at 37°C for 24 h, *S. aureus* colonies, ringed by an opaque halo and colored gray-black, were counted as specified in ISO method (Anonymous, 1997). The detection of staphylococcal enterotoxins A, B, C, D and E was carried out by enzyme immunoassay (RIDASCREEN® SET A,B,C,D,E; Art. No.: R 4101, R-Biopharm GmbH, Germany) (Park et al., 1994).

Listeria monocytogenes

Diluted sample (0.1 ml) was spread on ready-made commercial plates of Rapid'L. mono Medium (RLM, Cat. No. 63694, Sanofi, France). After 24 h of incubation at 37°C, positive control cultures of *L. monocytogenes* produced typical blue colonies without a yellow halo, having circular shape and smooth surface, with mean diameter of 1-2 mm (Heisick et al., 1995).

RESULTS AND DISCUSSION

Chemical parameters of commercial sufu

Table 3.1 shows chemical parameters of the tested sufu samples. There was no relation between the age after manufacturing date and pH value, which suggests that the pH does not change significantly during the sufu storage. Levels of free amino nitrogen in grey sufu were higher than in red and white sufu, and this was also reflected in higher pH values of grey sufu.

Since there is a wide choice of methods and recipes used in the production of sufu, it was according to expectation to find a range of parameter values such as shown in Table 3.1. Concentrations of NaCl, ethanol, glucose and fructose varied from 6.2 %, 0.5 %, 0 % and 0 % to 14.8 %, 6.3 %, 6.2 % and 4.8 % respectively. In addition, large variations of NaCl, ethanol, glucose, fructose and water contents among samples from the same brand indicate that most companies do not closely control the composition of their products.

Glucose and fructose were hardly found in white and grey sufu samples except in W4 and W6, to which Lao-Chao (a fermented rice product) (Wang & Hesseltine, 1970) had been added as an ingredient to the dressing mixtures. Sugar (sucrose) is commonly applied in the dressing mixture of red sufu (Han et al., 2001). However, sucrose was not found in any of the samples, suggesting that it was degraded by enzyme activities during the sufu ageing phase.

Table 3.1 Chemical parameters of commercial sufu (fresh sample)

Sample Code	Age[a] (month)	Moisture (g/100 g)	pH	Free Amino N (mM/g)	NaCl (g/100 g)	Ethanol (g/100 g)	Glucose (g/100 g)	Fructose (g/100 g)
R1	14	66.3	6.46	0.29	9.2	6.2	4.3	2.0
R2†	12	67.6	6.51	0.29	8.8	6.3	4.8	2.6
R3†	11	68.9	6.25	0.23	8.2	5.9	6.2	4.7
R4†	8	65.1	6.52	0.28	10.3	4.8	4.5	2.6
R5	4	60.5	6.02	0.24	9.6	5.5	5.4	4.8
R6‡	1	61.0	6.78	0.21	10.4	4.6	5.7	2.3
R7‡	6	63.2	6.20	0.33	9.6	4.6	4.0	1.5
R8‡	6	60.3	6.32	0.22	12.8	4.9	5.5	2.8
R9	6	58.6	6.30	0.22	14.8	5.1	4.1	2.3
R10	9	66.1	6.22	0.39	14.7	1.9	2.5	0.1
R11	5	55.1	5.68	0.42	14.4	1.1	0.8	0.0
R12	6	56.3	5.50	0.33	13.1	1.6	3.4	0.0
W1†	6	70.2	6.63	0.33	9.9	5.8	0.2	0.1
W2♀	10	72.4	6.90	0.47	7.5	6.2	0.1	0.0
W3♀	4	72.4	6.59	0.42	8.1	6.0	0.3	0.1
W4	12	58.7	5.54	0.26	13.0	2.3	4.9	4.5
W5	14	67.8	6.80	0.44	7.8	4.5	0.1	0.0
W6	6	63.6	5.25	0.29	10.2	3.5	5.3	0.0
W7	11	67.3	5.86	0.37	6.2	2.4	0.1	0.1
W8	12	70.1	6.06	0.39	8.2	4.0	0.1	0.0
G1†	11	70.8	7.32	0.55	11.8	1.9	0.0	0.1
G2†	6	73.1	7.45	0.51	10.2	2.2	0.0	0.0
G3	9	67.8	7.08	0.59	14.2	0.5	0.0	0.1

R – red sufu; W – white sufu; G – grey sufu; [a] Age after manufacturing date; †, ‡, ♀ Samples from the same manufacturer.

Microbiological analysis

High levels (> 10^5 cfu/g) of TMAB and B. endospores were found in all samples except in samples W7, W8 and G3 (Table 3.2). The similar levels of corresponding TMAB and B. endospore counts found in individual samples indicate that TMAB mainly consisted of B. endospores. About 85% of isolates taken from TMAB counting plates were indeed Gram-positive, and mostly rod-shaped. This was to be expected, since the resistance of Gram-positive bacteria to ethanol and salt is relatively stronger than that of Gram-negative bacteria (Seiler & Russell, 1991). THB10 and THB17.5 were detected in all samples, and the number of halotolerant bacteria was inversely proportional to the NaCl concentration in the media. Most of the isolates of THB10 grew after having been inoculated onto PCA without NaCl, indicating that these were not obligate halophilic organisms. On the other hand, most isolates transferred from THB17.5 failed to grow on PCA without NaCl, so these could be obligate halophiles.

Lactic acid bacteria are found in many fermented foods. All samples of white and grey sufu contained detectable levels of LAB, with considerable levels (~10^7 and ~10^5 cfu/g) in W7 and W8. Interestingly, there were no detectable spoilage phenomena in samples W7 and W8 with pH 5.86 and pH 6.06 that are not unusually low. Acid spoilage of these products by LAB may have been prevented by the lack of fermentable sugars (Table 3.1). In only 2 out of 12 red sufu samples, low levels (~10^2 cfu/g) of LAB could be detected. The apparent failure of LAB to spoil red sufu could be due to the use of ankak (red kojic rice) (Nout & Aidoo, 2002) in red sufu dressing. Antimicrobial effects of ankak have been associated with antibiotics such as ankalactone, monascorubrin and rubropunctatin (Nozaki et al., 1991); LAB in sufu may be sensitive to these antibiotics.

Isolates of presumptive LAB on MRS were Gram-positive, oxidase and catalase negative, non-sporeforming rod-shaped bacteria. They could grow in both aerobic and anaerobic conditions, produced acid from ribose but no gas from glucose, so they were initially classified as facultative heterofermentative lactobacilli. Carbohydrate assimilation data indicated that the lactobacilli isolated from sample R6 were most probably *Lb. curvatus* and those from samples W7 and W8 *Lb. casei*.

In all samples, fungi were absent (< 10 cfu/g). This appears to differ from the data of Pao (1994) who reported 10^2-10^6 cfu/g in sufu. Most likely the fungi, particularly the mould starters do not survive after the pehtze phase, due to the high levels of salt and/or ethanol in the dressing mixtures applied for ageing and storage.

In Table 3.2 it is shown that *B. cereus* was isolated from all samples. In three samples (R6, R11 and W5) ≥ 10^5 cfu/g of *B. cereus* were found, indicating a potential hazard for consumers. One third of commercial sufu samples contained less than 10^3 cfu/g. Some species of bacilli have been associated with incidents of foodborne disease related to consumption of unheated products. The enterotoxins

produced by *B. cereus* are responsible for the emetic and/or diarrhoea food poisoning (Granum, 1994). The fact that there are strains of *B. cereus* that may cause food poisoning with an infective dose as low as 10^3-10^4 cfu/g (Andersson et al., 1995), should be of concern for the sufu industry.

Table 3.2 Microbiological composition of commercial sufu (log cfu/g sample)

Sample Code	TMAB	B. endosp.	THB10	THB17.5	LAB	*B. cereus*	*C. perfri.*
R1	5.7	5.9	4.5	2.1	<1	2.5	1.3
R2†	6.0	5.7	4.3	1.8	<1	2.7	<1
R3†	6.2	6.6	5.1	2.3	<1	3.4	<1
R4†	6.6	5.7	5.6	2.6	<1	3.7	2.6
R5	5.2	5.2	4.1	1.9	<1	3.6	<1
R6‡	7.0	6.9	5.9	2.2	2.6	5.1	1.3
R7‡	6.8	6.9	6.2	3.3	<1	3.5	<1
R8‡	6.8	6.6	5.3	3.2	<1	3.2	<1
R9	6.7	6.4	6.6	3.8	<1	2.3	<1
R10	6.0	5.7	5.6	3.3	<1	2.2	<1
R11	7.0	7.0	5.9	3.6	2.3	5.3	5.1
R12	6.9	6.7	5.1	3.1	<1	4.6	<1
W1†	5.6	6.0	3.3	1.7	2.6	3.6	1.3
W2♀	5.3	5.4	4.7	4.4	2.7	3.4	<1
W3♀	6.0	5.9	4.9	4.6	2.6	2.7	<1
W4	7.8	7.8	7.0	2.7	3.7	3.5	<1
W5	5.5	5.4	3.5	3.6	2.2	5.0	<1
W6	5.1	4.9	4.0	3.9	3.5	3.6	1.6
W7	6.9	3.8	3.6	3.7	7.7	2.4	<1
W8	6.0	3.2	3.8	4.0	5.9	2.2	<1
G1†	6.6	6.2	4.1	3.7	2.8	4.3	<1
G2†	5.8	5.7	4.1	4.0	2.6	3.9	2.6
G3	4.7	2.8	2.9	2.8	2.2	1.3	<1

R – red sufu; W – white sufu; G – grey sufu.

†, ‡, ♀ Samples from the same manufacturer.

Abbreviations: TMAB: total count of mesophilic aerobic bacteria; B. endosp.: bacterial endospores; THB10: total count of halotolerant bacteria at 10% NaCl level; THB17.5: same, at 17.5% NaCl; LAB: lactic acid bacteria. *C. perfri.*: *C. perfringens.*

All analyses on duplicate subsamples.

When 58 presumptive *B. cereus* colonies were checked by the ISO method, 55 strains (94.8% of total) were confirmed to be *B. cereus*. Based on carbohydrate assimilation patterns, the remaining three strains (5.2% of total) were identified as

B. subtilis. This was also the main species found in a number of randomly picked isolates of sporeforming bacteria from sufu samples.

From Table 3.2, a quarter of the samples contained low numbers of *C. perfringens* (Log cfu/g < 3.0), except sample R11 (log 5.1 cfu/g). Sample R11 represents a rather unusual style of sufu, covered with fermented Chinese cabbage and packaged in a plastic vacuum-packed bag. Both the ingredients and the anaerobic packaging could have contributed to the comparitively high level of *C. perfringens*. This organism is known to occur frequently in spices and herbs, at levels of 10^2-10^4 cfu/g (Labbé, 2000). The dressing mixtures of some sufu products contain spices and/or herbs, which could also contribute to the presence of *C. perfringens*.

Most Enterobacteriaceae do not tolerate elevated salt levels. Whereas they were found at high levels (~10^7 cfu/g) in pehtze (Cao, 2001), they do not survive the salting and ageing steps. According to our expectation, no Enterobacteriaceae were detectable in any of the samples using VRBG agar with or without 2.5% NaCl. Similar results were observed in an earlier study (Pao, 1994), in which coliforms were not found in the investigated samples. Although Enterobacteriaceae are unlikely survivors of the final stages of the sufu process, their ability to grow and possibly produce endotoxins during the early pehtze making stage might constitute a hazard. Consequently, toxicological studies are required to assess the potential hazard posed by such toxins during sufu production.

S. aureus is often associated with human skin. It occurs in commercial tofu (Van Kooij & De Boer, 1985), and it is likely to be present in sufu as this involves manual operations on tofu. Due to its salt-tolerance, this pathogen expectedly survives in sufu. However, it was impossible to detect *S. aureus* in the samples tested due to the fact that there were no visible haloes surrounding the suspect colonies. The competitive microflora (mainly bacilli) prevented the formation of such haloes during the incubation of *S. aureus*, which we were able to confirm with artificially contaminated samples (data not shown). Further research is necessary to find a suitable method for enumeration of *S. aureus* in sufu samples. Qualitative detection tests on staphylococcal enterotoxins in sufu samples showed that some of the grey sufu contained enterotoxin A. Other enterotoxins (B, C, D, and E) were below level of detection.

Although *L. monocytogenes* is a salt tolerant pathogen being able to grow in 10% NaCl and to survive in the presence of 30% NaCl (Rocourt & Cossart, 1997), we could not detect the presence of this pathogen using Rapid'L mono Medium in any of the samples.

Microbiological guideline using attributes sampling plans

Presently, no microbiological guidelines exist for sufu. Based on the results of our study we propose the sampling plan (Adams & Moss, 2000) as shown in Table 3.3.

Total count of mesophilic aerobic bacteria was not included in the plan as we consider it to have little direct relevance to safety. Instead, we focussed on the pathogens that were encountered in some of the commercial sufu, notably *B. cereus*, *C. perfringens* and *S. aureus*. Enterobacteriaceae were included because of their potential of producing endotoxins.

Table 3.3 Attributes sampling plans for sufu.

Organisms	Plan class	*n*	*m* (cfu/g)	*M* (cfu/g)	*c*
Enterobacteriaceae	3	5	10	10^2	1
B. cereus	3	5	10^3	10^4	1
C. perfringens	3	5	10^2	10^3	1
S. aureus	3	5	10^3	10^4	1

n – the number of samples to be taken from a lot;

m – a count which separates good quality from marginal quality and which most test samples should not exceed;

M – a count which if exceeded by any of the test samples would lead to rejection of the lot;

c – the maximum number of test samples which may fall into the marginally acceptable category before the lot is rejected (Adams & Moss, 2000).

CONCLUSION

The microbiological composition of the sufu indicates that its manufacturing processes and recipes prevent survival or growth of fungi and Enterobacteriaceae. On the other hand, high levels of bacterial endospores and concomitant TMAB were observed in a majority of the samples, which indicates either that highly contaminated tofu and/or dressing mixture were used for sufu production, and/or that poor processing practices (e.g. inappropriate handling or unhygienic condition) were involved. Considering that no signs of spoilage were observed, it must be assumed that most bacterial endospores are not metabolically active in this product. In view of hygienic safety and to reduce microbiological counts in general, benefit could be expected from using fresh tofu for pehtze making, by using dressing mixtures with low microbiological load and by maximizing process sanitation.

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Chapter 4

Mucoraceous Moulds Involved in The Commercial Fermentation of Sufu Pehtze

Abstract

Sufu is a fermented cheese-like soybean product in China and Vietnam, obtained by fungal solid-state fermentation of soybean curd (tofu), which results in moulded tofu or "pehtze". The final product sufu is obtained by maturing pehtze in a brine containing alcohol and salt during a period of several months. The present report deals with the identity and phylogenetic relationships of mould starter cultures used for the preparation of pehtze. Starter cultures used in commercial pehtze fermentation were obtained from factories located in several provinces of China and Vietnam or isolated from their pehtze. They were identified as *Actinomucor repens*, *A. taiwanensis*, *Mucor circinelloides*, *M. hiemalis*, *M. racemosus*, and *Rhizopus microsporus* v. *microsporus*. Phylogenetic relations based on sequencing of genomic DNA of these starters and of relevant control strains from collections indicate that the genera *Mucor*, *Actinomucor* and *Rhizopus* form distinct and homogenous clusters, with *Mucor* and *Actinomucor* showing a slightly closer relationship with each other than with *Rhizopus*.

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INTRODUCTION

Sufu (*fu-ru*) is a popular fermented soybean food which has a characteristic flavour and which is commonly consumed as a side-dish during breakfast (Su, 1986). A very similar product is known as "Chao" in Vietnam. The preparation of sufu and chao takes place in stages: initially, soybeans are soaked, ground and filtered to obtain soymilk. Next, soy protein is coagulated from the milk, recovered, pressed into sheets and cut to dices of "tofu". Tofu is inoculated with a mould starter and incubated 2-7 d at 12-30ºC resulting in mycelium-covered pieces called "pehtze" (Han et al., 2001). The starter may either be a pure culture applied as a sprayed suspension, or house-flora which is inoculated onto the tofu by direct contact with utensils. The final stage consists of the maturation of pehtze during several months in a dressing mixture or brine containing alcohol (from rice wine), salt, and several product-specific ingredients for colour and flavour.

The pehtze fermentation is aimed at the formation of a white cover of biomass surrounding the piece of tofu; in addition several proteolytic, lipolytic and other enzymes are formed by the moulds and these will act upon the pehtze during the maturation causing softening and flavour development (Han et al., 2002). The properties of the mould(s) used for the pehtze fermentation are thus of importance for the quality of the final product. Descriptions of sufu (Han et al., 2001; Su, 1986) mention the use of *Actinomucor elegans*, *Actinomucor taiwanensis*, *Mucor* and *Rhizopus* spp. as starter cultures, but little recent information is available about starters that are presently applied in commercial practice. Whereas phylogenetic relations for clinically important Mucorales were reported (Voigt et al., 1999), the genus *Actinomucor* was not included.

The aim of our investigation was to obtain starter cultures used for commercial pehtze preparation, confirm their identity and study their phylogenetic relations in comparison with relevant strains from culture collections.

MATERIALS AND METHODS

Strains

Strains collected from commercial pehtze manufacturers included: *Actinomucor elegans* LU 2025/AS 3.27 from Beijing (LU – LandbouwUniversiteit; AS – Academia Sinica), *Mucor hiemalis* LU 2026/AS 3.2222 from Fujian, *Mucor wutungkiao* LU 2028/AS 3.25 from Sichuan, *Mucor sufu* LU 2027/AS 3.2233 from Jiangsu, and *Mucor rouxianus* LU 2029/AS 3.2545 from Guangdong, all in China. Unidentified strains isolated from commercial pehtze included: LU 2035/CTT3 from An Giang province, LU 2036/CKP2 from Soc Trang province, LU 2033/CVH1 from Dong Thap province, and LU 2034/CTH4 from Dong Thap province all in the Mekong Delta area of Vietnam. Pehtze making strains obtained

from mycological collection centers included: *Actinomucor taiwanensis* LU 2031/CCRC 31159 and *Actinomucor elegans* LU 2032/CCRC 31342 both purchased from the Food Industry Research and Development Institute, P.O.Box 246, Hsin Chu, Taiwan 30099, China. Control strains from CBS (Centraal Bureau voor Schimmelcultures) included: *Mucor circinelloides* v. *circinelloides* (CBS 478.70, CBS 394.68, CBS 195.68, CBS 192.68), *Mucor hiemalis* v. *corticolis* (CBS 106.09, CBS 366.68, CBS 363.68, CBS 365.68), *M. hiemalis* v. *hiemalis* (CBS 200.28, CBS 201.65, CBS 242.35), *M. hiemalis* v. *luteus* (CBS 244.35, CBS 243.35), *M. hiemalis* v. *silvaticus* (CBS 250.35, CBS 249.35), *M. racemosus* (ATCC 1216B), *Rhizomucor racemosus* (CBS 260.68), *Rhizopus microsporus* v. *chinensis* (CBS 537.80, CBS 338.34, CBS 394.34), *R. microsporus* v. *microsporus* (CBS 308.87, CBS 700.68, CBS 699.68), *R. microsporus* v. *oligosporus* (CBS 339.62, CBS 228.95, CBS 337.62), and *R. microsporus* v. *rhizopodiformis* (CBS 196.77, CBS 343.29).

Isolation and maintenance

All strains were isolated and/or grown on malt extract agar (Oxoid CM59) at 25 and/or 30ºC. They were maintained refrigerated (4ºC) or for longer periods they were stored in a mixture of equal volumes of malt extract broth (Oxoid CM57) and glycerol, stored at -80º. The cultures used for the molecular study were grown on Malt Peptone (MP) broth using 10% (v/v) of Malt Extract (Brix 10) and 0.1% (w/v) Bacto Peptone (Difco) in 2 ml of medium in 15 ml tubes. The cultures were incubated at 20°C for 5 d in darkness.

Identification

Identification was carried out in accordance with Benjamin and Hesseltine (1957), Hesseltine and Ellis (1973), Jong and Yuan (1985) and Scholer et al. (1983) based on macroscopic and microscopic morphology as well as relevant physiological distinctive features.

Extraction of DNA, sequencing, parsimony analysis of aligned sequences, UPGMA clustering method

The total fungal genomic DNA was isolated using FastDNA ® Kit from Bio 101 according to the manufacturer's instructions. Amplification of the ITS 1-5.8S-ITS 2 region was done by PCR using the primers LS266 (5'-GCATTCCCAAACAACTCGACTC-3') and V9G (5'-TGCGTTGATTACGTCCCTGC-3'). The PCR reaction mixture, total reaction mix is 50 µl, contained 2 µl of genomic DNA (10-20 ng/µl), 5 µl PCR buffer, 30 µl ultra pure sterile water, 10 µl 1 mM dNTP, 1 µl 50 pmol/µl LS266 primer, 1 µl 50

pmol/µl V9G primer and 1 µl 2.5 U/µl DNA Taq polymerase. Amplification was performed in a GeneAmp PCR system 9600 model (Perkin Elmer). The temperature cycling parameters were: denaturation at 94°C for 30 s followed by primer annealing at 55°C for 1 min and primer extension at 72°C for 1 min for 35 cycles plus a 4 min final elongation step at 72°C. After amplification of the ITS 1-5.8S-ITS 2 region, excess primers and dNTP's were removed from the reaction mixture using GFX-columns (Pharmacia Biotech, Uppsala, Sweden). The purified PCR fragments were resuspended in 50 µl of TE buffer. The PCR fragments were directly sequenced in both ways with the primers ITS 1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') performing a sequence PCR with a DNA sequencing kit (Applied Biosystems 4303152). The sequence PCR reaction mixture, total reaction mix is 10 µl, contained 2 µl of template DNA (15-45 ng/µl), 4 µl BigDye terminator RR mix, 3 µl ultra pure sterile water, 1 µl 4 pmol/µl ITS 1 primer or 1 µl 4 pmol/µl ITS 4 primer.

The reaction was performed in a GeneAmp PCR system 9600 model (Perkin Elmer). The temperature cycling parameters were: denaturation at 96°C for 10 s followed by primer annealing at 50°C for 5 s and primer extension at 60°C for 4 min for 25 cycles. After the sequence PCR the DNA fragments were being precipitated using 1 µl 3M Na-acetate, 25 µl 96% ethanol and 10 µl (total amount) reaction product. The mix was incubated on ice for 5-10 min and centrifuged (Eppendorf 5417R) for 20 min at 14000 rpm. The supernatant was carefully discarded from the tube and 250 µl 70% ethanol was added to the pellet. The tube was centrifuged again for 20 min at 14000 rpm and the supernatant was carefully discarded. The pellet was air dried until it was absolutely dry and 17 µl TSR buffer was added (Applied Biosystems 401956). The resuspended pellet was then transferred into a genetic analyzer vial, closed tightly with a septum, incubated at 95°C for 2 min and cooled on ice immediately. The samples were analyzed with the ABI PRISM 310 Genetic Analyzer (Perkin Elmer). The sequence data was optimized using the software package Seqman from DNAStar Inc. The alignments of the ITS 1-5.8S-ITS 4 sequence data were performed using the software package BioNumerics from Applied Maths.

RESULTS AND DISCUSSION

The identities of the collected strains are listed in Table 4.1. For some of the species names the current version as listed in the CBS catalogue were used and the original names were maintained in brackets. The majority of starters tested belonged to *Mucor* and *Actinomucor* genera. In addition, also one *Rhizopus* sp. was used for pehtze preparation. Considering the effect of climate conditions (temperature, relative humidity) on the distribution, growth and metabolic activity of fungi (Chou et al., 1988; Han et al., 2002) it was expected that *Mucor* and

Actinomucor spp. would abound in the North and *Rhizopus* spp. in the South. However, the limited numbers of strains tested do not enable a statistical approach of their distribution.

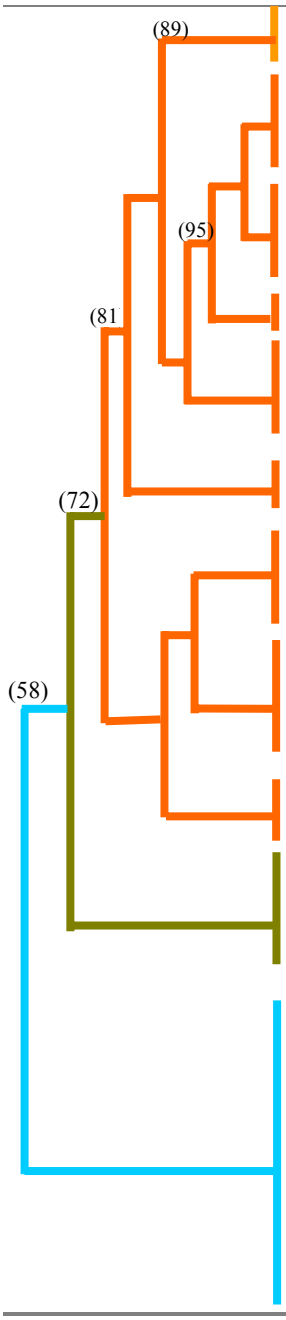
Table 4.1 Identities of moulds used for commercial sufu pehtze preparation.

Genus	Species	LU code	Other code	Origin
Actinomucor	repens (elegans)	2025	AS 3.27	Beijing
Actinomucor	repens (elegans)	2032	CCRC 31342	Taiwan
Actinomucor	repens (elegans)	2035	CTT3	An Giang
Actinomucor	taiwanensis	2031	CCRC 31159	Taiwan
Mucor	circinelloides (wutungkiao)	2028	AS 3.25	Sichuan
Mucor	hiemalis	2026	AS 3.2222	Fujian
Mucor	racemosus (sufu)	2027	AS 3.2233	Jiangsu
Mucor	racemosus (rouxianus)	2029	AS 3.2545	Guangdong
Mucor	racemosus	2033	CVH1	Dong Thap
Mucor	racemosus	2034	CTH4	Dong Thap
Rhizopus	microsporus v. microsporus	2036	CKP2	Soc Trang

in brackets: alternative species names

Among the cultures tested, distinct and homogenous groups of *Mucor*, *Actinomucor* and *Rhizopus* were observed in the phylogram (Figure 4.1). Within the genus *Actinomucor*, *A. taiwanensis* is not easily separated from *A. repens*. In *Mucor*, *M. hiemalis* is quite well separated whereas *M. racemosus* and *M. circinelloides* are somewhat mixed. Of the genus *Rhizopus*, only the species *R. microsporus* was included and it shows as a homogeneous group; however, the varieties *chinensis*, *microsporus*, *oligosporus* and *rhizoides* are not easily separated. Based on similarities *Mucor* and *Actinomucor* show a somewhat closer relationship with each other than with *Rhizopus*.

This would be in concordance with the observations by Schostakowitsch quoted by Benjamin and Hesseltine (1957) that *Actinomucor* is closely related to *Mucor* but distinct from *Rhizopus* and *Absidia*.



Relation (similarity%)	Genus	Species	Variety	Code
	Rhizomucor	*racemosus*		CBS 260.68
	Mucor	*circinelloides*		LU 2028
	Mucor	*circinelloides*	*circinelloides*	CBS 394.68
	Mucor	*circinelloides*	*circinelloides*	CBS 195.68
	Mucor	*hiemalis*		LU 2026
	Mucor	*circinelloides*	*circinelloides*	CBS 478.70
	Mucor	*racemosus*		LU 2034
	Mucor	*racemosus*		LU 2033
	Mucor	*circinelloides*	*circinelloides*	CBS 192.68
	Mucor	*racemosus*		LU 2029
	Mucor	*racemosus*		LU 2027
	Mucor	*racemosus*		ATCC
	Mucor	*hiemalis*	*silvaticus*	CBS 250.35
	Mucor	*hiemalis*	*silvaticus*	CBS 249.35
	Mucor	*hiemalis*	*hiemalis*	CBS 200.28
	Mucor	*hiemalis*	*corticolis*	CBS 106.09
	Mucor	*hiemalis*	*hiemalis*	CBS 201.65
	Mucor	*hiemalis*	*hiemalis*	CBS 242.35
	Mucor	*hiemalis*	*corticolis*	CBS 366.68
	Mucor	*hiemalis*	*corticolis*	CBS 363.68
	Mucor	*hiemalis*	*corticolis*	CBS 365.68
	Mucor	*hiemalis*	*luteus*	CBS 244.35
	Mucor	*hiemalis*	*luteus*	CBS 243.35
	Actinomucor	*repens*		LU 2035
	Actinomucor	*repens*		CCRC 31342
	Actinomucor	*repens*		LU 2025
	Actinomucor	*taiwanensis*		CCRC 31159
	Rhizopus	*microsporus*	*rhizopodiformis*	CBS 196.77
	Rhizopus	*microsporus*	*chinensis*	CBS 537.80
	Rhizopus	*microsporus*	*oligosporus*	CBS 339.62
	Rhizopus	*microsporus*	*oligosporus*	CBS 228.95
	Rhizopus	*microsporus*	*rhizopodiformis*	CBS 343.29
	Rhizopus	*microsporus*	*oligosporus*	CBS 337.62
	Rhizopus	*microsporus*	*chinensis*	CBS 388.34
	Rhizopus	*microsporus*	*microsporus*	CBS 308.87
	Rhizopus	*microsporus*	*chinensis*	CBS 394.34
	Rhizopus	*microsporus*	*microsporus*	LU 2036
	Rhizopus	*microsporus*	*microsporus*	CBS 700.68
	Rhizopus	*microsporus*	*microsporus*	CBS 699.68

Fig. 4.1 Phylogram inferred from the r-DNA sequence data, showing phylogenetic relationships of Mucoraceous moulds involved in sufu pehtze fermentation, compared with relevant collection strains.

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Chapter 5

Microbial Changes during the Production of Sufu – A Chinese Fermented Soybean Food

Abstract

Sufu is a Chinese soybean cheese obtained by solid-state fungal fermentation of tofu. The resulting "pehtze" is salted, followed by maturation in brine. The process of sufu manufacture is carried out under non-sterile conditions, so in addition to the mould starter, other microorganisms may be expected to be present in sufu. The quantitative evolution of microflora of sufu was studied throughout the production, with special attention for the effect of different salt contents during the ripening. Total counts of mesophilic aerobic bacteria (TMAB), bacterial endospores (B. endospores), *Bacillus cereus*, lactic acid bacteria (LAB), Enterobacteriaceae and fungi increased from around 10^4, 10^4, < 10, 10^4, < 10 and 10^3 cfu/g (in tofu) to around 10^8, 10^8, 10^3, 10^7, 10^7 and 10^7 (in pehtze), respectively. LAB, Enterobacteriaceae and fungi decreased 1-2 log cfu/g, and TMAB, B. endospores and *B. cereus* decreased < 1 log cfu/g after the salting of pehtze. TMAB and B. endospores in sufu with 8% and 11% salt content decreased to around 10^6 cfu/g during the ripening, and about 90% of B. endospores were identified as *Bacillus subtilis*. *B. cereus* remained at levels of around 10^3 cfu/g during the ripening, and 87.5% of presumptive *B. cereus* were confirmed as such. LAB in sufu with 8% and 11% salt content decreased gradually from 10^5-10^6 cfu/g to < 10^2 cfu/g. LAB in sufu with 5% salt content increased to 10^9 cfu/g during the ripening. These were identified as most probably *Lactobacillus curvatus*, and caused sour spoilage of sufu by pH decrease (from 6-7 to 4). Enterobacteriaceae and fungi decreased to the non-detectable level after 20 d and 30 d of maturation, respectively, in all samples. There was no obvious difference in changes of detected microflora (except for LAB) between different types of sufu (i.e. red and white) in this study.

Submitted for Publication

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INTRODUCTION

Sufu, *Fu-ru* written in hieroglyphics, is a fermented soybean food that originated in China. It is a soft creamy cheese-type product made from cubes of soybean curd (tofu) by the action of a mould (Su, 1986; Steinkraus, 1996). This fermented product with its characteristic flavour has been widely consumed by Chinese people as an appetiser for many centuries.

There are many different types of sufu, produced by various local processors in China (Wang & Du, 1998), with mould-fermented sufu being the most popular type of product (Han et al., 2001b). Four steps are normally involved in making this type of sufu: (1) Preparing tofu (soybean curd), (2) Preparing pehtze (*pizi*) by fungal solid-state fermentation of tofu using e.g. *Actinomucor elegans*, (3) Salting of pehtze, and (4) Ripening in dressing mixture (Wang & Hesseltine, 1970).

The sufu process and variations in recipes and compositions were described by Su (1986) and Han et al. (2001b). The pure culture starters mainly consist of moulds - Mucoraceae (*Actinomucor, Mucor* & *Rhizopus*) or bacteria - *Micrococcus* and *Bacillus* spp. Although a pure culture is used in the pehtze fermentation, the process of sufu manufacture itself is carried out under non-sterile conditions, and by consequence other functional microbes may be involved in sufu production, while microbial contamination will occur as well as was shown in commercial samples (Han et al., 2001a).

Like in some other fermented foods such as cheese (Messens et al., 1999) and miso (Chiou et al., 1999), salt plays a very important role in sufu. It imparts a salty taste to the products, and it serves to control the microbial growth and the enzyme activity as well.

Although most sufu contains considerable levels of the antimicrobial NaCl (6-15 % w/w) and ethanol (0-7% v/v) (Han et al., 2001a) that could prevent the survival or growth of contaminating microbes, it may be expected that the endospore-forming bacteria such as *Bacillus* spp. could grow since these vary greatly in their salt tolerance (Brewer, 2000). The fact that several bacteria have a remarkable ability to survive different environmental stress conditions makes it very difficult for the food industry to exclude them from their products (Andersson et al., 1995). The role of such microbes other than starters during sufu production is still unknown.

Tofu is a major precursor of sufu. The microbiological quality of tofu was studied by Tuitemwong and Fung (1991), and high levels of bacteria were observed. Foodborne pathogenic bacteria, such as Enterobacteriaceae, *B. cereus* and *S. aureus* were also found in commercial tofu (Ashraf et al., 1999; Van Kooij & De Boer, 1985; Rehberger et al., 1984). Earlier investigations of commercial sufu revealed the presence of high levels of bacteria (especially bacterial endospores) and considerable levels of foodborne pathogenic bacteria such as *Bacillus cereus* (Han et al., 2001a; Shi & Fung, 2000; Pao, 1994). But so far, much

less information is available about the microflora changes taking place during the production of red and white sufu.

From the microbiological point of view, it is of interest to investigate the microorganisms present in sufu production, with special attention to the effect of different salt contents.

MATERIALS AND METHODS

Microorganism

Actinomucor elegans (Academia Sinica AS 3.227) is commonly used as a starter in commercial sufu production in China. A pure culture inoculum of *A. elegans* AS 3.227 was prepared, starting from agar slant culture, by liquid substrate culture in Roux bottles as is practised in Chinese sufu factories. The medium consisted of soy whey (by-product from tofu manufacture) to which maltose (2-3% w/v) and peptone (1.5-2.0% w/v) were added prior to sterilisation by autoclaving. After incubation at 28°C for 72 h, medium and biomass were harvested and homogenized to obtain a spore suspension containing ~10^5 CFU/ml.

Sufu preparation

The tofu used as raw material for sufu was provided by Beijing WangZhiHe sufu manufacturer, and was cut into pieces (3.2*3.2*1.6 cm). The pieces were inoculated with *A. elegans* by spraying spore suspension onto the surface of tofu pieces. The inoculated pieces were placed evenly spaced, in plastic trays. The loaded trays were piled up in an incubation room with controlled temperature (around 25°C), relative humidity (around 90%) and air circulation to ensure adequate aeration. Fresh pehtze, i.e. tofu overgrown with *A. elegans* mycelium, was obtained after incubation for 48 h.

The pehtze was transferred into a container (20 liters) and salt was spread between layers of pehtze as they piled up in the container. During a period of 5 d, the pehtze absorbed the salt until the salt content of pehtze reached about 14-15% (w/w).

For the ripening of sufu, 12 pieces of salted pehtze (about 200 g fresh weight) were placed in individual wide-mouthed glass bottles with a capacity of 340 ml, after which dressing mixture (about 140 ml) was added to the pehtze. For red sufu, the dressing mixture consisted of angkak or kojic red rice (Han et al., 2001b), alcoholic beverage (rice wine) (Nout & Aidoo, 2002) to a final alcohol content of 5% (v/v), sugar, Chiang (wheat-based miso (Campbell-Platt, 1987)), and spices. For white sufu, the dressing mixture only consisted of alcoholic beverage (final alcohol content 5%). The filled bottles were incubated at 25-28°C for 80 d.

The whole procedure of sufu preparation was carried out under factory conditions in a factory.

Sampling for analysis

Two bottles were drawn randomly from each batch on each sampling day during the ripening. Sufu pieces of duplicate bottles were combined and homogenized aseptically using mortar and pestle prior to analysis in duplicate. A 5-g sample of sufu was homogenized with 45 ml demineralized water. The pH was measured in this suspension using a digital pH meter.

Representative 20 g portions of homogenized sufu were aseptically weighed, mixed with 180 ml peptone saline (0.1% neutral peptone, 2.5% NaCl) (except for tofu and pehtze samples) with higher than physiological salt concentration to minimise osmotic shock (Han et al., 2001a). Subsequent decimal dilutions were prepared with the same diluent, and in all cases, duplicate counting plates were prepared of appropriate dilutions.

Microbiological analysis

Total count of Mesophilic Aerobic Bacteria (TMAB)

TMAB was enumerated in pour-plates of Plate Count Agar (PCA, CM325, Oxoid, England) to which 25 g NaCl was added per litre, after incubation at 30°C for 2-3 d.

Bacterial endospores (B. endospores)

Samples were pasteurised (80°C, 10 min) and B. endospores were enumerated in pour-plates of PCA to which 25 g NaCl was added per litre, after incubation at 30 °C for 2-3 d.

Bacillus cereus

Selective enumeration was carried out on spread-plates of Mannitol Egg Yolk Polymyxin (MYP) (Cereus selective agar, Cat. No. 1.05267, Merck, Germany) to which 25 g NaCl was added per litre. The inoculated plates were incubated at 30°C for 24 h and examined for typical colonies. Presumptive colonies (pink colonies surrounded by a zone of precipitation) were counted. Carbohydrate metabolism by isolates of bacilli was determined using API 50 CH strips and API 50 CHB medium (bioMérieux, Lyon, France) according to the manufacturer's instructions. The resulting biochemical profiles were interpreted using APILAB software (Version 3.3.3, 1990, bioMérieux, Lyon, France).

Lactic acid bacteria (LAB)

LAB were enumerated in pour-plates of de Man, Rogosa and Sharpe medium (MRS, Cat. No. 1.10661, Merck, Germany) to which 12 g agar, 25 g NaCl and 2 g natamycin ("Delvocid", DSM, Delft, The Netherlands) were added per litre, after incubation at 30°C for 3-4 d. Initial confirmation was based on positive Gram stain, and negative catalase and oxidase tests. Carbohydrate metabolism of isolates of LAB was determined using API 50 CH strips and API 50 CHL medium (bioMérieux, Lyon, France) according to the manufacturer's instructions. Tentative identification based on phenotypical properties was done with IBIS software (Intelligent Bacteria Identification System, the Netherlands) (Wijtzes et al., 1997).

Enterobacteriaceae

Selective enumeration was carried out in pour-plates of Violet Red Bile Glucose agar (VRBG, CM485, Oxoid, England) with overlay to which 25 g NaCl was added, after incubation at 30°C for 24-36 h.

Yeasts and Moulds (Fungi)

Fungi were enumerated in pour-plates of standard and salt-enriched Oxytetracycline Glucose-Yeast Extract agar (OGYE, CM545, Oxoid, England) to which 0 g and 25 g NaCl was added per litre, respectively, after incubation at 30°C for 5 d.

RESULTS AND DISCUSSION

Figure 5.1 presents the microflora changes of tofu, pehtze and salted pehtze, which are intermediate products of sufu making, prior to the ripening procedure. Initial counts of TMAB, B. endospores and LAB were around 10^4 cfu/g, and *B. cereus* and Enterobacteriaceae were not detectable in tofu. After solid-state fermentation of tofu, high levels (10^7-10^9 cfu/g) of microflora (except for *B. cereus* 10^3 cfu/g) were found in pehtze as expected. Considerable decline of LAB, Enterobacteriaceae and fungi took place after salting of pehtze. On the other hand, B. endospores and *B. cereus* were less affected. This implies that the salting is a very useful step for inactivating contaminating microbes, such as LAB and Enterobacteriaceae during sufu production.

In Figure 5.2, the pH changes are shown. The pH of sufu with 8% and 11% salt content decreased slightly, and remained in the range of 6-7. But the pH of sufu with 5% salt content decreased rapidly during the first 20 d, and then decreased slowly to around 4, indicating that this sufu was spoiled after 20 d as evidenced by low pH (< 4.6) and off-flavour.

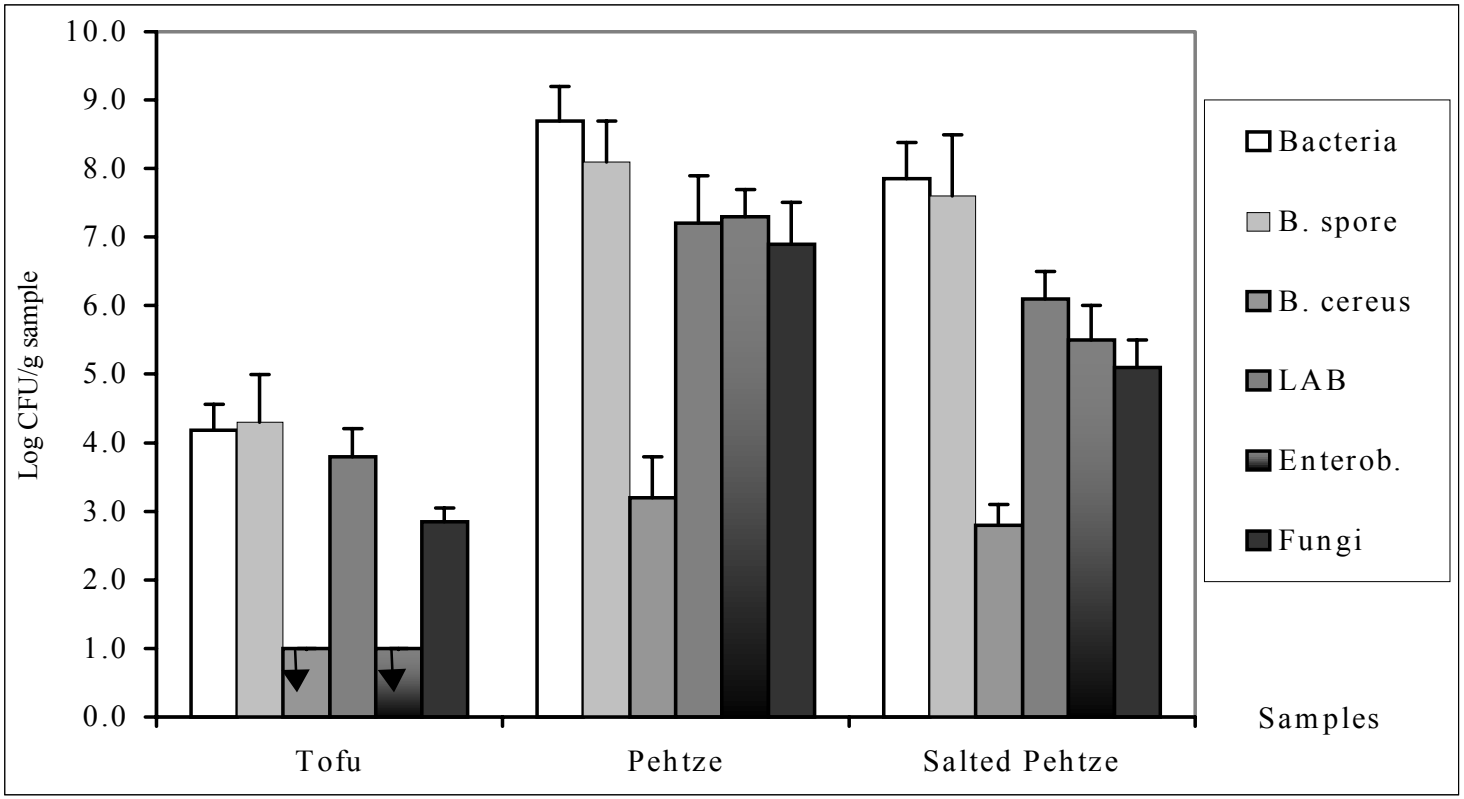

Fig. 5.1 Microflora of tofu, pehtze and salted pehtze.
B. spore: bacterial endospores; LAB: lactic acid bacterial; Entero.: Enterobacteriaceae.

In Figure 5.3, changes of TMAB during sufu ripening are shown. TMAB in sufu with 5% salt content increased over 10^9 cfu/g after ripening 30 d, and then remained at this level. TMAB in sufu with higher salt contents decreased to around 10^6 cfu/g and stabilized after ripening 30 d. The number of TMAB in sufu with 8% salt content was a little higher than that in sufu with 11% salt content. There was no apparent difference between red sufu and white sufu.

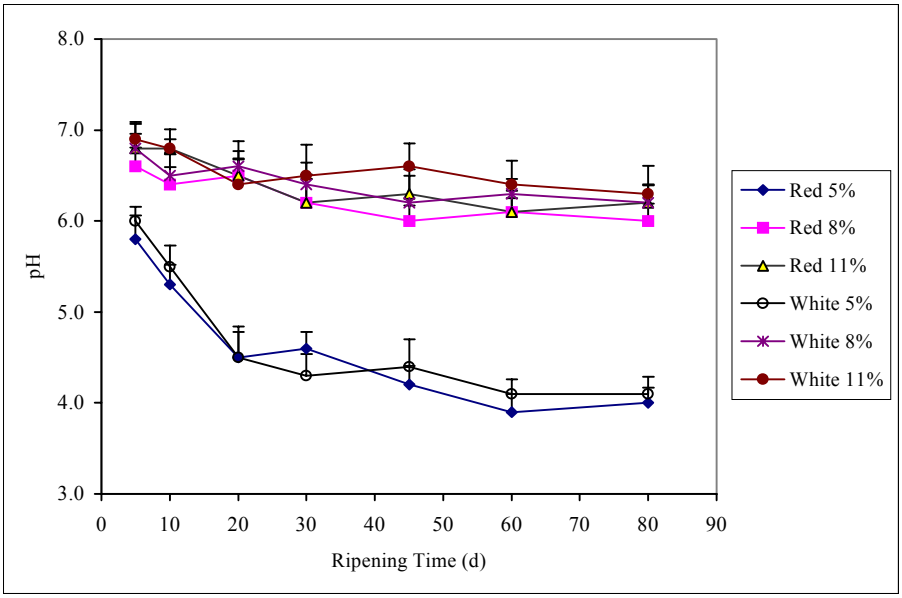

Fig. 5.2 pH changes during the ripening of sufu with different salt contents.

Changes of B. endospores during sufu ripening are presented in Figure 5.4. B. endospores changed in a similar way as the TMAB in sufu of higher salt content. However, they remained at high levels of around 10^7 cfu/g in sufu with 5% salt content during the ripening. B. endospores in low salt acidifying product did not germinate, and therefore surviving spores in neutral, high salt product apparently germinated and subsequently died.

From Figures 5.3 and 5.4, the similar levels of corresponding counts of TMAB and B. endospores at 8% and 11% salt content in the early phase of ripening indicate that TMAB mainly represented B. endospores. About 85% of isolates taken from TMAB counting plates were indeed Gram-positive, mostly rod-shaped bacteria. This was to be expected, since the resistance of Gram-positive bacteria to ethanol and salt is relatively higher than that of Gram-negative bacteria (Seiler & Russell, 1991). Over 90% of isolates taken from B. endospores counting plates were Gram-, oxidase- and catalase-positive, and rod-shaped. Based on their

carbohydrate assimilation patterns, the randomly picked isolates from B. endospores were identified as *B. subtilis*.

The presumptive colony counts of *B. cereus* on MYP were in the range of approximately 10^3 cfu/g during sufu ripening, indicating they could neither grow nor be inactivated, regardless of the salt content and type of sufu. When presumptive *B. cereus* isolates were checked by carbohydrate metabolism, 21 strains (87.5% of total) were confirmed to be *B. cereus*, and three strains (12.5% of total) were *B. subtilis*. The enterotoxins produced by *B. cereus* are responsible for emetic and/or diarrhoeal food poisoning (Granum, 1994). *B. cereus* is a potential hazard for consumers when its number is > 10^5 cfu/g (Andersson et al., 1995). Whereas the present study shows significantly lower levels (10^3 cfu/g), occasionally commercial samples exceed 10^5 cfu/g (Han et al., 2001a), which should be of concern to the sufu industry.

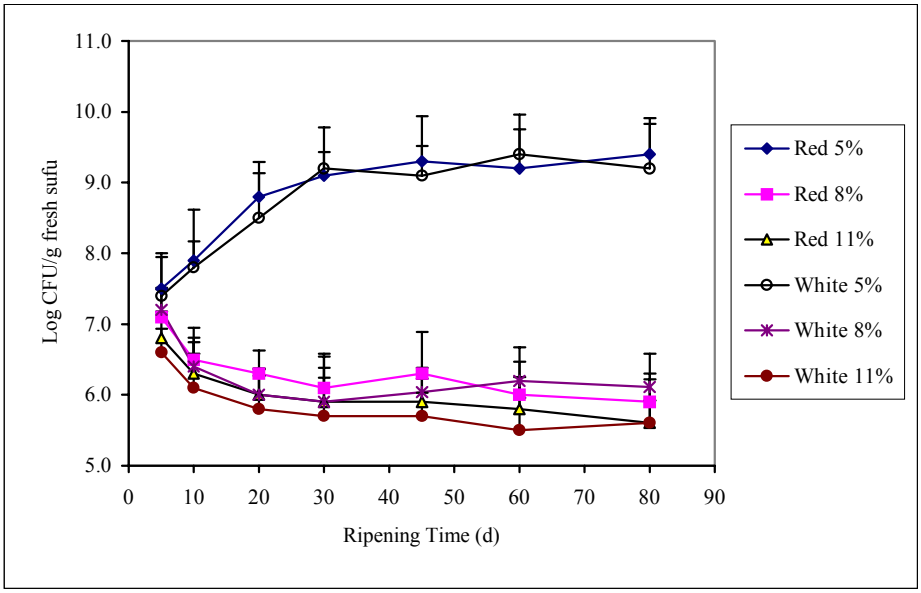

Fig. 5.3 Changes of total count of mesophilic aerobic bacteria (TMAB) during ripening of sufu with different salt contents.

Lactic acid bacteria are involved in many fermented foods. In red sufu with 8% and 11% salt content, LAB decreased gradually from 10^5-10^6 cfu/g to < 10 cfu/g (red sufu) and < 10^2 cfu/g (white sufu) after 60 d of ripening (Figure 5.5). This is in line with our earlier findings (Han et al., 2001a) that LAB were hardly found in commercial red and white sufu. On the other hand, high levels (> 10^6 cfu/g) of LAB were found in some commercial white sufu without any detectable spoilage phenomena (Han et al., 2001a). This may have been caused by production methods that allowed for survival of LAB in these specific samples.

The LAB found in sufu with 5% salt content increased during the first 30 d of the ripening, and stayed in the range of around 10^9 cfu/g (Figure 5.5), indicating that the combination of 5% salt and 5% alcohol was not adequate to inhibit the growth of LAB in sufu. Isolates of presumptive LAB on MRS were Gram-positive, oxidase and catalase negative, non-sporeforming rod-shaped bacteria. They could grow under both aerobic and anaerobic conditions, produced acid from ribose but no gas from glucose, and were initially classified as facultative heterofermentative lactobacilli (Wijtzes et al., 1997). Carbohydrate assimilation data indicated that these lactobacilli were most probably *Lb. curvatus*.

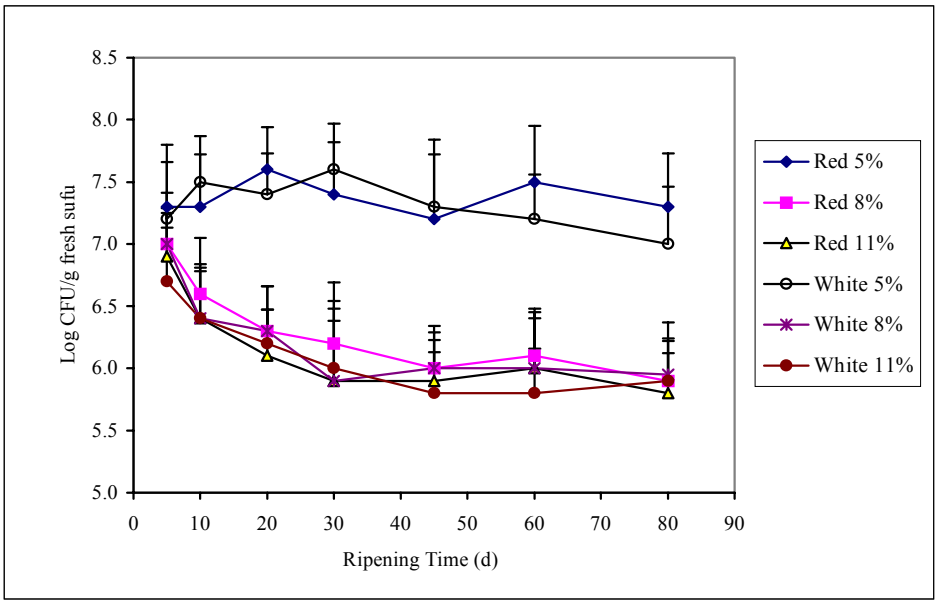

Fig. 5.4 Levels of bacterial endospores during ripening of sufu with different salt contents.

From Figures 5.3 and 5.5, the similar levels of corresponding TMAB and LAB counts found in sufu with 5% salt content, indicate that TMAB mainly represented lactobacilli including *Lb. curvatus*, and that they contributed to the pH decrease and the spoilage of sufu with low salt content (5%) during the ripening.

Most Enterobacteriaceae do not tolerate the combination of elevated levels of salt and alcohol. Whereas they were found at high levels (> 10^7 cfu/g) in pehtze, they were inactivated steadily during the salting and ageing steps. The levels of Enterobacteriaceae are presented in Table 5.1. As was expected, no Enterobacteriaceae were detectable after 20 d in any of the samples (even sufu with 5% salt) using VRBG agar with or without 2.5 % NaCl, which corresponded to the earlier studies (Han et al., 2001a; Shi & Fung, 2000; Pao, 1994). Coliforms were not found in the samples investigated by Pao (1994). Shi and Fung (2000) reported

that the foodborne pathogens *Escherichia coli* O157:H7, *Salmonella typhimurium*, and even *Staphylococcus aureus* and *Listeria monocytogenes* were reduced to non-detectable levels after one month of ripening.

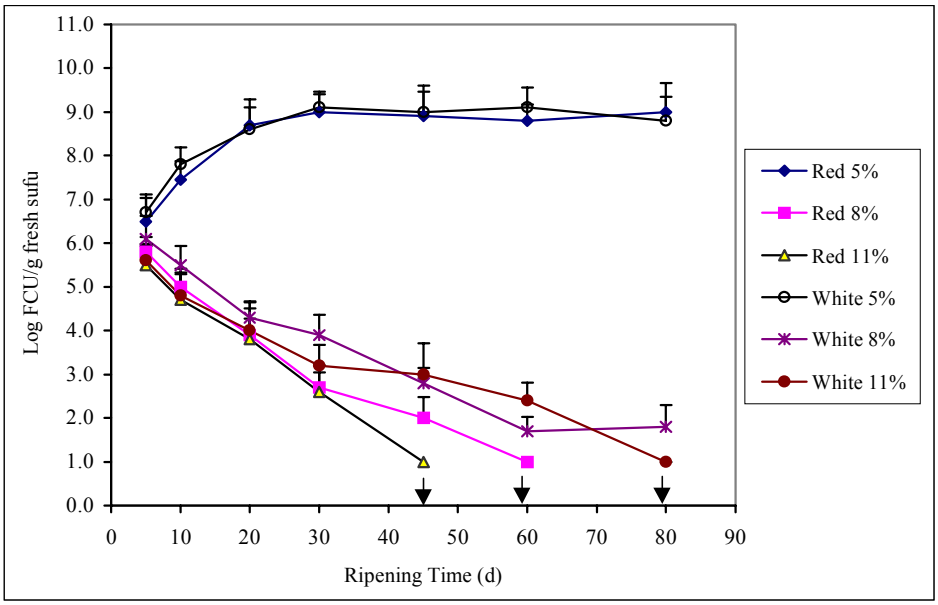

Fig. 5.5 Changes of lactic acid bacteria (LAB) during ripening of sufu with different salt contents.

Table 5.1 Changes of Enterobacteriaceae (log cfu/g fresh sufu) during ripening of sufu with different salt contents.

Ripening Time (d)	5	10	20	30
Red 5%	4.2 ±0.45*	2.3 ±0.57	<1	<1
Red 8%	3.5 ±0.71	1.7 ±0.22	<1	<1
Red 11%	<1	<1	<1	<1
White 5%	4.5 ±0.53	2.0 ±0.41	<1	<1
White 8%	3.3 ±0.69	<1	<1	<1
White 11%	<1	<1	<1	<1

* Mean of duplicate ± standard deviation.

Although Enterobacteriaceae are unlikely survivors of the final stages of the sufu process, their ability to grow and to possibly produce endotoxins during the early pehtze making stage might constitute a hazard. Consequently, toxicological studies are required to assess the potential hazard posed by such toxins during sufu production.

Table 5.2 shows that fungi were absent (< 10 cfu/g) in all samples after 30 d of ripening, as tested in OGYE agar with or without 2.5 % NaCl. This result differs from the data of Pao (1994) who reported 10^2-10^6 cfu/g of fungi in sufu. Shi and Fung (2000) mentioned that no moulds were detected in sufu samples after 1, 2 and 3 months of ripening. Most likely the fungi, particularly the mould starters do not survive after the pehtze preparation, owing to the combination of salt and ethanol in the dressing mixtures applied for the maturation of sufu.

In conclusion, sufu containing 8% salt or more and 5% ethanol during the ripening stage is microbiologically stable and safe.

Table 5.2 Changes of fungi (log cfu/g fresh sufu) during ripening of sufu with different salt content.

Ripening Time (d)	5	10	20	30	45
Red 5%	4.3 ±0.66*	3.2 ±0.43	2.1 ±0.34	<1	<1
Red 8%	4.0 ±0.75	2.5 ±0.37	1.8 ±0.29	<1	<1
Red 11%	3.6 ±0.43	2.8 ±0.42	<1	<1	<1
White 5%	4.5 ±0.52	3.4 ±0.44	2.3 ±0.26	<1	<1
White 8%	3.7 ±0.46	2.0 ±0.38	<1	<1	<1
White 11%	3.0 ±0.51	1.7 ±0.23	<1	<1	<1

* Mean of duplicate ± standard deviation

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Chapter 6

Effects of Temperature and Relative Humidity on Growth and Enzyme Production by *Actinomucor elegans* and *Rhizopus oligosporus* during Sufu Pehtze Preparation

Abstract

Sufu is a Chinese soybean cheese obtained after maturation of solid-state mould-fermented tofu. Ambient temperatures of 30-35ºC during the summer season prohibit the use of the usual starter *Actinomucor elegans*. We compared the properties of the latter with a potential alternative starter *Rhizopus oligosporus* that could be used at higher temperatures. The effects of temperature and relative humidity on growth rate of *Actinomucor elegans* and *Rhizopus oligosporus* were optimum at 25°C at RH 95-97%, and 35°C at RH 95-97%, respectively. Yields of protease (108 U/g pehtze), lipase (172 U/g) and glutaminase (176 U/g) by *A. elegans* were maximum after 48 h at 25°C and RH 95-97%, and for α-amylase (279 U/g pehtze) and α-galactosidase (227 U/g) at 30°C and RH 95-97% after 48 h and 60 h of incubation. Highest protease (104 U/g pehtze), and lipase (187 U/g) activities of *R. oligosporus* were observed after 48 h at 35°C and RH 95-97%, while maximum α-amylase (288 U/g pehtze) and glutaminase (187 U/g) activities were obtained after 36 h at 35°C and RH 95-97%. Maximum α-galactosidase activity (226 U/g) by *R. oligosporus* was found after 36 h at 30°C and RH 95-97%. It is concluded that *R. oligosporus* is a potential alternative to *A. elegans* as sufu pehtze starter during hot seasons.

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INTRODUCTION

Sufu, *Fu-ru* written in hieroglyphics, is a traditional Chinese fermented soybean curd and a highly flavoured, soft creamy cheese-type product, which can be used in the same way as cheese (Su, 1986; Steinkraus, 1996). This fermented product has been widely consumed by Chinese people as an appetizer for many centuries.

There are many different types of sufu, produced by different local processes in China (Wang & Du, 1998); mould-fermented sufu is the most popular type (Han et al., 2001). Four stages are involved in preparing this type of sufu: (1) Preparing tofu (soybean curd), (2) Preparing pehtze (*pizi*) by fungal solid-state fermentation of tofu, (3) Salting of pehtze, (4) Ripening in dressing brine (Wang & Hesseltine, 1970).

Pehtze, fresh soybean curd overgrown with fungal mycelium, is produced by means of solid-substrate fermentation after inoculation (about 48 h) with pure culture moulds. In commercial practice, *Actinomucor* spp., *Mucor* spp and *Rhizopus* spp. are used for sufu preparation. Among them, *Actinomucor elegans* and *Actinomucor taiwanensis* seem to be the most frequently used for commercial sufu production in China. However, these two mould species only grow well at 25-30°C, so it is impossible to produce sufu during the hot summer with indoor factory temperatures reaching 35ºC or even higher (Han et al., 2001). Since *Rhizopus oligosporus* grows well at higher temperatures (up to 40ºC) (Han & Nout, 2000), it might be used as a starter to produce sufu during this season.

Protein is the main component in tofu. Among the fungal enzymes formed on tofu, proteases have received much attention. Using wheat bran, wheat, and soybean as substrates, Wang et al. (1974) investigated the incubation conditions for maximum production of acid protease by *Mucor dispersus, A. elegans* and *R. oligosporus*. Whereas Chou et al. (1988) studied the enzymes produced by *A. taiwanensi*s on tofu, there are few published data describing the enzymes produced by *A. elegans* and *R. oligosporus* during sufu pehtze preparation.

In the present study, we determined the effects of physical process parameters that can be monitored and controlled easily under factory conditions. As criteria for the comparison of the two fungal species, we selected relative humidity, incubation temperature and time, and studied their relation with the growth of *A. elegans* and *R. oligosporus* by measuring their biomass increment, and their enzyme production during the preparation of pehtze. Protease, lipase, α-amylase, glutaminase, and α-galactosidase, affecting the flavour and texture of sufu during the ripening period, were assessed.

MATERIALS AND METHODS

Microorganisms

Actinomucor elegans (Academia Sinica (AS) 3.227) and *Rhizopus oligosporus* (NRRL 5905) isolated originally from commercial sufu and tempe, respectively, were grown on malt extract agar (MEA, Oxoid CM 59) and maintained at 4°C. After incubation at 30°C for 5 d, spore suspensions (~10^5 CFU/ml) were harvested by scraping the sporangia off the agar and suspending them into sterile distilled water with 0.85% salt, 0.1% peptone and 0.05% Tween 80. For each series a fresh spore suspension was prepared.

Determination of biomass formation rate [$d(x^{1/3})/dt$] on membrane-covered tofu

The tofu used for biomass monitoring was provided by Beijing WangZhiHe sufu manufacturer, and was cut into circular pieces (diam 8.5 cm x height 0.8 cm) and then immersed in 1% w/v citric acid solution for 5 min to inhibit growth of contaminating microbes. Proximate analysis of the tofu revealed that it contained approximately 76.8% moisture, 12.5% crude protein, 5.7% crude lipid, and 3.2% carbohydrate. The piece of tofu was fitted into a sterile glass Petri dish, and a membrane (Nylon Transfer Membrane, Schleicher & Schuell, No: 10416116, Pore hole 0.45 μm) was placed onto the tofu (Nagel et al., 1999). The membrane-covered tofu was inoculated at the centre of the membrane with a drop (0.02 ml) of spore suspension, and then incubated in a ventilated climate incubator at controlled temperature (20°C, 25°C, 30°C, 35°C, or 40°C) and controlled relative humidity (RH) (73-75%, 84-86%, or 95-97%).

At regular intervals duplicate Petri dishes were removed, and mycelium with membrane was taken off from a tofu. Subsequently, the mycelium was dried for 2 d at 80°C, and the mycelial dry weight was determined gravimetrically.

Growth curves based on the cubic root of mycelial dry weights were fitted using linear regression (Microsoft Excel) to determine the biomass formation rate [$d(x^{1/3})/dt$] (Han & Nout, 2000).

Pehtze preparation

Tofu, as mentioned above, was cut into pieces (3.2*3.2*1.6 cm) for pehtze preparation. The pieces were inoculated with *A. elegans* and *R. oligosporus,* respectively, by spraying spore suspension onto the surface of each. The inoculated tofu was placed evenly spaced in plastic trays, and incubated in a ventilated climate incubator, at controlled temperature (25°C, 30°C, or 35°C) and controlled RH (73-75%, 84-86%, or 95-97%). Fresh pehtze (or tofu if not real pehtze) were

obtained for analyses after being incubated for 12, 24, 36, 48, and 60 h.

Enzyme assays

Crude extracts

Duplicate pehtze samples (about 10 g) were mixed with 50 ml of 0.3 M NaCl and 0.2 M phosphate buffer (pH 6.8) and homogenized in a blender. They were kept at room temperature for 60 min with frequent stirring, and then centrifuged at 2100 x *g* for 5 min. The supernatant was used as crude enzyme extract. All samples were analyzed in duplicate ($n = 4$).

Protease activity

The method mentioned by Kruger (1973) was used. 2 ml of crude enzyme extract and 2 ml of 2.4% azocasein (Sigma A-2765) dissolved in 0.05 M McIlvaine citric acid-disodium phosphate buffer (pH 6.0) were mixed. The mixture was then shaken gently for 2 h in a shaking bath at 35°C. The reaction was terminated by addition of 5 ml of 10% TCA, and the mixture was filtered. 5 ml of 0.5 M NaOH was added to 5 ml of the filtrate and after 20 min the absorbance of the solution was read at 440 nm. One unit of protease activity is defined as an absorbance (440 nm) change of 0.01 after 2 h at 35°C (UV-VIS Spectrophotometer, UV mini 1240, SHIMADZU, Japan).

Lipase activity

Titrimetric determination of lipase (Lipase kit, Sigma Catalog No. 800-B) was carried out. A mixture of 1.0 ml crude enzyme extract, 2.5 ml water, 3.0 ml Sigma Lipase Substrate (Catalog No.800-1), and 1.0 ml TRIZMA Buffer (Sigma Catalog No. 800-2) was shaken at 37°C for 6 h. The reaction was stopped by adding 3.0 ml 95% ethanol, and 4 drops of Thymolphthalein Indicator Solution (Catalog No. 800-3) were added, followed by titration with 0.05 M NaOH solution until a slight blue colour was observed. Sigma-Tietz Units of lipase are equal to the volume (ml) of 0.05 M NaOH required to neutralize the fatty acids liberated during the incubation. Lipase activity in Sigma-Tietz Units/ml was converted to International Units multiplying by 280.

α-Amylase

The activity of α-amylase was determined by a colorimetric method as follows. The reaction mixture, which contained 10 ml of 1% soluble starch and 5 ml of 0.1 M phosphate buffer (pH 6.0), was equilibrated at 60°C for 5 min. Then, 1.0 ml of the crude enzyme extract was added into the mixture and it was kept at 60°C for 10 min.

Next, 1 ml of the mixture was added to 5 ml of a solution containing 0.44 mg iodine and 0.2 g KI. The absorbance of the solution was measured at 660 nm, and α-amylase activity was calculated using a standard curve made with commercial α-amylase (Microbiologial Media Product, Beijing). For practical reasons we defined one unit of α-amylase as the activity catalysing the hydrolysis of 1 ml of 1% starch solution in 1 h under the assay conditions, in comparison with α-amylase of known activity.

Glutaminase

A modification of the method of Moriguchi et al. (1994) was used, monitoring the formation of L-glutamate with L-glutamate dehydrogenase. The reaction mixture contained 100 mM Tris-HCl buffer (pH 7.5), 30 mM L-glutamine (Sigma G-3126), 5% NaCl and crude enzyme extract (0.1 ml) in a final volume of 0.5 ml. After being allowed to react for 10 min at 30°C, the reaction was terminated by boiling for 3 minutes. 50 mM Tris-hydrazine buffer (pH 9.0), 1.5 mM NAD^+ (Sigma N-7004), 0.5 mM ADP (Sigma A-2754) and 5 units/ml of glutamate dehydrogenase (Sigma G-2501) were added in a total volume of 1.0 ml to this supernatant after centrifugation (Centrifuge CT4D, HITACHI, Japan). The absorbance at 340 nm was measured after incubating the mixture for 1 h at 30°C. One unit of glutaminase activity was defined as a change in the absorbance of 0.1 units at 340 nm at 30°C.

α-Galactosidase

The combined method described by van den Broek et al. (1999) and Chou et al. (1988) was used. The reaction mixture, which contained 0.1 ml of 0.01 M *p*-nitrophenyl-α-D-galactopyranose (PNPG, Sigma N-0877), 0.3 ml of 0.2 M phosphate buffer (pH 6.4), and 0.1 ml of crude enzyme extract, was incubated at 37°C for 20 min. The reaction was stopped by adding an equal volume of 0.5 M glycine/NaOH buffer (pH 9) containing 2 mM EDTA. The colour formation was measured at 400 nm. The α-galactosidase activity was then assayed by measuring the amount of *p*-nitrophenol liberated from PNPG at 37°C in 20 min using a standard curve. One unit of α-galactosidase was defined as the activity liberating 1 μg *p*-nitrophenol per min under the specified conditions.

RESULTS AND DISCUSSION

Effect of incubation temperature and RH on fungal growth rate on membrane-covered tofu

Previously, Chou et al. (1988) and Wang et al. (1974) monitored the growth by visual estimation, which only provides an indication of mycelial formation. In this

study, a nylon membrane separated the mycelium from the tofu substrate. Previously, we found that fungal growth is not affected by the presence of a membrane, whereas this technique offers the possibility to separate the biomass and determine its weight. Similar experiments were reported by Mitchell et al. (1989) and Nagel et al. (1999).

Figure 6.1 shows the effect of incubation temperature and RH on the biomass formation rate expressed as $[d(x^{1/3})/dt]$. RH levels varied from 73-75% to 95-97% and temperatures from 20°C to 40°C. The optimum growth temperatures and RH for *A. elegans* and *R. oligosporus* were 25°C at 95-97%, and 35°C at 95-97%, respectively. At constant temperature, the formation rates were positively correlated with RH. The formation rate of *A. elegans* declined sharply at temperatures exceeding 30°C. On the other hand, the formation rate of *R. oligosporus* was still quite high at 40°C.

Effect of incubation temperature and RH on enzyme activities expressed in pehtze

Protease production by *A. elegans* and *R. oligosporus* is presented in Table 6.1. The protease activities produced by the tested mould strains on tofu were considerably affected by the incubation temperature and humidity. At higher RH, higher protease activities were obtained. The highest protease activity (108 U/g of pehtze d.m.) of *A. elegans* was found after 48 h incubation at 25°C and RH 95-97%. For *R. oligosporus* the highest activity (104 U/g of pehtze d.m.) was obtained after 48 h incubation at 35°C and RH 95-97%.

A. elegans and *R. oligosporus* produced relatively high lipase activities on tofu under optimum conditions (Table 6.2). The highest lipase activity (172 U/g pehtze d.m.) produced by *A. elegans* was found after 48 h incubation at 25°C and RH 95-97%. Although lipase activity produced by *R. oligosporus* was not strongly affected by incubation temperature and RH, the maximum activity (187 U/g pehtze d.m.) was observed after 48 h incubation at 35°C and RH 95-97%.

Similarly to the production of protease and lipase, *A. elegans* produced more α-amylase activity at higher RH levels (Table 6.3). Although the optimum growth temperature for *A. elegans* was 25°C, the highest yield of α-amylase (279U/g pehtze d.m.) was observed after 48 h incubation at 30°C and RH 95-97%. The enzyme activity declined after 60 h. *R. oligosporus* produced maximum α-amylase activity (288 U/g pehtze d.m.) after 36 h of incubation at 35°C and RH 95-97%; the enzyme activity decreased after 48 h.

Glutaminase is considered an important key enzyme for the palatable taste of fermented soybean foods (Lu et al., 1996), and expectedly, the predominance of glutamate in sufu (Liu & Chou, 1994) results from glutaminase activity. Glutamate in combination with salt (NaCl) contributes to the flavour and hedonic characteristics of foods (Halpern, 2000). Table 6.4 shows that the highest glutaminase activity (176 U/g pehtze d.m.) of *A. elegans* was formed at 25°C and

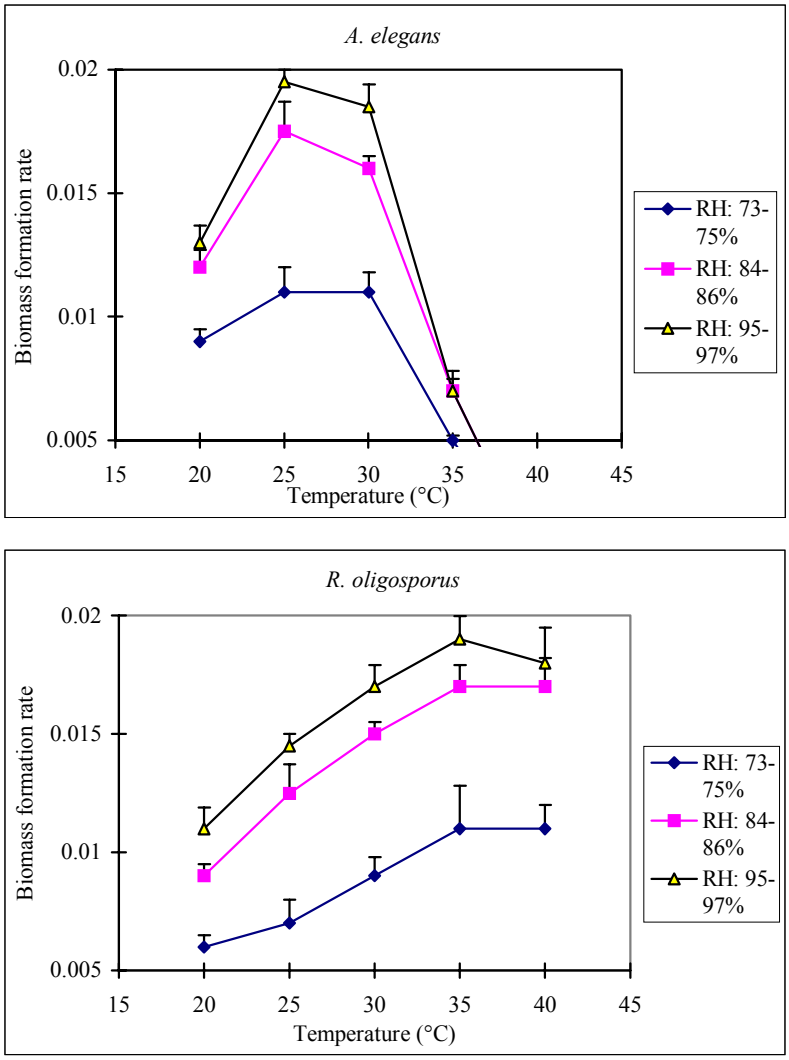

Fig. 6.1 Biomass formation rate [$d(x^{1/3})/dt$] of *Actinomucor elegans* AS 3.227 and *Rhizopus oligosporus* NRRL 5905 as a function of temperature and relative humidity.

RH 95-97% after 48 h of incubation. The highest yield of glutaminase (187 U/g pehtze d.m.) by *R. oligosporus* was observed at 35°C and RH 95-97% after 36 h of cultivation. Large amounts of glutaminase are a favourable prerequisite for production of pehtze with highly appreciated palatability.

Table 6.1 Protease activity (U/g pehtze dry matter) production by *Actinomucor elegans* AS 3.227 and *Rhizopus oligosporus* NRRL 5905 at various incubation temperatures and relative humidities (RH).

Mould	RH (%)	Temperature (°C)														
		25					30					35				
		Incubation time (h)					Incubation time (h)					Incubation time (h)				
		12	24	36	48	60	12	24	36	48	60	12	24	36	48	60
A. elegans	73-75	4.4 ±0.8	36.4 ±8.8	58.3 ±10.0	61.2 ±4.2	60.3 ±5.6	6.4 ±1.3	43.4 ±7.4	52.3 ±8.4	61.2 ±5.4	60.1 ±3.4	2.3 ±0.1	8.1 ±2.0	13.3 ±3.0	14.5 ±1.3	14.3 ±1.1
	84-86	5.2 ±0.7	52.1 ±5.7	64.0 ±5.1	82.3 ±5.5	75.2 ±4.7	8.1 ±1.1	53.1 ±3.0	64.2 ±0.1	81.9 ±11.2	73.3 ±4.2	2.2 ±0.3	7.4 ±0.9	11.4 ±1.6	16.2 ±2.8	12.3 ±0.5
	95-97	5.2 ±0.8	58.3 ±4.2	80.4 ±5.9	108 ±13.8	90.3 ±8.6	12.1 ±0.5	56.3 ±1.2	85.4 ±4.6	96.3 ±5.9	81.3 ±7.9	2.1 ±0.2	10.5 ±1.1	12.3 ±1.1	15.4 ±1.3	16.4 ±1.6
R. oligosporus	73-75	5.2 ±1.8	38.4 ±4.0	46.2 ±5.6	61.9 ±1.9	72.2 ±7.9	6.3 ±1.6	46.2 ±5.9	69.5 ±4.5	77.3 ±5.8	58.7 ±6.7	11.8 ±1.6	57.9 ±4.9	76.2 ±5.2	86.8 ±6.2	77.2 ±7.4
	84-86	5.6 ±0.7	46.1 ±5.7	58.7 ±6.6	78.4 ±3.1	77.9 ±9.3	6.2 ±0.9	58.6 ±1.9	78.3 ±1.2	91.8 ±8.5	87.7 ±7.5	19.2 ±2.4	67.5 ±1.2	81.3 ±4.6	97.1 ±3.5	74.8 ±4.8
	95-97	7.4 ±1.0	39.3 ±4.2	63.9 ±1.1	72.3 ±8.3	72.7 ±4.7	7.2 ±1.3	56.1 ±1.9	82.5 ±6.2	97.9 ±4.3	91.4 ±5.6	20.4 ±2.5	64.5 ±1.5	94.4 ±6.7	104 ±8.8	103 ±9.8

Data represent averages ± standard deviations of duplicate analyses of duplicate samples.

Table 6.2 Lipase activity (U/g pehtze d.m.) production by *Actinomucor elegans* AS 3.227 and *Rhizopus oligosporus* NRRL 5905 at various incubation temperatures and relative humidities (RH).

Mould	RH (%)	Temperature (°C)														
		25					30					35				
		Incubation time (h)					Incubation time (h)					Incubation time (h)				
		12	24	36	48	60	12	24	36	48	60	12	24	36	48	60
A. elegans	73-75	15.6 ±2.3	56.2 ±7.4	113 8.1	123 ±9.9	105 ±7.0	14.2 ±1.7	62.3 ±6.1	96.4 ±7.3	131 ±5.7	105 ±6.1	10.2 ±0.9	35.2 ±2.6	44.6 ±7.1	56.4 ±3.1	55.3 ±5.1
	84-86	14.3 ±1.6	88.3 ±5.5	122 ±9.6	146 ±5.7	143 ±8.2	16.7 ±2.3	91.2 ±5.9	118 ±7.4	154 ±7.1	137 ±4.2	9.8 ±1.1	37.3 ±3.5	42.1 ±6.6	62.8 ±3.8	60.8 ±4.1
	95-97	17.4 ±2.3	93.6 ±6.7	135 ±10.0	172 ±11.3	168 ±9.4	17.3 ±1.1	102 ±8.5	120 ±5.8	147 ±4.2	135 ±10.0	11.7 ±1.5	31.2 ±2.8	45.9 ±3.6	59.2 ±4.1	61.6 ±7.8
R. oligosporus	73-75	12.3 ±1.2	78.4 ±4.8	90.6 ±7.7	119 ±5.7	123 ±9.3	13.3 1.6	84.3 ±6.1	93.3 ±7.7	128 ±8.4	119 ±6.1	12.5 ±1.0	87.2 ±4.0	99.1 ±9.7	129 ±7.8	115 ±5.1
	84-86	16.8 ±1.4	83.9 ±1.8	127 ±3.8	154 ±5.7	158 ±6.9	18.4 ±1.2	96.8 ±4.0	125 ±7.5	169 ±11.3	172 ±8.4	19.7 ±2.3	96.4 ±5.1	143 ±12.4	156 ±8.5	155 ±9.7
	95-97	15.8 ±1.7	92.2 ±5.1	133 ±10.2	179 ±9.9	170 ±6.4	18.9 ±1.6	106 ±5.7	144 ±7.2	177 ±10.1	183 ±8.1	20.1 ±2.6	95.1 ±5.0	148 ±11.6	187 ±5.7	178 ±9.9

Data represent averages ± standard deviations of duplicate analyses of duplicate samples.

Table 6.3 α-Amylase activity (U/g pehtze d.m.) production by *Actinomucor elegans* AS 3.227 and *Rhizopus oligosporus* NRRL 5905 at various incubation temperatures and relative humidities (RH).

Mould	RH (%)	Temperature (°C)														
		25					30					35				
		Incubation time (h)					Incubation time (h)					Incubation time (h)				
		12	24	36	48	60	12	24	36	48	60	12	24	36	48	60
A. elegans	73-75	56.4 ±3.8	157 ±4.2	198 ±9.9	231 ±4.2	167 ±17.0	67.4 ±3.5	162 ±5.7	206 ±5.7	248 ±18.4	187 ±9.9	12.5 ±3.9	34.3 ±4.2	58.1 ±4.4	79.2 ±11.8	75.8 ±4.2
	84-86	64.5 ±4.0	167 ±5.7	219 ±4.2	251 ±4.2	213 ±9.9	76.2 ±9.9	173 ±7.1	227 ±4.2	267 ±4.4	235 ±14.1	13.4 ±3.7	38.6 ±1.1	64.2 ±5.7	86.7 ±6.4	76.1 ±6.1
	95-97	76.2 ±8.8	178 ±5.7	236 ±14.1	269 ±5.7	223 ±11.3	88.4 ±4.5	187 ±8.5	243 ±4.2	279 ±12.7	226 ±5.7	11.4 ±1.7	33.9 ±2.6	65.2 ±4.1	57.2 ±2.6	67.9 ±10.6
R. oligosporus	73-75	34.6 ±3.5	126 ±5.7	176 ±9.9	204 ±8.5	187 ±7.1	56.3 ±3.4	187 ±2.8	238 ±8.5	212 ±11.3	209 ±4.2	49.5 ±5.5	197 ±7.1	249 ±4.2	225 ±11.3	217 ±4.3
	84-86	46.5 ±5.9	134 ±5.7	198 ±9.9	226 ±5.6	223 ±4.2	57.7 ±5.2	198 ±9.9	265 ±9.9	243 ±21.2	224 ±18.3	68.7 ±3.3	228 ±8.5	282 ±17.0	264 ±25.5	245 ±14.1
	95-97	58.4 ±4.5	149 ±5.7	213 ±9.9	238 ±11.3	225 ±5.7	67.5 ±3.8	176 ±8.5	287 ±9.9	257 ±5.7	253 ±8.5	76.1 ±5.8	249 ±7.1	288 ±17.0	274 ±8.5	259 ±12.7

Data represent averages ± standard deviations of duplicate analyses of duplicate samples.

Table 6.4 Glutaminase activity (U/g pehtze d.m.) production by *Actinomucor elegans* AS 3.227 and *Rhizopus oligosporus* NRRL 5905 at various incubation temperatures and relative humidities (RH).

Mould	RH (%)	Temperature (°C)														
		25					30					35				
		Incubation time (h)					Incubation time (h)					Incubation time (h)				
		12	24	36	48	60	12	24	36	48	60	12	24	36	48	60
A. elegans	73-75	25.3 ±1.8	57.1 ±4.2	91.2 ±5.5	105 ±7.7	110 ±5.4	24.5 ±2.7	68.1 ±3.7	95.7 ±8.9	111 ±4.8	121 ±10.4	8.35 ±1.1	23.8 ±1.6	36.4 ±3.1	31.5 ±1.6	32.6 ±3.3
	84-86	34.2 ±1.5	76.4 ±7.9	128 ±9.4	147 ±9.6	136 ±9.3	35.4 ±1.9	77.6 ±7.1	128 ±5.6	167 ±13.2	155 ±9.8	8.26 2.0±	24.6 ±3.3	28.4 ±2.0	27.8 ±2.4	24.5 ±2.8
	95-97	49.2 ±3.4	95.8 ±7.7	158 ±11.1	176 ±12.0	167 ±7.1	36.5 ±3.6	72.3 ±4.0	139 ±10.2	168 ±11.8	159 ±4.7	8.41 ±0.8	31.2 ±2.1	33.4 ±1.9	36.1 ±3.7	35.6 ±4.2
R. oligosporus	73-75	16.2 ±1.2	48.7 ±6.4	98.3 ±4.3	106 ±9.4	103 ±5.0	19.3 ±1.7	55.8 ±2.5	110 ±6.1	118 ±6.9	116 ±9.3	34.2 ±4.5	67.5 ±3.9	127 ±9.1	145 ±8.4	138 ±11.2
	84-86	27.1 ±1.8	61.4 ±3.8	119 ±2.8	128 ±6.7	118 ±10.4	43.6 ±2.8	71.5 ±3.8	146 ±11.3	154 ±10.9	136 ±13.2	46.3 ±5.3	97.5 ±7.9	167 ±12.0	178 ±14.7	164 ±8.6
	95-97	27.9 ±2.6	79.4 ±6.9	164 ±10.3	169 ±9.4	171 ±12.3	58.4 ±4.4	83.2 ±7.7	179 ±8.9	170 ±11.0	176 ±12.1	58.4 ±3.2	106 ±6.5	187 ±11.8	176 ±12.6	177 ±11.5

Data represent averages ± standard deviations of duplicate analyses of duplicate samples.

Table 6.5 α-Galactosidase activity (U/g pehtze d.m.) production by *Actinomucor elegans* AS 3.227 and *Rhizopus oligosporus* NRRL 5905 at various incubation temperatures and relative humidities (RH).

Mould	RH (%)	Temperature (°C)														
		25					30					35				
		Incubation time (h)					Incubation time (h)					Incubation time (h)				
		12	24	36	48	60	12	24	36	48	60	12	24	36	48	60
A. elegans	73-75	23.2 ±2.1	58.8 ±6.3	98.3 ±6.4	129 ±9.4	124 ±9.5	41.6 ±3.6	87.2 ±6.7	119 ±9.1	167 ±10.7	174 ±7.3	12.4 ±2.2	26.1 ±2.1	41.3 ±2.9	32.7 ±3.0	34.6 ±2.5
	84-86	31.2 ±3.0	84.5 ±7.5	137 ±7.3	181 ±13.5	184 ±10.8	48.1 ±2.9	91.2 7.9±	164 ±9.9	210 ±15.5	216 ±15.5	7.2 ±1.6	24.1 ±1.5	35.4 ±1.8	31.3 ±2.4	32.4 ±2.4
	95-97	42.1 ±1.9	95.4 ±4.7	158 ±11.0	197 ±12.1	184 ±7.6	56.2 ±4.8	110 ±5.4	189 ±16.3	225 ±12.4	227 ±14.3	7.2 ±1.0	21.5 ±1.1	38.4 ±1.7	24.1 ±1.3	26.4 ±2.8
R. oligosporus	73-75	35.7 ±4.2	65.2 ±6.1	158 ±14.3	179 ±13.4	142 ±10.8	34.6 ±4.4	120 ±7.8	189 ±12.8	187 ±10.6	178 ±11.8	58.4 ±2.6	91.2 ±4.9	178 ±11.2	192 ±11.7	176 ±15.4
	84-86	48.3 ±2.3	87.2 ±5.8	149 ±10.6	197 ±9.4	168 ±13.0	56.2 ±3.3	91.4 ±6.9	167 ±10.3	215 ±11.9	208 ±12.9	69.3 ±4.7	98.1 ±5.6	188 ±9.9	216 ±16.5	213 ±12.6
	95-97	57.4 ±3.5	130 ±9.0	189 ±11.2	178 ±12.6	168 ±11.3	42.6 ±2.9	140 ±12.0	226 ±16.6	208 ±10.8	201 ±9.1	57.2 ±3.5	133 ±10.4	210 ±13.5	198 ±13.8	188 ±11.9

Data represent averages ± standard deviations of duplicate analyses of duplicate samples.

Soybean contains low-molecular-weight saccharides, such as stachyose and raffinose, which may be involved in flatulence resulting from ingestion of soybean products. Hydrolysis of stachyose and raffinose by α-galactosidase has been suggested as a way to resolve the flatulence problem (Hayakawa et al., 1990; Sugimoto & van Buren, 1970). Therefore, it is of interest to investigate α-galactosidase production by *A. elegans* and *R. oligosporus.*

Within the conditions investigated (Table 6.5), *A. elegans* produced the highest α-galactosidase activity (227 U/g pehtze d.m.) at 30°C and RH 95-97% after 60 h of incubation. *R. oligosporus* yielded the highest α-galactosidase activity (226 U/g pehtze d.m.) at 30°C and RH 95-97% after 36 h of incubation. The optimum temperature for production of α-galactosidase by both moulds was 30°C, which did not exactly follow the trends of optimum growth temperatures.

We observed that *A. elegans* grows well at 25-30°C and RH 84-97%, and produces considerable enzyme activities after 48 h, whereas it poorly tolerates higher temperatures (35°C). On the other hand, *R. oligosporus* could grow very well at 35-40°C, and yield a very similar pattern of enzyme activity when compared with *A. elegans*. We conclude that, from the point of view of growth and extracellular enzyme production, *R. oligosporus* is able to produce similar levels of biomass and enzyme activities as *A.elegans* at temperatures that are about 10ºC higher than those tolerated by the latter. Therefore, *R. oligosporus* could be an alternative for *A. elegans* as the starter of sufu production during hot seasons. It remains to be established whether or not sufu produced with *R. oligosporus* has a flavour and taste similar to sufu from *A. elegans*.

The flavour and texture of sufu that develop during the aging are determined by the enzymes produced by the mould in pehtze. There is so far no indication of metabolites also contributing to the flavour. The data obtained in this study demonstrate that growth and enzyme production by *A. elegans* and *R. oligosporus* on tofu were influenced by incubation temperature, humidity, and time. From these results it is obvious that sufu manufacturers should pay attention to the control of these conditions. The fluctuation of incubation conditions could result in sub-optimum flavour and texture, or even in putrefaction. Control of incubation conditions will also contribute to reduce the variability in quality from batch to batch.

In conclusion, *R. oligosporus* has been shown to have similar growth and enzyme production abilities as *A. elegans*. Consequently, it will now be of interest to evaluate the feasibility and acceptability of the use of *Rhizopus* strains in sufu production.

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Chapter 7

Effect of NaCl on Textural Changes and Protein and Lipid Degradation during the Ripening Stage of Sufu, a Chinese Fermented Soybean Food

Abstract

Sufu is made by solid-state fungal fermentation (using *Actinomucor elegans*) of tofu, followed by salting and maturation in dressing mixtures containing salt, alcohol and various other ingredients. NaCl in dressing mixtures strongly affected the changes of textural properties and the hydrolysis of protein and lipid of sufu. Higher salt contents (14% w/w) resulted in increased hardness (+ 100%) and elasticity (+ 18%), and reduced adhesiveness (- 30%). Hardness and elasticity could be used to judge the extent of sufu ripening. SDS-PAGE showed disappearance of all protein subunits at 8% and 11% salt content; however, some protein subunits were still detectable at 14% salt content after 60 d ripening. Higher ratios of free amino nitrogen to total nitrogen (FAN/TN = 0.4-0.45) and free amino acids to crude protein (FAA/CP = 0.24-0.26) were observed in sufu with lower (8%) salt content. Ratios of FAN/TN and FAA/CP in white sufu (obtained with dressing mixtures containing only salt and alcohol) were higher than those in red sufu (dressing mixtures containing red kojic rice "angkak") due to different dressing mixture compositions. Increases of free fatty acid (FFA) were also observed during ripening. FFA levels in sufu with lower salt content increased rapidly during the first 30-40 d, and then increased slowly, probably resulting from the formation of fatty acid esters. Lowering salt content (8%) can shorten the ripening time to 40 d, which is of benefit to manufacturers. However, sufu will spoil, i.e. undergo souring, during the ripening stage at salt contents of 5% or lower.

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INTRODUCTION

Sufu, *Fu-ru* written in hieroglyphics, is a traditional Chinese fermented soybean curd and a highly flavoured, soft creamy cheese-type product, which can be used in the same way as cheese (Su, 1986; Steinkraus, 1996). This fermented product with a relatively high protein and lipid content has been widely consumed by Chinese people as an appetizer for many centuries.

There are many different types of sufu, produced by various local processes in China (Wang & Du, 1998), with mould-fermented sufu being the most popular type of product (Han et al., 2001b). Four steps are normally involved in making this type of sufu: (1) Preparing tofu (soybean curd), (2) Preparing pehtze (*pizi*) by fungal solid-state fermentation of tofu using e.g. *Actinomucor elegans*, (3) Salting of pehtze, and (4) Ripening in dressing mixture (Wang et al., 1970). Hydrolysis of protein and lipid occurs mainly during the ripening stage that usually takes 3-6 months. Traditionally, the ripening stage took over 6 months since the salt content in some sufu exceeded 14%. Presently, the salt content in most products, especially red sufu, is still > 10% (Han et al., 2001a) which results in ripening periods of three months or longer. A reduction of salt content would have the combined advantages of shortening the ripening periods as well as reducing dietary sodium intake. Hardness and smoothness were identified as important factors influencing consumer acceptability of tofu (Ji et al., 1999). These textural properties are also important factors influencing consumer acceptability of sufu, and they could also be used as parameters for judging the extent of ripening of sufu during its production.

Like in other fermented foods such as cheese (Messens et al., 1999) and miso (Chiou et al., 1999), salt has a multiple role in sufu. During the salting period, the pehtze absorbs the salt and free water until the salt content of pehtze reaches an equilibrium level. The absorbed salt will later impart a salty taste to the sufu, but it will control the microbial growth and enzyme activity in sufu as well. In addition, salt influences physical and biochemical changes in the product. Not surprisingly, the salt concentration in sufu is considered one of the most important factors affecting its quality. However, little quantitative information on its effect on the hydrolysis of protein (the major component of tofu) and lipid is available.

Chou and Hwan (1994) investigated the effect of ethanol in dressing mixtures on some biochemical changes in white sufu during ripening, and showed that the added alcohol delayed the degradation of soybean proteins. In the present study, the effect of salt on such biochemical changes as hydrolysis of protein and lipid during the ripening of red and white sufu prepared with *Actinomucor elegans* was investigated.

MATERIALS AND METHODS

Microorganism

Actinomucor elegans (Academia Sinica AS 3.227) is commonly used as a starter in commercial sufu production in China. Starting from an agar slant culture, a pure culture inoculum of *A. elegans* AS 3.227 was prepared by liquid substrate culture in Roux bottles, as is common practice in Chinese sufu factories. The medium consisted of soy whey (by-product from tofu manufacture) to which maltose (2-3%) and peptone (1.5-2%) were added prior to sterilization by autoclaving. After incubation at 28°C for 72 h, medium and biomass were harvested and homogenized to obtain a spore suspension containing ~10^5 CFU/ml.

Sufu preparation

The tofu used as raw material for sufu was provided by Beijing WangZhiHe sufu manufacturer, and was cut into pieces (3.2*3.2*1.6 cm). The pieces were inoculated with *A. elegans* by spraying spore suspension onto the surface of each. The inoculated tofu pieces were placed evenly spaced, in plastic trays. The loaded trays were piled up in an incubation room with controlled temperature (around 25°C), relative humidity (around 90%) and air circulation to ensure adequate aeration. Fresh pehtze, i.e. tofu overgrown with *A. elegans* mycelium, was obtained after incubation for 48 h.

The pehtze was transferred into a container (20 liters) and salt was spread between layers of pehtze as they piled up in the container. During a period of 5 d, the pehtze absorbed the salt until salt content of pehtze reached about 14-15%.

For the ripening of sufu, 12 pieces of salted pehtze (about 200 g fresh weight) were placed in individual wide-mouthed glass bottles with a capacity of 340 ml, after which dressing mixture (about 140 ml) was added to the pehtze. In order to reach the required final salt level, salt was also added in some dressing mixtures to obtain sufu with 11% and 14% salt content. For red sufu, the dressing mixture consisted of angkak or kojic red rice alcoholic beverage (rice wine) (Nout & Aidoo, 2002) to a final alcohol content of 5%, sugar, Chiang (wheat-based miso) (Campbell-Platt, 1987), and spices. For white sufu, the dressing mixture only consisted of alcoholic beverage (final alcohol content 5%). The filled bottles were closed and incubated at 25-28°C for 80 d.

Sampling for analysis

Two bottles were drawn randomly from each batch on each sampling day during the ripening. The ripening dressing mixture was decanted and sufu pieces were tested.

Determination of textural properties

Pieces of tofu, pehtze and sufu were tested in triplicate. The Texture Profile Analysis (TPA) of sufu was measured with a Rheometer (NRM 2002J, Fudo Kabushiki Kaishia, Japan). A cylindrical plunger with 8 mm diameter and a weight beam of 2 kg was used. The plunger travelled 75% depth into a sufu sample. The speeds of the crosshead and the recording chart were set at 60 mm/min and 100 mm/min, respectively. The textural parameters including hardness (firmness), elasticity (springiness) and adhesiveness of sufu were calculated from the curve according to Bourne (1978).

The hardness was reported as the force required to compress sufu, measured as the maximum height of the curve during the first compression. The elasticity was the extent to which sufu returned to its original shape after it had been decompressed; elasticity was expressed as the horizontal distance between the point when the second curve started and the point when the second curve reached the peak. The adhesiveness was defined as the negative force area following the first compression, representing the work necessary to pull the compressing plunger away from the sufu sample.

Biochemical analyses

Sufu pieces of duplicate bottles were homogenized using mortar and pestle prior to analysis in duplicate. Contents of total nitrogen, free amino nitrogen and crude lipid in each sample were analysed according to the Kjeldahl method, the formol titration method and the Soxhlet extraction method, respectively, as described by Nielsen (1998).

Free fatty acid (FFA) content was determined according to Pike (1998). To the extracted sufu lipid sample, neutralized 95% ethanol and phenolphthalein indicator was added. The sample then was titrated with NaOH and the percent FFA calculated as oleic acid.

Determination of total free amino acid content was performed with a modified method according to Niven et al. (1998). The lyophilised sample homogenates were dissolved in sulphosalicylic acid, and supernatants were applied to the amino acid analyser (HITACHI 835-50, Japan) for determination of total free amino acids.

SDS-PAGE profile

SDS-PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis) was performed using the Phast System from Amersham Pharmacia Biotech AB (Kiers et al., 2000). The markers used were those of the LMW calibration kit that contained proteins with 14.4, 20.1, 30, 43, 67 and 94 KDa molecular weights. Samples were mixed with sample buffer at the concentration of 6 mg lyophilised

sample powder/ml. The prepared samples and markers were then heated at 100°C for 5 min. The SDS-PAGE was performed on PhastGel™ Gradient 8-25% using SDS buffer strips. The heated samples were applied using 8/1 (8 wells each 1µl) sample applicator/comb. The separation and visualization were performed according to the manufacturer's manual.

RESULTS AND DISCUSSION

Properties of tofu, pehtze and salted pehtze

Table 7.1 presents the textural properties and chemical parameters of tofu, pehtze and salted pehtze, which were intermediate products of sufu prior to the ripening procedure. Considerable differences can be observed between tofu, pehtze and especially salted pehtze, since the added salt has great impact on the composition of the dry matter.

Chemical parameters of sufu during ripening

During the experiments, since sufu was ripened in closed bottles, the moisture contents of sufu remained stable in the ranges of 64%, 61% and 59% for red sufu, and of 71%, 67% and 64% for white sufu, containing 8%, 11% and 14% salt, respectively. pH values decreased slightly from 6.6~6.8 after 10 d, to pH 5.9~6.1 after 80 d of ripening time.

Sufu with 5% salt content was also included in the experiments. However, this sufu was spoiled after 20 d as evidenced by low pH (< 4.6) and off-flavour. This suggests that for stability, sufu should have > 5% salt content during the ripening.

Change in textural properties

The Texture Profile Analysis (TPA) of red and white sufu with various salt contents is shown in Figure 7.1. All starting points were at 10 d instead of 0 d because salted pehtze without dressing mixture cannot be compared with ripening sufu. Sufu at the ripening age of 10 d was considered to be a representative starting sample for this study. Hardness, elasticity and adhesiveness of red sufu were slightly higher than that of white sufu at the same salt content; this is probably due to the different composition of dressing mixtures for red and white sufu. The salt levels in sufu greatly affected the textural changes. Higher salt contents resulted in higher values for hardness and elasticity, with lower adhesiveness. Hardness of sufu with 8% salt decreased rapidly from ~600 g to ~100 g within 45 d, followed by a slow decrease. Even at the lowest (8%) salt content, elasticity values did not decrease below 0.5.

Table 7.1. Textural properties and chemical parameters of tofu, pehtze and salted pehtze

Sample	Hardness (g)	Elasticity (-)	Adhesiveness (J)	pH	FAN (mM/g)[a]	Moisture (% w/w)[a]	C Protein (% w/w)[b]	FAA (% w/w)[b]	C Lip (% w/w)[b]	FFA (% w/w)[c]
Tofu	560 ± 63[d]	1 ± 0	0.1 ± 0	6.87 ± 0.21	0.035 ± 0.002	74.3 ± 4.2	63.1 ± 4.5	0.13 ± 0.01	30.1 ± 1.8	3.7 ± 0.16
Pehtze	860 ± 55	1 ± 0	0.2 ± 0.03	7.03 ± 0.35	0.36 ± 0.02	70.3 ± 3.7	62.8 ± 3.8	1.8 ± 0.11	30.8 ± 2.1	6.4 ± 0.29
Salted Pehtze	1380 ± 103	1 ± 0	3.8 ± 0.21	6.96 ± 0.27	0.26 ± 0.01	56.3 ± 4.1	37.5 ± 2.6	1.1 ± 0.09	18.4 ± 1.2	5.9 ± 0.33

a: fresh weight basis; b: dry matter basis; c: % of crude lipids; d: averages ± standard deviations.

FAN = Free Amino Nitrogen; C Protein = Crude Protein; FAA = Free Amino Acid; C Lip = Crude Lipids; FFA = Free Fatty Acids.

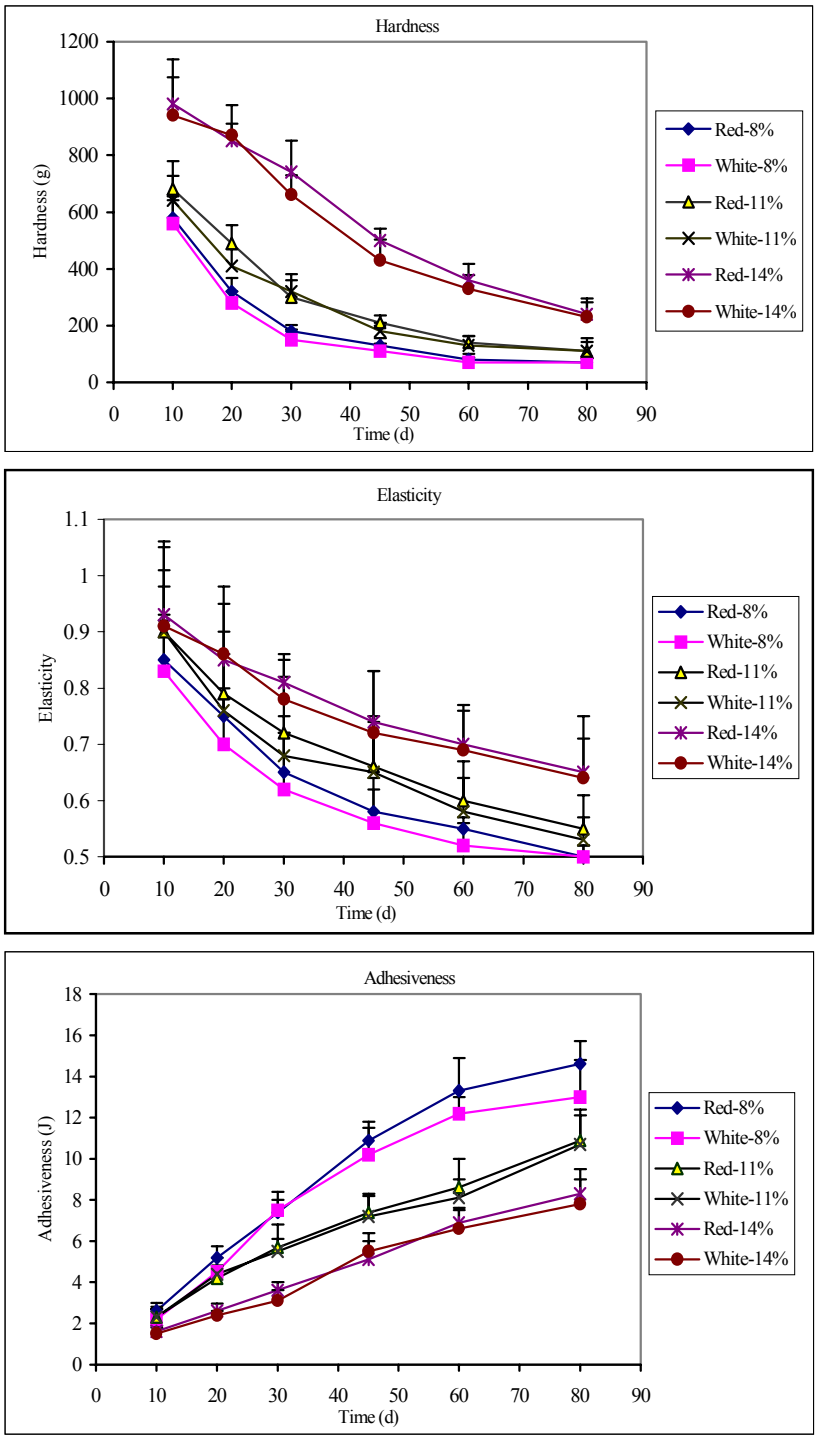


Fig. 7. 1 Texture profile analysis of sufu during ripening.
Rheometer, probe diameter 8 mm. Triplicate analysis of duplicate samples.
Error bars indicate Standard Deviation.
Legend coding: Red-8% = red sufu with 8% total NaCl content.

The TPA was widely used to evaluate the textural properties of tofu (Ji et al., 1999; Hou et al., 1997). However, no published data refer to the textural properties of sufu. In the absence of comparative standards and based on limited subjective evaluations by sufu manufacturing experts, we propose that sufu may be considered ready for packing and distribution when hardness and elasticity have decreased to around 100 g and 0.55, respectively.

Degradation of protein during the ripening of sufu

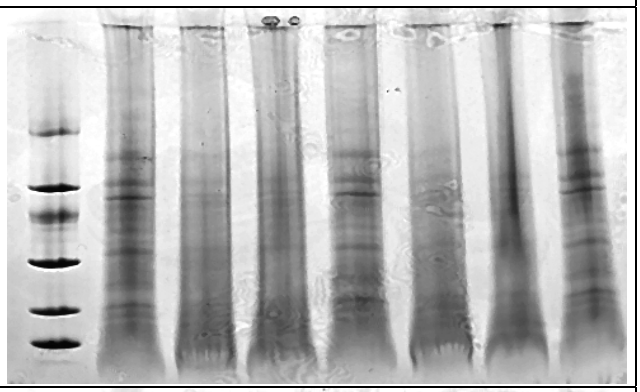
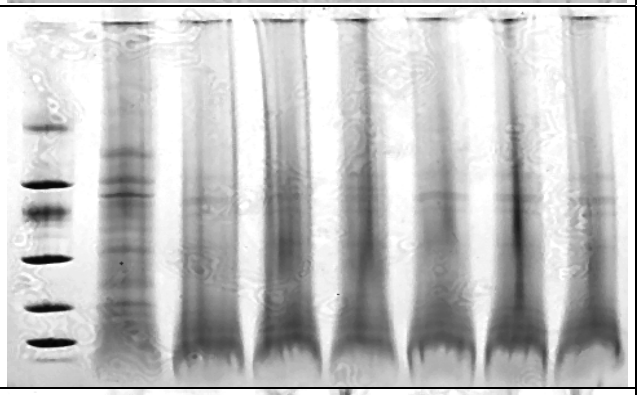
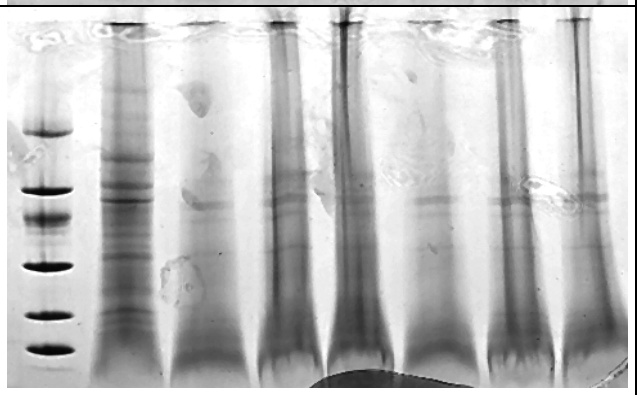
Proteolysis is the principal and most complex biochemical event that occurs during the maturation of most cheese varieties (McSweeney & Fox, 1997). Sufu is a cheese-like product, and proteolysis plays an equally important role during its ripening.

SDS-PAGE can easily separate major proteins, and it has been applied to monitor the hydrolysis of cheese (Dewettinck et al., 1997) and fermented soybean (Kiers et al., 2000). In Figure 7.2 the SDS-PAGE profiles are shown for salted pehtze and subsequent red and white sufu with different salt contents at various ripening stages. The major protein subunits can be clearly identified in salted pehtze (lane 2) and in sufu containing 14% salt after 10 d ripening (lanes 5 and 8). Nevertheless, after 10 d, most protein subunits had been degraded to a large extent in sufu containing 8% (lanes 3 and 6) and 11% salt (lanes 4 and 7). All protein subunits had disappeared in sufu (lanes 3 and 6) containing 8% salt at the age of 60 d. Some protein subunits were still visible in sufu (lanes 5 and 8) with 14% salt content at the same age.

The increase of free amino nitrogen gives a further indication of the hydrolysis of protein. Figure 7.3 shows the change of the ratio of free amino nitrogen to total nitrogen (FAN/TN) during sufu ripening. FAN/TN in white sufu was higher than that in red sufu with the same salt content, probably owing to different chemical compositions of dressing mixtures and microflora present. The FAN/TN in white sufu with 8% salt content reached approximately 0.45 after 80 d, i.e. about 2 times higher than in sufu containing 14% salt.

Ultimately, the degradation of protein in sufu leads to the liberation of free amino acids that are considered to be important flavour enhancing compounds in many fermented foods. In addition, volatile compounds formed from amino acids by decarboxylation, deamination, transamination and other transformations can make substantial contributions to sufu flavour. This is a major reason for the many different volatile compounds (Chung, 1999, 2000) encountered in sufu.

The ratio of free amino acid to crude protein (FAA/CP) is shown in Figure 7.4 as an indicator of protein degradation. FAA/CP in white sufu was larger than that in red sufu of the same salt content, which was similar to corresponding FAN/TN. The FAA/CP in white sufu of a lower salt content (8%) exceeded 0.25 and that in sufu with 14% salt was less than 0.15 after 80 d.

Ripening time	Molecular weight of Marker (KDa)	SDS-PAGE profile
10 d	94 67 43 30 20.1 14.4	
30 d	94 67 43 30 20.1 14.4	
60 d	94 67 43 30 20.1 14.4	

Lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
	Marker	Salted Pehtze	Red sufu			White sufu		
			8%	11%	14%	8%	11%	14%

Fig. 7.2 SDS-PAGE profiles of red and white sufu with different salt content during ripening stage.

Comparing FAN/TN with FAA/CP in sufu, FAN/TN was much higher than FAA/CP. As an example, taking into account the absence of protein bands in SDS-PAGE, data for white sufu with 8% salt content after 80 d indicate that approximately 27% of N occurs as monomeric amino acids, and remaining N as peptides and amino acid degradation products.

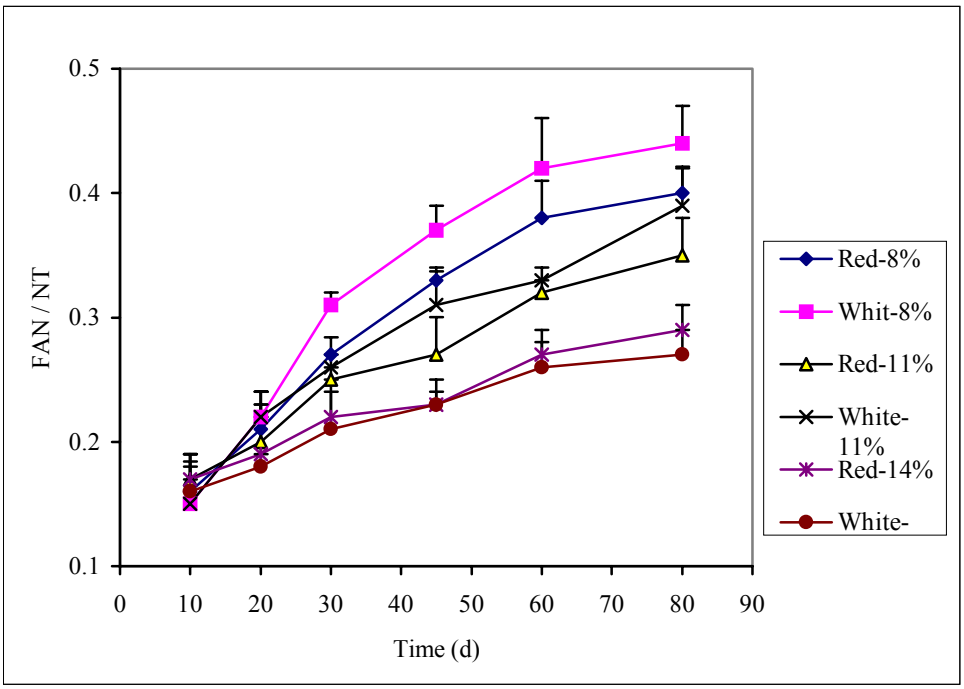


Fig. 7.3 Ratio Free Amino Nitrogen / Total Nitrogen (FAN/TN) during sufu ripening as an indicator of protein degradation.
Averages of duplicate analysis of pooled duplicate samples.
Legend coding: Red-8% = red sufu with 8% total NaCl content.

Lipolysis during sufu ripening

Lipids form the second largest substance in sufu after protein. Crude lipids slightly decreased (< 2% of 18.4% in salted pehtze) during the sufu ripening, regardless of red or white sufu with various salt contents. Fungal lipases can decompose crude lipid into free fatty acids and di- and monoglycerides.

The change of free fatty acid content expressed as fraction of crude lipid during sufu ripening is presented in Figure 7.5. Obviously, salt retarded the hydrolysis of lipids resulting in higher free fatty acid content in sufu containing less salt. Free fatty acid in sufu containing 8% and 11% salt increased rapidly during the first 40 d of ripening, after which time it continued to increase slowly.

This trend probably results from the formation of fatty acid esters from free fatty acids and alcohol present in the dressing mixtures (Hwan & Chou, 1999). Chou and Hwan (1994) reported that free fatty acid content in sufu ripened in brine containing ethanol increased during the first 30 d, after which period it decreased as ripening progressed. We did not observe a decrease even after 80 d although 5% alcohol was present in the dressing mixtures we used for sufu.

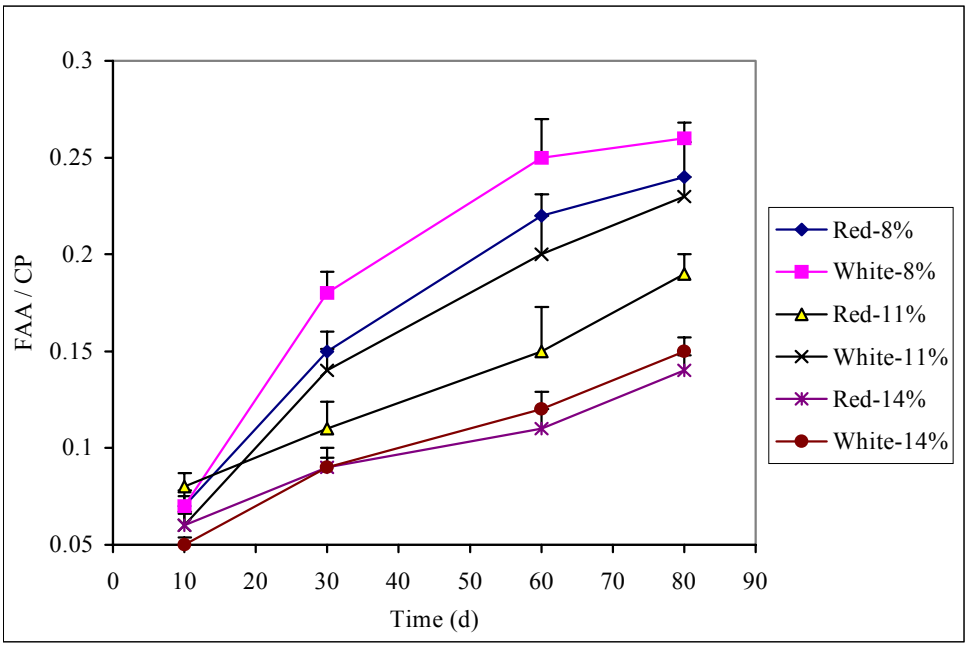


Fig. 7.4 Ratio Free Amino Acids / Crude Protein (FAA/CP) during sufu ripening as an indicator of protein degradation.
Averages of duplicate analysis of pooled duplicate samples.
Legend coding: Red-8% = red sufu with 8% total NaCl content.

CONCLUSIONS

Salt inhibits the enzymic ripening processes resulting in the hydrolysis of protein and lipid. Lower salt content in sufu resulted in reduced hardness and elasticity, and higher extent of degradation of protein and lipid. The ripening time of sufu can be shortened by reducing the salt content, which is of benefit to manufacturers. However, sufu will spoil during the ripening stage at a salt content of 5% or below.

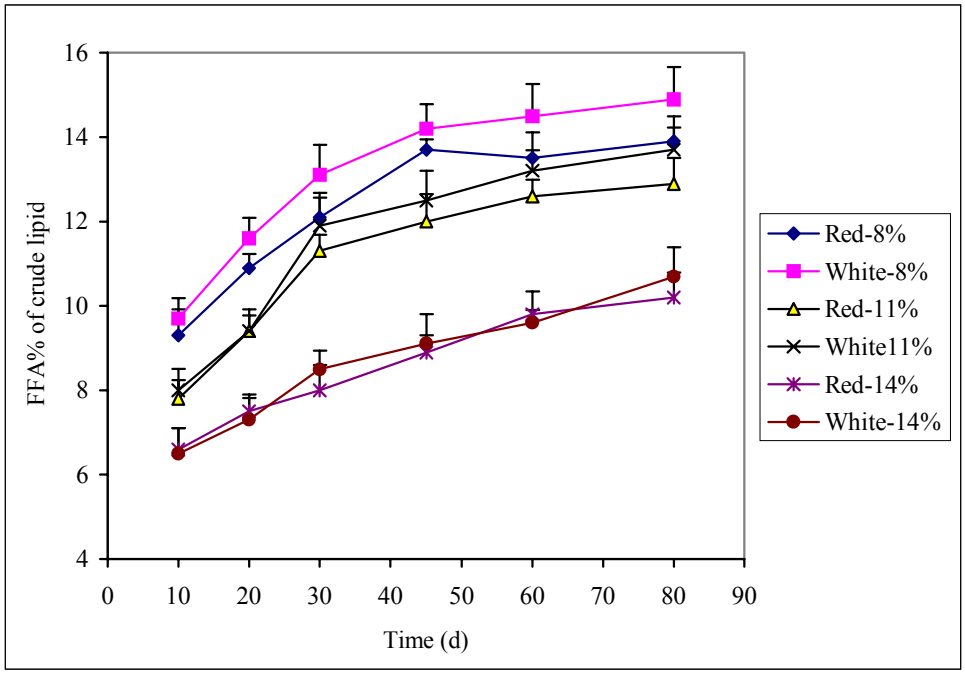


Fig. 7.5 Ratio Free Fatty Acids / Crude Lipid (FFA/CL) during sufu ripening as an indicator of lipid degradation.
Averages of duplicate analysis of pooled duplicate samples.
Legend coding: Red-8% = red sufu with 8% total NaCl content.

ACKNOWLEDGMENT

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Chapter 8

Amino Acid Profiles of Sufu, a Chinese Fermented Soybean Food

Abstract

Total (TAA) and free amino acid (FAA) profiles were determined during consecutive stages of sufu manufacture, i.e. tofu, pehzte (fungal fermented tofu), salted pehtze and in white, red and grey sufu, ripening in dressing mixtures of different salt content. TAA in tofu, pehtze and salted pehtze differed. FAA was high in pehtze and salted pehtze after fermentation of tofu by *Actinomucor elegans*. FAA and NH_3 in white sufu were a little higher than in red sufu of the same age and salt content. FAA increased gradually during ripening and was higher at lower salt content. While FAA increased during ripening of red and white sufu, the ratio of each amino acid remained essentially constant, and glutamic acid, leucine, aspartic acid, alanine, phenylalanine and lysine were found in large quantities. However, in grey sufu the ratio was different, with large proportions of leucine, alanine, isoleucine, valine and phenylalanine found after ripening.

Submitted for Publication
B.-Z. Han, F.M. Rombouts & M.J.R. Nout

INTRODUCTION

Sufu, *Fu-ru* written in hieroglyphics, is a fermented soybean food that originated in China. It is a soft creamy cheese-type product made from cubes of soybean curd (tofu) by the action of a mould (Su, 1986; Steinkraus, 1996). This fermented product with its characteristic flavour has been widely consumed by Chinese people as a salty appetizer for many centuries.

There are different types of sufu, produced by various local processors in China (Wang & Du, 1998), with mould-fermented sufu being the most popular type (Han et al., 2001b). Four steps are normally involved in making this type of sufu: (1) Preparing tofu, (2) Preparing pehtze (*pizi*) by fungal solid-state fermentation of tofu using e.g. *Actinomucor elegans*, (3) Salting of pehtze, and (4) Ripening in dressing mixture (Wang et al., 1970). According to the colour and flavour, sufu can be be classified into four types, i.e. red sufu, white sufu, gray sufu and others, which are mainly based on the different ingredients of dressing mixtures in the ripening.

Hydrolysis of protein occurs mainly during the ripening stage that usually takes 3-6 months. During this stage, protein is hydrolyzed and converted to smaller nitrogen compounds such as peptides, amino acids, amines and ammonia, resulting in flavour increase.

Traditionally, the ripening stage took over 6 months since the salt content in some sufu exceeded 14%. Presently, the salt content in most products, especially red and grey sufu, is still > 10% (Han et al., 2001a), with corresponding ripening periods of three months or longer. A reduction of salt content would have the combined advantages of shortening the ripening periods as well as reducing dietary sodium intake.

Like in other fermented foods such as cheese (Messens et al., 1999) and miso (Chiou et al., 1999), salt has a multiple role in sufu. It imparts a salty taste to the sufu, controls enzyme activity and influences biochemical changes in the product.

Since soybean foods serve as a source of protein in the Chinese diet, its amino acid content and pattern is important from nutritional point of view. In addition, amino acids contribute to the taste of foodstuffs (Kirimura et al., 1969; Nishimura & Kato, 1988) and influence the consumers' acceptance of sufu. Although sufu contains about 40% (dry matter basis) crude protein (Su, 1986), there is little published information on its amino acid profile. Therefore, the objective of this investigation was to determine the levels of total and free amino acids of sufu so as to provide data of nutritional and sensory relevance.

From the biochemical point of view, it is of interest to investigate amino acid profiles and chemical changes during red, white and grey sufu production, with special reference to the effect of different salt contents in the dressing mixtures.

MATERIALS AND METHODS

Microorganism

Actinomucor elegans (Academia Sinica AS 3.227) is commonly used as a starter in commercial sufu production in China. Starting from a malt extract agar slant culture, a pure culture inoculum of *A. elegans* AS 3.227 was prepared by liquid substrate culture (approximately 1 cm depth) in Roux bottles as is common practice in Chinese sufu factories. The medium consisted of soy whey (by-product from tofu manufacture) to which maltose (2-3% w/v) and peptone (1.5-2.0% w/v) were added prior to sterilization by autoclaving. After incubation at 28°C for 72 h, medium and biomass were harvested and homogenized to obtain an inoculum suspension containing ~10^5 colony forming units (CFU)/ml.

Sufu preparation

The tofu used as raw material for sufu was provided by Beijing WangZhiHe sufu manufacturer, and was cut into pieces (3.2*3.2*1.6 cm). The pieces were inoculated with *A. elegans* by spraying inoculum suspension onto their surface. The inoculated tofu pieces were placed evenly spaced, in plastic trays. The loaded trays were piled up in an incubation room with controlled temperature (around 25°C), relative humidity (around 90%) and air circulation to ensure adequate aeration. Fresh pehtze, i.e. tofu overgrown with *A. elegans* mycelium, was obtained after incubation for 48 h.

The pehtze was transferred into a container (20 liters) and salt was spread between layers of pehtze as they were piled up in the container. During a period of 5 d, the pehtze absorbed the salt until salt content of pehtze reached about 14-15% (w/w).

For the ripening of sufu, 12 pieces of salted pehtze (about 200 g fresh weight) were placed in individual wide-mouthed glass bottles with a capacity of 340 ml, after which dressing mixture (about 140 ml) was added to the pehtze. In order to reach the required final salt level, salt was also added in some dressing mixtures to obtain sufu with 11% and 14% (w/w) salt content. For red sufu, the dressing mixture consisted of angkak or kojic red rice (Han et al., 2001), alcoholic beverage (rice wine) (Nout & Aidoo, 2002) to a final alcohol content of 5%, sugar, Chiang (wheat-based miso) (Campbell-Platt, 1987), and spices. For white sufu, the dressing mixture only consisted of alcoholic beverage (final alcohol content 5%). For grey sufu, the dressing mixture has a special recipe without alcohol (not specified due to confidentiality). The filled bottles were incubated at 25-28°C for 80 d.

Sampling for analysis

Two bottles were drawn randomly from each batch on each sampling day during the ripening. The ripening dressing mixture was decanted and sufu pieces were lyophilized.

Biochemical analyses

Crude protein was analysed according to the Kjeldahl method. The lyophilized sample homogenates were hydrolysed in 6 N HCl under vacuum for 24 h at 110°C, and then the hydrolysates were applied to an Automatic Amino Acid Analyser (HITACHI 835-50, Japan) for determination of total amino acids (Wu & Ding, 2002). The tryptophan was analysed after hydrolysing in 4.2 N NaOH. The lyophilized sample homogenates were dissolved in sulphosalicylic acid, and supernatants were applied to the analyser for free amino acids (Niven et al., 1998).

Amino acid groupings

Amino acids were grouped (Sarkar et al., 1997; Nelson & Cox, 2000) as acidic (aspartic acid + glutamic acid), basic (lysine + histidine + arginine), total charged (basic + acidic), hydrophilic (total charged + threonine + serine), hydrophobic (valine + leucine + isoleucine + phenylalanine + tyrosine + tryptophan + methionine) and apolar (hydrophobic – tyrosine).

RESULTS AND DISCUSSION

Changes of pH and crude protein

pH values in tofu, pehtze and salted pehtze, which were intermediate products of sufu prior to the ripening stage, were around 6.9-7.0. During the ripening of sufu, pH values of red and white sufu decreased slightly from 6.6-6.8 after 10 d, to pH 5.9-6.1 after 80 d of ripening time. pH values of grey sufu increased slowly from 6.8 after 10 d, to 7.1 after 80 d of ripening time.

Crude protein contents in tofu, pehtze and salted pehtze were 63.1%, 62.8% and 37.5% (dry matter basis), respectively. Crude protein in sufu decreased 3-6% gradually during the ripening, regardless of the type of sufu. This may have been caused by diffusion out of the sufu, into the dressing mixture.

Amino acid profiles of tofu, pehtze and salted pehtze

Table 8.1 shows profiles of total amino acid (TAA), free amino acid (FAA) and NH_3 in tofu, pehtze and salted pehtze. Differences were observed between tofu,

pehtze and especially salted pehtze, since the added salt has great impact on the composition of the dry matter. But the ratio of amino acids remained similar. FAA content of tofu was low, but after fermentation a considerable amount of FAA was obtained in pehtze and salted pehtze. Most FAA in salted pehtze was lower than that in pehtze due to the change of the dry matter caused by salt. However, some FAA such as serine, isoleucine, leucine, tyrosine and phenylalanine increased after the salting stage, which implies that degradation of protein took place in the salting stage.

Table 8.1 Profiles of total amino acid (TAA), free amino acid (FAA) and NH_3 in tofu, pehtze and salted pehtze (mg/g sample dm)

	TAA			**FAA**		
Amino acid	Tofu	Pehtze	Salted P.	Tofu	Pehtze	Salted P.
Asp	65.9*	66.9	42.6	0.04	0.86	0.74
Thr	21.6	22.1	14.1	0.01	0.46	0.40
Ser	30.6	30.2	19.4	0.06	0.25	0.44
Glu	102.9	101.2	66.1	0.09	4.64	3.00
Gly	24.1	23.8	15.4	0.02	0.50	0.31
Ala	24.0	28.2	17.6	0.04	3.71	2.30
Val	21.5	22.1	14.2	0.01	0.68	0.53
Met	4.2	4.4	2.6	0.01	0.08	0.08
Ile	22.1	25.1	15.5	0.01	0.28	0.39
Leu	42.5	44.9	29.0	0.02	0.34	0.52
Tyr	21.7	21.4	14.1	0.04	0.58	0.63
Phe	31.7	30.8	20.2	0.04	0.36	0.68
Lys	33.3	32.6	21.1	0.04	0.81	0.68
His	13.3	13.2	8.6	0.10	0.78	0.35
Arg	44.2	40.9	25.8	0.76	0.34	0.30
Pro	30.1	29.7	18.8	0.02	0.94	0.59
Trp	6.1	6.5	2.2			
Cys	6.9	7.2	4.0			
NH_3	*10.3*	*11.5*	*7.9*	*0.13*	*3.63*	*1.64*

* Mean of duplicate; Salted P.: salted pehtze.

Amino acid profiles during the ripening of red and white sufu

Total amino acid profile and NH_3 during the ripening of red sufu with 8% salt content, as an example, is presented in Table 8.2. There was no great change for TAA during the ripening. Interestingly, some hydrophobic amino acids such as valine, isoleucine and leucine increased, but all hydrophilic amino acids decreased slightly during the ripening. Probably these amino acids diffuse into the dressing mixture more easily and are therefore lost from the product.

Table 8.2 Total amino acid (TAA) changes during the ripening of red sufu with 8% (w/w) salt content (mg/g sufu dm)

TAA	10 d	30 d	60 d	80 d
Asp	39.4*	34.2	31.0	32.0
Thr	13.3	11.3	10.6	11.0
Ser	17.4	14.0	13.1	12.9
Glu	58.1	55.0	51.7	54.1
Gly	13.5	12.9	12.1	12.6
Ala	15.1	14.7	15.1	15.8
Val	12.9	13.5	15.4	15.8
Met	1.8	3.0	2.4	1.8
Ile	12.9	15.4	15.1	17.6
Leu	24.7	25.1	26.1	27.7
Tyr	13.3	11.3	11.4	11.8
Phe	19.1	17.0	18.7	19.7
Lys	17.4	16.0	15.7	16.0
His	7.3	6.7	6.5	6.3
Arg	21.1	14.9	12.0	12.3
Pro	17.2	16.0	16.2	16.6
Trp	4.1	4.5	3.4	3.8
Cys	4.2	3.9	6.0	6.9
NH_3	*7.3*	*7.8*	*9.7*	*8.6*

* Mean of duplicate

Tables 8.3 and 8.4 present free amino acid (FAA) and NH_3 changes during the ripening of red and white sufu with 8%, 11% and 14% salt content. Both total FAA and NH_3 in white sufu were a little higher than those in red sufu with comparable salt content. As could be expected, FAA in sufu with a lower salt content increased faster than that in sufu with higher salt content, regardless of red or white sufu, since the salt inhibits the enzyme activity in sufu. When FAA increased with the ripening time of the sufu the relative proportion of individual amino acid remained generally constant, which was similar to the situation during the ripening of Cheddar cheese (Puchade et al., 1989).

The very high acidic amino acid content of red and white sufu was of particular interest. Glutamic acid was the most abundant acid, followed by aspartic acid, together representing around 30% of total FAA. The total basic amino acids (i.e. lysine, histidine and arginine) constituted only <10% of the total (arginine was even not detected in white sufu after 80 d of ripening). These results are in agreement with those of Chou and Hwan (1994). Glutamic acid in combination with salt (NaCl) contributes to the flavour and hedonic characteristics of foods (Halpern, 2000), also referred to as the umami taste. The predominance of glutamic acid has been reported in sufu (Liu & Chou, 1994), in cheese (Omar, 1984) as well as in several fermented soybean foods (Sarkar et al., 1997; Yamaguchi & Ninomiya, 2000). In addition, volatile compounds formed from amino acids by decarboxylation, deamination, transamination and other transformations can make substantial contributions to sufu flavour. This is a major reason for the many different volatile compounds (Chung, 1999, 2000; Hwan & Chou, 1999) encountered in sufu.

Free amino acid profile during the ripening of grey sufu

Changes of free amino acid in grey sufu are summarized separately in Table 8.5, since they evolved in a totally different pattern compared with red and white sufu. All hydrophobic amino acids (except for methionine) increased to a large extent, which dominated the proportion of total free amino acid. On the other hand, hydrophilic amino acids, especially acidic amino acids that were main amino acid in red and white sufu, decreased to a very low content. The dramatic decrease in some amino acids during ripening suggests that they were consumed or transformed somehow at a greater rate than they were formed by proteolytic activity. So far, there is no published information about grey sufu. This makes it hard to explain what happened to these amino acids during the ripening of grey sufu. Grey sufu has a strong, offensive odour, and the grey (or blue) colour was formed during 5-15 d of ripening. Possibly, a relatively high turn-over of amino acids into volatile nitrogen compounds is associated with this smell and the depletion of FAA.

Table 8.3 Free amino acid (FAA) changes during the ripening of red sufu with different salt content (mg/g sufu dm)

FAA		8% (w/w) salt content				11% salt content				14% salt content			
	Time (d)	10	30	60	80	10	30	60	80	10	30	60	80
Asp		1.60*	4.04	6.40	6.96	1.79	2.80	3.60	5.20	1.65	1.90	2.28	3.10
Thr		1.00	2.19	3.30	3.42	1.02	1.40	1.80	2.40	0.92	1.10	1.32	1.60
Ser		1.00	2.00	3.40	3.10	1.02	2.00	2.04	2.60	0.87	1.40	1.68	2.20
Glu		7.20	14.28	19.60	21.29	6.46	9.10	11.76	14.40	6.05	6.80	8.16	9.30
Gly		0.80	1.76	2.70	3.10	0.77	1.20	1.56	2.20	0.73	0.80	0.96	1.10
Ala		2.70	3.99	5.90	6.63	2.64	3.00	3.69	4.90	2.14	2.30	2.76	3.30
Val		1.40	3.14	4.80	4.92	1.53	2.20	2.52	3.50	1.34	1.50	1.80	2.30
Met		0.40	0.81	1.40	1.50	0.43	0.60	0.84	1.00	0.40	0.40	0.48	0.60
Ile		1.40	3.36	4.90	5.53	1.53	2.20	2.64	3.70	1.33	1.50	1.80	2.50
Leu		2.30	5.19	8.00	8.77	2.55	3.70	4.68	6.10	2.39	2.60	3.12	4.10
Tyr		1.80	3.44	4.20	4.49	1.96	2.60	3.96	4.10	1.82	2.00	2.40	2.70
Phe		2.30	4.56	6.30	6.31	2.38	3.30	3.84	4.80	2.39	2.60	3.12	3.40
Lys		1.50	3.43	5.30	5.56	1.70	2.60	3.12	4.00	1.46	1.70	2.04	2.80
His		0.50	0.89	1.40	1.39	0.51	0.80	0.96	1.00	0.53	0.60	0.72	0.80
Arg		0.60	0.93	1.20	1.18	0.55	0.50	0.84	0.20	0.56	0.50	0.60	0.40
Pro		1.30	2.56	3.70	4.07	1.28	1.70	2.16	2.90	1.15	1.30	1.56	1.80
NH_3		*2.30*	*3.51*	*4.80*	*5.46*	*1.87*	*2.80*	*3.00*	*3.20*	*1.99*	*2.00*	*2.40*	*2.80*

* Mean of duplicate

Table 8.4 Free amino acids (FAA) and NH_3 changes during the ripening of white sufu with different salt content (mg/g sufu dm)

FAA		8% (w/w) salt content				11% salt content				14% salt content			
	Time (d)	10	30	60	80	10	30	60	80	10	30	60	80
Asp		2.30*	5.60	5.15	6.40	1.98	4.82	6.77	7.90	1.26	2.70	4.17	6.40
Thr		1.40	3.50	5.15	5.30	1.15	2.18	4.03	4.70	0.70	1.40	2.18	3.20
Ser		1.10	0.10	0.09	0.50	0.90	2.37	0.12	0.20	0.84	1.90	2.66	4.50
Glu		5.80	14.70	21.62	22.10	4.76	12.10	16.91	18.50	3.16	4.90	7.86	11.20
Gly		1.10	2.70	4.51	4.80	0.90	2.00	3.11	3.70	0.45	1.00	1.40	2.50
Ala		3.80	6.80	11.32	9.50	3.12	4.46	7.82	7.90	2.30	3.00	4.20	5.70
Val		2.00	4.90	7.27	7.50	1.64	3.19	5.64	6.50	1.00	2.00	2.80	4.80
Met		0.50	1.20	1.39	2.00	0.41	0.91	1.38	1.70	0.22	0.50	0.70	1.30
Ile		1.90	5.20	8.00	8.20	1.56	3.37	5.98	7.30	0.86	2.00	3.20	5.30
Leu		3.20	7.90	11.13	11.50	2.26	5.55	9.09	10.30	1.53	3.60	5.04	7.80
Tyr		2.10	4.00	1.38	1.90	1.72	3.73	4.60	0.90	1.31	2.40	3.36	4.20
Phe		3.10	6.70	8.10	8.20	2.54	4.37	7.71	7.40	1.87	3.60	5.04	6.30
Lys		2.30	5.10	8.37	8.60	1.89	3.64	5.87	7.50	1.21	2.10	2.94	5.40
His		0.60	1.50	2.12	1.50	0.49	0.91	1.73	1.80	0.41	0.70	0.98	1.10
Arg		0.70	1.40	0.20	0.00	0.57	0.18	1.61	0.00	0.32	0.80	1.02	0.00
Pro		1.40	3.70	5.98	5.80	1.15	2.64	4.06	5.20	0.97	1.40	2.06	3.50
NH_3		*2.40*	*5.00*	*5.70*	*5.80*	*1.97*	*2.91*	*5.75*	*4.80*	*1.65*	*1.80*	*2.52*	*3.30*

* Mean of duplicate

In conclusion, total free amino acids generally increased during the ripening and were higher in the lower salt content than in the higher salt content. While free amino acids increased with the age of red and white sufu, the relative proportion of each amino acid remained essentially constant. Acidic amino acids (i.e. glutamic acid, aspartic acid), leucine, alanine, phenylalanine and lysine were found in large quantities. But grey sufu showed a different pattern of changes, with leucine, alanine, isoleucine, valine and phenylalanine as the dominant amino acids at the end of ripening. It is a challenge to speculate further about the different FFA profile of grey sufu. Was the proteolysis not inhibited, or were more amino acids converted into volatiles? Obviously, more research into this fermentation will be needed to gain a better understanding of this product.

Table 8.5 Free amino acids (FAA) and NH_3 changes during the ripening of grey sufu with 11% (w/w) salt content (mg/g sufu dm)

FAA	10 d	30 d	60 d	80 d
Asp	0.51*	0.81	0.80	0.70
Thr	0.38	0.06	0.00	0.10
Ser	0.09	0.00	0.17	0.20
Glu	5.07	1.17	0.30	0.10
Gly	0.84	0.10	0.00	0.00
Ala	2.70	5.20	7.40	8.90
Val	1.24	3.84	5.30	6.80
Met	0.18	0.05	0.10	0.00
Ile	1.13	3.56	5.80	7.10
Leu	1.73	4.83	7.80	9.60
Tyr	0.98	2.14	3.20	3.70
Phe	1.61	3.60	5.40	6.50
Lys	0.06	0.08	0.20	0.20
His	0.33	0.11	0.06	0.05
Arg	0.37	0.12	0.10	0.00
Pro	1.27	0.22	0.00	0.00
NH_3	*2.90*	*5.45*	*6.40*	*7.80*

* Mean of duplicate

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Chapter 9

General Discussion

To provide the increasing population with a source of protein other than meat is a worldwide challenge. Alternative protein sources are legumes and cereals. But plant protein by itself is poorly digestible and often lacks desirable flavours. Fermentation can improve digestibility and either add desirable flavours or diminish unpleasant ones. This is especially true in soybean products made by fermentation.

Fermented soybean foods are attractive products in view of organoleptic quality, nutritional properties and functionalities; in addition, their industrial manufacture can be a bonus for developing economies. Sufu is one of the most famous Chinese fermented soybean foods. It has been consumed for many centuries in China. Its production is mastered at an artisanal level. Future technological development requires a better understanding of the effect of processing conditions on product formation and its quality and safety.

MICROBIOLOGICAL CHARACTERIZATION

Microbiological safety and quality of sufu

Sufu is manufactured in China both commercially and domestically employing various traditional processes. The quality of product varies with different localities and even differs between batches from the same producer. Not surprisingly, a wide range of chemical parameter values was found in commercial sufu as shown in Chapter 3. In addition, large variation of chemical composition, such as NaCl, ethanol, glucose, fructose and water contents of samples from the same company indicate that there is scope to establish quality control management within the companies.

Based on microbiological analyses of sufu, most samples contained high levels of bacterial endospores and concomitant mesophilic aerobic bacteria, which may originate either from highly contaminated tofu and/or dressing mixture, and/or from poor hygiene during processing practices (Chapter 3).

Subsequently, changes of microbial composition during the production of sufu were studied (Chapter 5). The high levels of bacterial endospores and mesophilic aerobic bacteria found in all process stages from pehtze to the final product are not surprising, considering the open-air and non-sterile conditions in the sufu manufacturing process.

Enterobacteriaceae were found at high levels in pehtze, but did not survive the salting and ageing steps since most of them are sensitive to salt and alcohol. Consequently, no Enterobacteriaceae were detectable in any of the experimental

sufu products. Also, Shi and Fung (2000) reported that regular sufu fermentation and ageing conditions are adequate to control common foodborne pathogens, supporting our view that regular sufu is a safe product even though its preparation does not include pasteurization.

From a microbiological safety point of view, sufu products are stable and safe when they are produced under conditions of controlled hygiene and using dressing mixtures resulting in final product concentrations of at least 5% v/v ethanol and 8% w/w NaCl. The microbiological guideline proposed in Chapter 3 may serve as a criterion for safety assessment.

Starter strains and feasible alternatives

A pure-culture fermentation has been used in sufu production since a mould named as *Mucor sufu* was isolated by Wai (1929). Nowadays, several kinds of starters are used in sufu production. Some of them, such as *Actinomucor elegans* and *Mucor hiemalis* conform with internationally accepted nomenclature, whereas others such as *Mucor sufu* and *Mucor wutunkiao* cannot be found in international mycological catalogues.

Eleven moulds used for commercial sufu production were collected from manufacturers located in China and Vietnam or isolated from their pehtze. *Mucor sufu*, *M. wutunkiao* and *M. rouxianus* named by Chinese investigators were identified as *M. racemosus*, *M. circinelloides* and *M. racemosus*, respectively, based on macroscopic and microscopic morphology as well as relevant physiological distinctive features (Chapters 2 and 4). Presently, we use the scientifically accepted names for international exchanges.

The starter used in fermentation of sufu pehtze is crucial and must possess certain characteristics. Mould-fermented sufu is the most popular product among the different known types of sufu. Moulds involved are mainly *Actinomucor, Mucor* and *Rhizopus* spp. Among these, *A. elegans* and *A. taiwanensis* are the most frequently used for commercial sufu pehtze production in China, especially in Beijing and Taiwan. However, also other moulds such as *M. racemosus* (*M. sufu*) and *M. circinelloides* (*M. wutunkiao*) have been mentioned as popular starter cultures. Nevertheless, most of *Actinomucor* spp. and *Mucor* spp. only grow well at 25-30°C, so it is impossible to produce sufu during the hot summer with indoor factory temperatures reaching 35°C or even higher (Han, et al., 2001).

Traditionally, winter is the peak season for consuming sufu as a side dish because of shortage of vegetables, especially in the north of China. Obviously, taking into account the production cycle, summer is the best time commercially for sufu production. Unfortunately, most manufacturers in China, if not all, are forced to stop preparing sufu pehtze in the summer since the moulds do not perform well at the high temperature.

Rhizopus oligosporus grows well on soybean substrate at higher temperatures

(up to 40°C) (Han & Nout, 2000). Might it be used as a starter to produce sufu during the hot season? In Chapter 6, growth and enzyme production by *A. elegans* and *R. oligosporus* during sufu pehtze preparation were studied. The two cultures have similar growth and enzyme production abilities in sufu pehtze.

Based on the results of preliminary laboratory experiments, three batches of sufu were made using both *R. oligosporus* and *A. elegans* as fermentation starters at a pilot-scale in a commercial plant during the hot summer. According to the compositional analyses, there was no significant difference between the products fermented with the two cultures. A sensory evaluation was carried out by an expert panel. The sample made with *R. oligosporus* had a black appearance occasionally, which was attributed to the sporulation of the culture after pehtze making. When cultivation time for pehtze was shortened to avoid black sporulation, the mould mycelial mats were not strong enough to wrap sufu tightly in shape.

In general, it is really necessary to find an alternative starter mould in the hot summer. Consequently, it will be of interest to further evaluate the feasibility and acceptability of the use of *Rhizopus* spp. in sufu production.

Bacteria-fermented sufu is produced only in some locations, using specially adapted production processes. Two bacterial cultures were collected from manufacturers in Heilongjiang and Hubei in China, respectively. The former was probably *Micrococcus luteus* based on the morphology and some physiological properties, and the report from a local company. The latter was identified as *Bacillus subtilis* based on morphology and carbohydrate assimilation patterns.

BIOCHEMICAL CHARACTERIZATION AND PRODUCT INNOVATION

Low-salt sufu

Traditional sufu fermentation requires extravagant space, time and labour, which all three are increasingly expensive throughout the world. Especially the ripening period of sufu, which takes about 3-6 months, should be shortened without affecting the quality of sufu.

Epidemiological surveys show that essential hypertension has become a major health problem not only in industrialized nations, but also in some developing countries, such as China (Tian, 1996). Of the related dietary factors, the effects of dietary sodium and potassium on blood pressure are relatively well established, and indicate that high intake of sodium and low intake of potassium are related to high blood pressure.

Salt inhibits the enzymatic ripening processing of sufu. The salted pehtze with a high salt content in the dressing mixture therefore requires longer periods of ripening. In order to decrease the dietary intake of sodium and shorten the sufu maturation time, low-salt sufu products could be a healthy and profitable option.

Chapters 5 and 7 report microbial changes and protein and lipid degradation in

sufu production, with special attention to the effect of different salt contents. Microbial spoilage was observed in sufu with 5% salt content after 20 d of ripening as evidenced by souring and off-flavour. Sufu with 8% salt was similar to sufu with 11% salt and had a stable and inactive microflora. However, in the 8% salt sufu protein and lipid were degraded to a higher extent.

Conventional red sufu has a salt content of over 11%, and takes more than 3 months for ripening. Based on the relevant distinctive features, sufu with 8% salt content would be qualified to be termed "finished product" after only 45 d of ripening.

In an industrial setting, pilot-scale trials were carried out with sufu having 7% salt content. Judged by an expert panel, sufu with 7% salt content at the age of 45-180 d fulfilled all criteria of conventional sufu, and was highly appreciated by all panelists. The only problem was that due to its on-going enzymatic degradation, 8 months after the manufacturing date the sufu became too soft to be taken out of the container using a chopstick. Pasteurization of sufu at the end of ripening could be an option to inactivate the enzymes involved. Further evaluation of the feasibility and acceptability of pasteurized sufu would be required.

Biochemical changes in sufu production

During the fungal fermentation process a substantial breakdown occurs of the soybean storage proteins, lipids and carbohydrates due to the production of fungal proteases, lipases and carbohydrases (De Reu, 1995). Proteolysis is the principal and most complex biochemical event that occurs during ripening of sufu. The SDS-PAGE profiles (Chapter 7) showed that after 60 d of maturation, all protein subunits had disappeared in sufu containing 8% salt, which indicates that most proteins were degraded into peptides and free amino acids. Chapter 8 confirms that a large amount of free amino acids is found in matured sufu.

It is commonly assumed that the flavour, aroma and texture of sufu that mainly develop during the ripening are determined by the enzymes produced by the mould in pehtze preparation. However, significant levels of bacteria (mainly *Bacillus* spp.) were also present in pehtze (Chapter 5). It has been shown that *Bacillus* spp. show considerably higher and different proteolytic activity compared to e.g. *Rhizopus* spp., resulting in the degradation of virtually all major soy protein subunits (Kiers, et al., 2000). So *Bacillus* spp. could also contribute to the formation of hydrolytic enzymes that benefit the sufu ripening. The function of microbes other than fungal starter cultures should be investigated in the future. In view of the observations mentioned above it would be worthwhile to study the possibilities of producing a sufu with 5% or less salt in which bacilli also play a role in the ripening process. Obviously, such a product would require pasteurization when the ripening process is at its optimum.

NUTRITION AND FUNCTIONALITY

Proteins are an essential component of the diet needed for the survival and growth of animals and humans. Their function in nutrition is to supply adequate amounts of needed amino acids. Since soybean foods serve as a source of protein in the Chinese diet, their content and distribution pattern of amino acids is important from a nutritional point of view. Sufu contains a great amount of free amino acids (Chapter 8); in particular, the very high acidic amino acid content of red and white sufu is of interest.

Soybean contains high levels of oligosaccharides, notably α-galactosides of sucrose, such as raffinose and stachyose. These may have prebiotic properties, but excessive levels may also result in intestinal gas production (flatulence). These oligosaccharides are mainly removed by soaking and cooking of soybeans (Ruiz-Teran & Owens, 1999) and are probably further degraded by moulds since *Actinomucor elegans* and *Rhizopus oligosporus* can produce α-galactosidase in pehtze preparation (Chapter 6).

Consumption of soybean foods is increasing because of reported beneficial effects on nutrition and health (Liu, 2000). These effects include lowering of plasma cholesterol, prevention of cancer, diabetes, and obesity (Friedman & Brandon, 2001).

Isoflavones are a type of phytoestrogen, plant-based compounds with weak estrogenic activity. Asian countries have seen a low incidence of several chronic diseases mentioned above that plague the Western countries. Possibly, the ingestion of soybean isoflavones contributes to this. It has been reported that the level of some isoflavones (i.e., genistein and daidzein) increased by about 20 times after 50 d of sufu ripening, which thus offers an easily absorbable source of isoflavones (Zhang, et al., 2002).

Angkak (red kojic rice), an important ingredient in dressing mixture of red sufu as a natural colorant, is traditionally obtained by fungal solid-state fermentation of cooked rice, mainly with *Monascus* spp. (Nout & Aidoo, 2002). Angkak is also regarded to be a health-promoting food ingredient (Wang, et al., 1997). It was reported that the angkak mould *Monascus ruber* (ATCC 96218) produced the mycotoxin citrinin at levels up to 6.8 mg per g glucose when grown in aerated shaking flask cultures in a chemically defined liquid growth medium (Hajjaj, et al., 1999, 2000). We did a limited sampling of angkak produced traditionally on rice. Samples from different regions (Guang dong, Jiangsu, Hunan, Fujian and Beijing) did not contain detectable levels (< 1 ppb) of citrinin (courtesy by K.E. Aidoo, Glasgow Caledonian University, UK).

CONCLUDING REMARK

Understanding of the overlapping microbiological, biochemical, and nutritional aspects of sufu will add valuable insight into the indigenous fermented soybean foods in China. This, in turn, can lead to better and safer foods and improved human health. As is shown in this thesis, this important goal can be achieved by research in which different disciplines are integrated.

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Summary

Over the centuries, Chinese people have consumed soybeans in various forms of traditional fermented soybean foods. Sufu (*Furu*), a cheese-like product originating in China, is one of the most popular fermented soybean foods in China, and is becoming popular in Chinese shops all over the world. The main objective of this thesis is to understand the microbiological and biochemical characteristics of sufu and the sufu production process, and to create a basis for improvement of processing technology to obtain high quality products.

In Chapter 2 the nature of sufu and its manufacture are reviewed based on scientific publications. Sufu is produced by fungal solid-state fermentation of tofu followed by salting and ripening in dressing mixture containing various ingredients. For the first time, sufu is classified in English language into several types, according to the processing method employed, or according to colour and flavour of the final product. Processing can be directed to result in mould fermented, naturally fermented, bacterially fermented, or enzymatically ripened sufu. Depending on the choice of dressing mixture, red, white or grey sufu may be obtained. Regardless of the traditional and innovated commercial processes, four main steps are involved: (1) preparation of tofu, (2) preparation of pehtze (moulded tofu), (3) salting, and (4) ripening.

In Chapter 3 the microbiological safety and quality of commercial sufu from all over China are investigated. Chemical parameters of collected sufu samples were analysed, and the concentration of NaCl, ethanol, glucose and fructose varied from 6.2%, 0.5%, 0% and 0% to 14.8%, 6.3%, 6.2% and 4.8%, respectively. Microbiological analyses were done for total count of mesophilic aerobic bacteria (TMAB), bacterial endospores (B. endospores), total count of halotolerant bacteria at 10% and at 17.5% NaCl, lactic acid bacteria (LAB), fungi, Enterobacteriaceae, and the following pathogens: *Bacillus cereus*, *Clostridium perfringens*, *Staphylococcus aureus* and *Listeria monocytogenes*. High levels (> 10^5 cfu/g) of TMAB and B. endospores were found in most samples, and considerable levels (10^5 and 10^7 cfu/g) of LAB were detected in two samples of white sufu. One third of the samples contained less then 10^3 cfu/g *B. cereus*, but three samples had over 10^5 cfu/g indicating a potential hazard to consumers. All samples had less than 10^3 cfu/g *C. perfringens,* except one red sufu sample (~10^5 cfu/g). Fungi, Enterobacteriaceae, and *S. aureus*, *L. monocytogenes* were not detected in any of the samples. A microbiological guideline for safe commercial sufu is proposed based on these results.

Chapter 4 deals with the identity and phylogenetic relationships of mould starter cultures used for the preparation of pehtze. Starter cultures used in commercial pehtze fermentation were obtained from factories located in several provinces of China and Vietnam or isolated from their pehtze. They were identified

as *Actinomucor repens*, *A. taiwanensis*, *Mucor circinelloides*, *M. hiemalis*, *M. racemosus*, and *Rhizopus microsporus* v. *microsporus*. Phylogenetic relations based on sequencing of genomic DNA of these starters and of relevant control strains from collections indicate that the genera *Mucor*, *Actinomucor* and *Rhizopus* form distinct and homogenous clusters, with *Mucor* and *Actinomucor* showing a slightly closer relationship with each other than with *Rhizopus*.

In Chapter 5 the quantitative evolution of the microflora of sufu was studied throughout its production, with special attention for the effect of different salt contents during the ripening. TMAB, B. endospores, *B. cereus*, LAB, Enterobacteriaceae and fungi increased from around 10^4, 10^4, < 10, 10^4, < 10 and 10^3 cfu/g (in tofu) to around 10^8, 10^8, 10^3, 10^7, 10^7 and 10^7 (in pehtze), respectively. LAB, Enterobacteriaceae and fungi decreased 1-2 log cfu/g, and TMAB, B. endospores and *B. cereus* decreased < 1 log cfu/g after the salting of pehtze. TMAB and B. endospores in sufu with 8% and 11% salt content decreased to around 10^6 cfu/g during the ripening. *B. cereus* remained at levels of around 10^3 cfu/g during the ripening. LAB in sufu with 8% and 11% salt content decreased gradually from 10^5-10^6 cfu/g to < 10^2 cfu/g. LAB in sufu with 5% salt content increased to 10^9 cfu/g during the ripening. These were identified as most probably *Lactobacillus curvatus*, and caused sour spoilage of sufu by pH decrease (from 6-7 to 4). Enterobacteriaceae and fungi decreased to the non-detectable level after 20 d and 30 d of maturation, respectively, in all samples.

Chapter 6 compares the effects of temperature and relative humidity on growth and enzyme production by the two fungi *A. elegans* and *R. oligosporus* during sufu pehtze preparation. The optimum conditions for growth of *A. elegans* and *R. oligosporus* were at 25°C at RH 95-97%, and 35°C at RH 95-97%, respectively. Yields of protease (108 U/g pehtze), lipase (172 U/g) and glutaminase (176 U/g) by *A. elegans* were maximum after 48 h at 25°C and RH 95-97%, and for α-amylase (279 U/g pehtze) and α-galactosidase (227 U/g) at 30°C and RH 95-97% after 48 h and 60 h of incubation. Highest protease (104 U/g pehtze), and lipase (187 U/g) activities of *R. oligosporus* were observed after 48 h at 35°C and RH 95-97%, while maximum α-amylase (288 U/g pehtze) and glutaminase (187 U/g) activities were obtained after 36 h at 35°C and RH 95-97%. Maximum α-galactosidase activity (226 U/g) by *R. oligosporus* was found after 36 h at 30°C and RH 95-97%. It is concluded that *R. oligosporus* could be a potential alternative to *A. elegans* as sufu pehtze starter during hot seasons.

In Chapter 7 the effect of NaCl on textural changes and protein and lipid degradation during the ripening stage of sufu is studied. NaCl in dressing mixtures strongly affected the changes of textural properties and the hydrolysis of protein and lipid of sufu. Higher salt contents (14% w/w) resulted in increased hardness (+ 100%) and elasticity (+ 18%), and reduced adhesiveness (- 30%). Hardness and elasticity could be used to judge the extent of sufu ripening. SDS-PAGE showed the disappearance of all protein subunits at 8% and 11% salt content; however, some

protein subunits were still detectable at 14% salt content after 60 d ripening. Higher ratios of free amino nitrogen to total nitrogen (FAN/TN = 0.4-0.45) and free amino acids to crude protein (FAA/CP = 0.24-0.26) were observed in sufu with lower (8%) salt content. Ratios of FAN/TN and FAA/CP in white sufu were higher than those in red sufu due to different dressing mixture compositions. Increases of free fatty acid (FFA) were also observed during ripening. FFA levels in sufu with lower salt content increased rapidly during the first 30-40 d, and then increased slowly, probably resulting from the formation of fatty acid esters. Lowering the salt content (8%) can shorten the ripening time to 40 d, which is of benefit to manufacturers. However, sufu will spoil during the ripening stage at salt contents of 5% or lower.

In Chapter 8 total amino acid (TAA) and free amino acid (FAA) profiles are determined during consecutive stages of sufu manufacture, i.e. tofu, pehzte, salted pehtze and in white, red and gray sufu ripening in dressing mixtures of different salt content. TAA levels in tofu, pehtze and salted pehtze differed. FAA was high in pehtze and salted pehtze after fermentation of tofu by *A. elegans*. FAA and NH_3 in white sufu were a little higher than in red sufu of the same age and salt content. FAA increased gradually during ripening and was higher at a lower salt content. While FAA increased during ripening of red and white sufu, the ratio of each amino acid remained essentially constant, and glutamic acid, leucine, aspartic acid, alanine, phenylalanine and lysine were found in large quantities. However, in grey sufu the ratio was different, with large proportions of leucine, alanine, isoleucine, valine and phenylalanine found after ripening.

Samenvatting

Sinds mensenheugenis eten Chinezen allerlei traditionele gefermenteerde sojaproducten. Eén van de populairste van deze sojaproducten is Sufu (*Furu*), een soort sojakaas die met de migratie van Chinezen geleidelijk ook over de gehele wereld in Chinese winkels te koop is. Het hoofddoel van dit proefschrift is een bijdrage te leveren aan de kennis en het begrip van de microbiologische en biochemische eigenschappen van sufu en het sufubereidingsproces, opdat een grondslag wordt gelegd voor technologische ontwikkeling en productverbetering.

Hoofdstuk 2 behandelt het product sufu en het bereidingsproces op grond van wetenschappelijke literatuurbronnen. Sufu wordt gemaakt door tofu (blokjes soja-eiwitcoagulaat) te laten beschimmelen en vervolgens te zouten en te laten "rijpen" in een opgiet die diverse karakteristieke ingrediënten bevat. Dit is de eerste keer dat een Engelstalige beschrijving wordt gegeven van de verschillende soorten sufu, al naar gelang bereidingsproces, of naar kleur en aroma van het eindproduct. Processen kunnen zodanig worden gekozen dat schimmelgefermenteerde, natuurlijk gefermenteerde, bacterieel gefermenteerde of enzymatisch gerijpte sufu ontstaat. Door de keuze van de opgietsamenstelling kan rode, witte of grijze sufu worden verkregen. Er zijn in het bereidingsproces altijd vier stadia te onderscheiden: (1) maken van tofu, (2) maken van pehtze (beschimmelde tofu), (3) zouten, en (4) rijpen.

Hoofdstuk 3 betreft een onderzoek van de veiligheid en kwaliteit van handels-sufu uit diverse provincies van China. Het gehalte aan zout (NaCl), ethanol glucose en fructose liep uiteen van respectievelijk 6,2%, 0,5%, 0% en 0% tot 14,8%, 6,3%, 6,2% en 4,8%. Microbiologisch onderzoek richtte zich op het vóórkomen van de volgende (groepen) micro-organismen: totaal mesofiele aerobe bacteriën (TMAB), bacteriesporen (B.sporen), halotolerante bacteriën bij 10% en 17,5% NaCl, melkzuurbacteriën (MZB), gisten en schimmels, Enterobacteriaceae, en de pathogenen *Bacillus cereus*, *Clostridium perfringens*, *Staphylococcus aureus* en *Listeria monocytogenes*. In de meeste sufumonsters werden veel (>10^5 kve/g) TMAB en B.sporen gevonden, terwijl in twee monsters tamelijk veel (10^5 en 10^7 kve/g) MZB werden aangetroffen. Een derde van de monsters bevatte minder dan 10^3 kve/g *Bacillus cereus*, terwijl veel hogere aantallen (>10^5 kve/g) in drie monsters op een mogelijk veiligheidsrisico zouden kunnen duiden. Behalve in één monster rode sufu (waarin 10^5 kve/g), waren in alle andere sufumonsters minder dan 10^3 kve/g *C. perfringens* aantoonbaar. In geen van de monsters werden gisten, schimmels, Enterobacteriaceae, *S.*

aureus of *L. monocytogenes* aangetroffen. Op basis van deze resultaten werd een microbiologische richtlijn voor veilige handels-sufu opgesteld.

In hoofdstuk 4 wordt aandacht besteed aan de identiteit en phylogenetische verwantschap van de schimmels die als startercultures worden gebruikt bij het maken van pehtze. Een aantal startercultures werden geleverd door sufufabrikanten uit diverse delen van China en uit Vietnam, terwijl ook een aantal schimmels uit pehtze werden geïsoleerd. De schimmels werden geïdentificeerd als *Actinomucor repens*, *A. taiwanensis*, *Mucor hiemalis*, *M. racemosus* en *Rhizopus microsporus* v. *microsporus*. Op grond van genoom DNA sequenties en phylogenetische verwantschapsanalyse bleek dat de geslachten *Mucor*, *Actinomucor* en *Rhizopus* homogene en duidelijk verschillende groepen vormen, waarbij *Mucor* en *Actinomucor* onderling meer verwantschap hebben dan met *Rhizopus*.

Hoofdstuk 5 beschrijft het onderzoek naar de veranderingen in microbiologische samenstelling van sufu tijdens het productieproces en als gevolg van verschillende zoutgehalten tijdens de rijping. Aantallen van TMAB, B.sporen, *B. cereus*, MZB, Enterobacteriaceae, gisten en schimmels namen toe van respectievelijk 10^4, 10^4, $<10^4$, <10 en 10^3 kve/g in tofu tot ca. 10^8, 10^8, 10^3, 10^7, 10^7 en 10^7 kve/g in pehtze. MZB, Enterobacteriaceae, gisten en schimmels namen 1-2 logeenheden kve/g af, en TMAB, B.sporen en *B. cereus* namen <1 logeenheid kve/g af na het zouten van de pehtze. TMAB en B.sporen in sufu met 8% en 11% zout namen af tot ongeveer 10^6 kve/g tijdens de rijping, terwijl *B. cereus* constant bleef op ongeveer 10^3 kve/g. In sufu met 8% en 11% zout namen de aantallen MZB geleidelijk af van 10^5-10^6 kve/g tot $<10^2$ kve/g, terwijl bij een zoutgehalte van 5% het aantal MZB toenam tot 10^9 kve/g. Deze MZB werden geïdentificeerd als *Lactobacillus curvatus* en veroorzaakten bederf door verzuring waarbij de pH daalde van 6-7 tot circa 4. Enterobacteriaceae, gisten en schimmels konden in alle experimentele monsters na respectievelijk 20 en 30 dagen rijping niet meer worden aangetoond.

In hoofdstuk 6 wordt de invloed van kweektemperatuur en relative luchtvochtigheid op de groei en enzymvorming van twee schimmels, *A. elegans* en *R. oligosporus* tijdens de vorming van pehtze gemeten. De optimale omstandigheden voor de groei van *A. elegans* en *R. oligosporus* waren respectievelijk 25ºC bij RV 95-97%, en 35ºC bij RV 95-97%. *A. elegans* vormde de maximale activiteit van protease (108 Eenheden/g pehtze), lipase (172 E/g) en glutaminase (176 E/g) na 48 uur bij 25ºC en RV 95-97%, van α-amylase (279 E/g pehtze) en α-galactosidase (227 E/g) bij 30ºC en RV 95-97% na resp. 48 en 60 uur. *R. oligosporus* daarentegen vormde de maximale activiteit van protease (104 E/g pehtze) en lipase (187

E/g) na 48 uur bij 35ºC en RV 95-97%, terwijl α-amylase (288 E/g pehtze) en glutaminase (187 E/g) maximaal waren na 36 uur bij 35ºC en RV 95-97%. *R. oligosporus* vormde maximale α-galactosidase activiteit (226 E/g) na 36 uur bij 30ºC en RV 95-97%. Geconcludeerd werd dat bij zeer warm zomerweer in plaats van *A. elegans, R. oligosporus* als alternatieve startercultuur gebruikt zou kunnen worden.

Hoofdstuk 7 beschrijft het onderzoek naar de invloed van zout op textuurveranderingen en de afbraak van eiwit en vet tijdens de rijping van sufu. Het zoutgehalte bleek een grote invloed op deze factoren te hebben. Hoge (14% w/w) zoutgehalten leidden tot verdubbelde hardheid en 18% toegenomen elasticiteit maar tot 30% minder plakkerigheid. Hardheid en elasticiteit bleken goede eigenschappen waarmee men het verloop van de rijping zou kunnen volgen. Met behulp van SDS-PAGE werd aangetoond dat bij 8% en 11% zoutgehalte alle eiwitmoleculen worden afgebroken; bij 14% waren echter na 60 dagen rijping nog steeds intacte eiwitten aantoonbaar. Bij lagere (8%) zoutgehalten werden in sufu hogere verhoudingen vrije amino-stikstof tot totaal stikstof (VAS/TS = 0,4-0,45), en vrije aminozuren tot ruw eiwit (VAZ/RE = 0,24-0,26) waargenomen. In witte sufu waren de verhoudingen VAS/TS en VAZ/RE hoger dan in rode sufu; dit werd toegeschreven aan de verschillende ingrediënten in de opgiet. Tijdens de rijping werd ook een toename in vrije vetzuren (VVZ) waargenomen. VVZ in sufu met laag zoutgehalte nam snel toe tijdens de eerste 30-40 dagen rijping en daarna langzamer, vermoedelijk wegens de vorming van vetzuuresters. Verlaging van het zoutgehalte verkort het rijpingsproces tot 40 dagen hetgeen aantrekkklijk is voor producenten. Nog lagere zoutgehalten (5% of minder) leiden echter tot bederf.

Een verdere studie van totaal aanwezige aminozuren (TAZ) en vrije aminozuren (VAZ) tijdens de opeenvolgende stadia van de sufubereiding, namelijk tofu, pehtze, gezouten pehtze en witte, rode en grijze sufu rijpend in hun opgiet met verschillende zoutgehalten, werd beschreven in hoofdstuk 8. Hoeveelheden TAZ in tofu, pehtze en gezouten pehtze zijn verschillend. Na de fermentatie met *A. elegans* is het gehalte aan VAZ in pehtze en gezouten pehtze toegenomen. Gehalten aan VAZ en NH_3 waren hoger in witte dan in rode sufu met hetzelfde zoutgehalte en van dezelfde rijpingstijd. VAZ nemen geleidelijk toe tijdens de rijping en dit gebeurt sneller bij lagere zoutgehalten. Tijdens deze toename in rode en witte sufu bleef het aminozurenpatroon practisch gelijk waarbij glutaminezuur, leucine, asparaginezuur, alanine, fenylalanine en lysine de meerderheid vormen. In grijze sufu daarentegen was dit patroon verschillend en dit werd gedomineerd door leucine, alanine, isoleucine, valine en fenylalanine na afloop van de rijping.

概　要

大豆发酵食品种类繁多，在中国已有数百年的历史。腐乳起源于中国，其生产方法和食用方式与西方的干酪相似，它丰富了全球华人的饮食，受到了广大消费者的关注。本论文的主要目的是：在认识和阐明腐乳的微生物学和生物化学特性，及其生产过程的基础上，改进其生产工艺，提高产品质量。

第二章回顾了腐乳的历史及其生产工艺发展过程，并首次用英文对腐乳产品进行了分类。根据生产方法的不同，腐乳分为霉菌型、细菌型和腌制型；根据色泽和风味的不同，分为红腐乳、白腐乳和青腐乳。 主要生产步骤包括：（1）豆腐坯制作；（2）前期培菌（毛坯制作）；（3）腌制；（4）后期发酵。本章对腐乳的化学成分，氨基酸含量和不同腐乳的风味组成也进行了讨论和总结。

第三章对腐乳的微生物安全性和产品质量进行了调查研究。首先对收集的 26 个不同样品的化学组分进行了分析，其中，氯化钠、乙醇、葡萄糖和果糖的含量范围分别为：6.2-14.8%，0.5-6.3%，0-6.2% 和 0-4.8%。微生物检测项目主要包括总细菌、芽孢菌、耐盐菌（10% 和 17.5% 盐分）、乳酸菌、真菌、肠道菌群和下列病原菌：蜡样芽孢杆菌、产气梭状芽孢杆菌、金黄色葡萄球菌和单核细胞利斯特氏菌。多数样品中含有大量的总细菌和芽孢菌（> 10^5 cfu/g），两个白腐乳样品中含有大量的乳酸菌（10^5 和 10^7 cfu/g）。三分之一的样品中蜡样芽孢杆菌数少于 10^3 cfu/g，但是有三个样品中的数量超过 10^3 cfu/g，表明该产品对消费者有潜在的危害。除一个红腐乳样品含有约 10^5 cfu/g 产气梭状芽孢杆菌外，其它样品中的数量均小于 10^3 cfu/g。真菌、肠道菌、金黄色葡萄球菌和单核细胞利斯特氏菌在所有样品中均未检出。根据上述分析结果，提出了腐乳产品的微生物安全指标。

第四章对腐乳生产常用的菌种进行了鉴定，并研究了其种系关系。生产菌株由中国和越南的不同地区收集和分离而得，它们分别被鉴定为：雅致放射毛霉、台湾放射毛霉、、冻土毛霉、总状毛霉和小孢根霉。根据 DNA 序列分析，对它们的种系关系与标准菌株的关系进行了分析对照。

第五章研究了腐乳生产过程中微生物菌群的变化，特别是食盐在后期发酵中对微生物菌群的影响。总细菌、芽孢菌、蜡样芽孢杆菌、乳酸菌、肠道菌和真菌分别由豆腐坯中的 10^4、10^4、< 10、10^4、< 10 和 10^3 cfu/g 增长为毛坯中的 10^8、10^8、10^3、10^7、10^7 和 10^7 cfu/g。腌制后，腌制毛坯中的乳酸菌、肠道菌和真菌下降了 1-2 log cfu/g，而总细菌、芽孢菌和蜡样芽孢杆菌仅下降了不到 1 log cfu/g。含盐量为 8%和 11%的腐乳，在后期发酵中，其总细菌和芽孢菌大约降至 106 cfu/g，蜡样芽孢杆菌仍为 10^3 cfu/g，乳酸菌降至< 10^2 cfu/g，而含盐量为 5%的腐乳，在后期发酵中，乳酸菌数量增长为 10^9 cfu/g，它们被鉴定为弯曲乳杆菌，是低盐腐乳酸败的主要原因。在后期发酵 30 天后，所有样品中均未检出肠道菌和真菌。

第六章比较了在制备毛坯时，温度和相对湿度对雅致放射毛霉和少孢根霉生长和产酶的影响。上述两个菌种的最适生长条件分别为 25°C，95-97% 和 35°C，95-97%。雅致放射毛霉最大产酶量及其条件分别为：蛋白酶（108 U/g 毛坯）、脂肪酶（172 U/g）、谷氨酰胺酶（176 U/g），条件（25°C，95-97%，48 h）；α-淀粉酶（279 U/g）、α-半乳糖苷酶（227 U/g），条件（30°C，95-97%，48 h 和 60 h）。少孢根霉最大产酶量及其条件分别为：蛋白酶（104 U/g 毛坯）、脂肪酶（187 U/g），条件（35°C，95-97%，48 h）；α-淀粉酶（288 U/g）、谷氨酰胺酶（187 U/g），条件（35°C，95-97%，36 h）；α-半乳糖苷酶（226 U/g），条件（30°C，95-97%，36 h）。由上述可知，少孢根霉可代替雅致放射毛霉作为夏天生产腐乳的耐热菌种。

第七章研究了后期发酵过程中食盐对腐乳质地变化的影响和对蛋

白质和脂肪水解的影响。研究表明，高盐（14% NaCl）导致腐乳硬度（+ 100%）和弹性（+ 18%）增强、粘着力（- 30%）下降。食盐影响腐乳质地的变化，而硬度和弹性可用于评定腐乳的成熟期。SDS-PAGE 证明，在后期发酵 60 天后，低盐（8%和 11% NaCl）腐乳中的蛋白质全部被分解，而高盐（14% NaCl）腐乳中仍然有蛋白质存在。低盐（8% NaCl）腐乳中的游离氨基氮对总氮的比率（0.4-0.45）和游离氨基酸对粗蛋白质的比率（0.24-0.26）均较高，白腐乳中上述比率比红腐乳中高。游离脂肪酸在后期发酵中不断增加，在低盐腐乳后酵前期（30-40 天），游离脂肪酸迅速增长，而随后增长速度减慢，可能是游离脂肪酸形成酯类物质所致。

第八章报道了腐乳生产过程中各阶段总氨基酸和游离氨基酸的变化。白坯、毛坯和腌坯中总氨基酸含量有所不同；经雅致放射毛霉发酵后，毛坯和腌坯中游离氨基酸含量高于白坯。白腐乳中游离氨基酸和氨的含量高于同期红腐乳的含量；后期发酵中，游离氨基酸逐渐增加，增加速度与腐乳中的食盐含量成反比。红、白腐乳中游离氨基酸增加，各氨基酸的组成比率基本相同，其中有大量的谷氨酸、亮氨酸、天门冬氨酸、丙氨酸、苯丙氨酸和赖氨酸。而在青腐乳中，随着游离氨基酸的增加，其氨基酸的组成比率却发生了变化，其中主要氨基酸为亮氨酸、丙氨酸、异亮氨酸，缬氨酸和苯丙氨酸。

Curriculum Vitae

Bei-Zhong HAN was born in Taiyuan City, Shanxi province, China on February 2nd, 1961. After primary, secondary and high school education in Taiyuan, he entered the Department of Food Engineering, Tianjin Institute of Light Industry in 1979, and received his Bachelor degree in July 1983. From 1983 to 1985, he worked as a technician in Shanxi XingHuaCun Sorghum Liquor Factory. He returned again to the Department of Food Engineering, Tianjin Institute of Light Industry, and obtained his Master Degree in Science for Food Fermentation in 1988. He started his teaching career in the Department of Food Engineering, Beijing Agricultural Engineering University in the same year. He was promoted to lecturer in the University in 1989, and then as an associate professor in 1996 in College of Food Science and Engineering, China Agricultural University. He has been mainly teaching Food Microbiology, Food Fermentation, Fermentation Engineering, Food Technology, and Sensory Evaluation in Food. From 1997 to 1998, supported by the Sino-Dutch Exchange Programme, he worked for one year in the Laboratory of Food Microbiology of Wageningen Agricultural University. In 1999, he started the PhD research project, which is described in this thesis, at the Laboratory of Food Microbiology, Department of Agrotechnology and Food Sciences, Wageningen University and Research Centre.

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