Asian fungal fermented food

The Mycota X, Industrial Applications

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2 Asian Fungal Fermented Food

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CONTENTS

I. Introduction

A. Food Fermentation

The fermentation of food is often defined as the manufacture of foods employing the action of micro-organisms and their enzymes. This would ideally result in changes in the flavour, texture, colour and other quality attributes that are considered desirable by the consumer, all within the context of socio-cultural patterns of food preferences. The origin and attractiveness of several fermented foods is due to their prolonged shelf life, reduced volume, shorter cooking times and superior nutritive value as compared to the non-fermented ingredients. Fermented foods are encountered worldwide, and they are prepared from a wide variety of foods of animal and plant origin and micro-organisms.

Traditionally, food fermentation is carried out at a household scale. Whereas a considerable number of fermentation processes have been scaled-up for commercial purposes, it may be safely stated that most types of fermented foods are still manufactured at home-scale under conditions of variable hygiene, using relatively simple processing facilities. Such products often contain mixed microbial populations because of the lack of sterility and the use of natural (spontaneous) fermentation or mixed-culture fermentation starters.

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For the purpose of this chapter, fungal fermented foods are defined as those foods in which fungi (yeasts and mycelial fungi) predominate and play a functional role, i.e., they contribute to the desirable attributes of the fermented product.

B. Useful Fungi

The number of fungal species encountered in fermented foods is relatively limited, but they belong to various orders. Table 2.1 shows some major examples and the products in which they are predominant.

C. Fungal Fermented Foods World-Wide

Fungal fermentation is practised in nearly all continents. In African traditional culture, cherished fermented cereal products include beers and fermented porridges, most of which are fermented by a natural mixed culture of lactic acid bacteria and yeasts. The use of mycelial fungi for fermentation in Africa is less common, but is practised in the fermentative detoxification of bitter cassava roots in, e.g., Mozambique and Uganda.

In the European region, the major traditional uses of yeasts include the production of alcoholic beverages and the fermentation of leavened bread whereas specific uses of mycelial fungi are mould-ripened cheeses and meat products. In several other products, such as in fermented olives, yeasts belong to the functional flora in combination with lactic acid bacteria.

The Asian continent provides the greatest variety of fungal fermented foods. The potential of yeasts to contribute flavour, nutritive value, alcohol and gas is evident in both starchy and leguminous foods. Mycelial fungi are used for their enzymatic ability to degrade polymeric substances, as well as for texture-forming properties.

There is an increasing international interest in the contribution to health by mould-fermented foods. In particular, fermented soybean foods attract world-wide attention for their content of bioactive peptides derived from glycinin (Gibbs et al. 2004); fermentation strongly improves digestibility, protection against diarrhoea and chronic

Table 2.1. Major functional fungal species in Asian fermented foods; adapted and enlarged from Samson (1993a)

Zygomycetes	
Actinomucor	*A. elegans*, *A. taiwanensis* (sufu, tou-fu-ru)
Amylomyces	*A. rouxii* (ragi)
Mucor	*M. circinelloides*, *M. rouxii*, *M. indicus* (ragi, murcha, tempe, pehtze)
Rhizopus	*R. microsporus* (tempe), *R. oligosporus* (tempe), *R. oryzae* (koji, nuruk, chu, murcha, tempe)
Ascomycetes	
Monascus	*M. purpureus*, *M. ruber* (angkak)
Neurospora	*N. sitophila*, *N. intermedia* (oncom)
Deuteromycetes	
Aspergillus	*A. oryzae* (koji), *A. sojae* (koji), *A. glaucus*, *A. melleus*, *A. repens*, *A. candidus* (katsuobushi), *A. niger* (koji)
Penicillium	*P. glaucum* (katsuobushi)
Yeasts	
Brettanomyces	*B. anomalus* (kumiss)
Candida	*C. javanica* (idli, kombucha, murcha)
Endomyces	*E. fibuliger* (murcha, ragi)
Hansenula	*H. anomala* (saké, koji)
Hyphopichia	*H. burtonii* (ragi)
Saccharomyces	*S. cerevisiae* (nan, toddy, murcha, kombucha), *S. dairensis* (tempe), *S. globosus* (kumiss), *S. kluyveri* (nan), *S. saké* (saké)
Torulopsis	*T. versatilis* (idli, kombucha, soy sauces, pastes)
Trichosporon	*T. pullulans* (idli), *T. beigelii* (tempe)
Zygosaccharomyces	*Z. rouxii*, *Z. sojae* (soy sauces, soy pastes)

degenerative diseases (Nout 2005). Obviously, as a result of today's intensive international commerce, an increasing number of formerly regional products are finding their way to foreign markets, making it even more important to educate the public about the properties and values of exotic foods.

D. Categories of Asian Fungal Fermented Foods

Considering the numerous regional names of fungal fermented foods, some attempt of classification is worthwhile. According to the type of products obtained, a distinction can be made of beverages (alcoholic and non-alcoholic), condiments or flavourings (including, e.g., soy sauces and pastes), protein-rich meat substitutes (tempe-like products) and bread- or cake-like products (e.g., idli; Lim 1991). Ko (1986) and Nout et al. (2007) distinguished various products on the basis of the functional micro-organisms during the fermentation such as chiefly by mycelial fungi, mycelial fungi and yeasts and a sequence of mycelial fungi followed by yeasts and bacteria. We will approach the issue from a technological angle, and distinguish three principally different fermentation processes, as follows:

1. Natural fermentation: the simplest type of process, whereby uncooked ingredients are mixed and are allowed to undergo an uncontrolled, "spontaneous" fermentation, i.e., without added starter. Usually the functional micro-organisms are present in the substrate or are provided by the environment (the utensils, the house atmosphere, etc.). In many cases these fermented products are cooked after fermentation. Examples are idli and nan (Table 2.2).
2. Starter-mediated single-stage fermentation: ingredients to be fermented are cooked first, followed by the addition of a specific starter (concentrate of viable spores or mycelium). During incubation the starter micro-organism (s) multiplies and modifies the food. After this stage, the fermentation is completed. Usually, the fermented product is cooked again prior to consumption. Examples include kumiss and oncom (Table 2.2).
3. Multiple-stage fermentation: this type of fermentation can be characterized by two or more fermentation stages, for example a first stage of solid-substrate fermentation followed by a liquid fermentation. The objective of the first stage is to produce a high concentration of polymer-degrading fungal enzymes. During the second stage these enzymes degrade polymers such as starch (for beer or wine-making) and proteins (soy sauces and pastes). Examples are rice wines, soy sauces and pastes and vinegar (Table 2.2).

II. Tempe

A. The Product

Tempe or tempeh is a collective name for fungally fermented beans, cereals or some other food processing by-products. Tempe most probably originates from the island of Java, Indonesia. Yellow-seeded soy beans are the most common and preferred raw material to make tempe. Figure 2.1 shows soy bean tempe (in full: tempe kedele) being sold at a market of Malang, East Java, Indonesia. Tempe is a highly nutritious, easily digestible and delicious product, and as such it meets an increasing demand from consumers looking for high-quality meat replacers. Scientific reports were published already during the 1800s, and this early and subsequent literature has been covered in the excellent review by Ko and Hesseltine (1979). The overview of the major scientific literature was complemented in the review by Nout and Rombouts (1990). The review by Hachmeister and Fung (1993) is of interest because of its coverage of the use of various leguminous seeds and cereals for tempe making. More recently, the functionality of tempe was reviewed (Nout and Kiers 2005). The present section on tempe addresses the aspects of relevance for the functionality of the product and the control of the fermentation process.

B. Traditional Manufacturing Process

Figure 2.2 summarizes the major process unit operations involved in making tempe. In most small-scale Indonesian tempe workshops, the soy beans are dehulled in a wet process. If sufficient water and cheap labour is available, wet dehulling has the advantage that no major equipment is essential and the beans suffer very little mechanical

Table 2.2. Selected Asian fungal fermented foods

Product	Main ingredients		Functional microflora	References
Natural fermentation				
Idli (India): beakfast steamed cakes	Rice and black gram dal		*Torulopsis*, *Candida*, *Trichosporon pullulans*, lactic acid bacteria	Batra (1986)
Nan (India): leavened bread	Wheat flour		*Saccharomyces cerevisiae*, *S. kluyveri*, *Lactobacillus*	Batra (1986)
Tou-shi, hamanatto (China): condiment	Soy beans		Asp. oryzae, Mucor	Wang and Fang (1986)
Red kojic rice, angkak (China): colourant	Rice		*Monascus purpureus*	Lim (1991)
Toddy (Malaysia): palm wine	Palm sap		*Saccharomyces* spp.	Lim (1991)
Starter-mediated, single-stage fermentation				
Tapé ketan (Indonesia): sweet snack	Glutinous rice	Ragi tapé (rice)	*Amylomyces rouxii*, *Endomyces fibuliger*, *Hyphopichia burtonii*	Ko (1986), Nout (1995)
Oncom (Indonesia): side dish	Groundnut press cake	Mycelium grown on cassava fibre	*Neurospora sitophila*	Ko (1986)
Tempe bongkrek (Indonesia): side dish	Coconut press cake	Usar	*Rhizopus* spp.	Ko (1986)
Katsuobushi (Japan): fish	Bonito fish	Fermentation vessel, or pure cultures	*Asp. glaucus*, *Pen. glaucum*, *Asp. melleus*, *Asp. repens*, *Asp. candidus*	Cook and Campbell-Platt (1994)
Airag (kumiss)	Milk	Starter cultures pre-grown in milk	*Saccharomyces globosus*, *Brettanomyces anomalus*, lactic acid bacteria	Naersong et al. (1996)
Kombucha (tea)	Tea, sugar	Cellulosic microbial film	*Saccharomyces* spp., *Candida* spp., *Torulopsis* spp., acetic acid bacteria	Nout (1992)
Multiple-stage fermentation				
Tempe kedele (Indonesia): side dish	Soya	Usar	*Rhizopus oligosporus*, *R. oryzae*, *Mucor indicus*, various yeasts and bacteria	Ko (1986), Nout et al. (1992), Nout (1995), data in this chapter
Yakju and takju (Korea): wines	Rice	Nuruk (wheat)	*Asp.oryzae*, *Asp. sojae*, *Rhizopus*	Mheen et al. (1986), data in this chapter
Huan-jiu (China): wine	Rice	Chu (wheat)	*Asp. oryzae*, *Asp. sojae*, *Rhizopus*	Mheen et al. (1986), Fukushima (1998), data in this chapter
Sake (Japan): wine	Rice	Koji (rice)	*Asp. oryzae*, *Hansenula anomala*, *Sacch. sake*	Nout (1995), Fukushima (1998)
Schochu (Japan): spirit	Barley, rice or sweet potato	Koji (barley or rice)	Black Aspergilli	Fukushima (1998)
Bai-jiu (China): spirit	Kaoliang, wheat, rice or other cereals	Chu (wheat)	*Rhizopus* spp.	Fukushima (1998)
Chiang, jnard (India, Nepal): beer	Barley, millet	Murcha (rice)	*Mucor circinelloides*, *Mucor rouxii*, *Rhizopus oryzae*, *Candida javanica*, *Endomyces fibuliger*, *Saccharomyces cerevisiae*	Batra (1986), Nout (1992)
Kochujang (Korea): spicy catsup	Rice, wheat flour and meju	Meju (soy beans)	*Asp. oryzae*, *Asp. sojae*, *Bacillus subtilis*	Mheen et al. (1986)

continued

Table 2.2. continued

Product	Main ingredients		Functional microflora	References
Chiang-yu, shi-tche (China), kecap (Indonesia), shoyu (Japan): soy sauces	Soy beans, wheat	Koji (soy beans and wheat)	*Asp. oryzae*, *Asp. sojae*, *Rhiz. oryzae*, *Zygosaccharomyces rouxii*, *Z. sojae*, *Hansenula* spp., *Torulopsis* spp., lactic acid bacteria	Ko (1986), Wang and Fang (1986), Nout (1995), Fukushima (1998), data in this chapter
Chiang (China), taoco (Indonesia), miso (Japan): soy paste	Soy beans, wheat, rice, barley	Koji (rice, barley or soy beans)	*Asp. oryzae*, *Asp. sojae*, *Rhiz. oryzae*, *Z. rouxii*, *Torulopsis versatilis*, lactic acid bacteria	Ko (1986), Wang and Fang (1986), Nout (1995), Fukushima (1998)
Tou-fu-ru (= sufu, furu) (China): soy paste	Soy bean curd (tofu)	Pehtze (tofu)	*Actinomucor*, *Mucor*	Wang and Fang (1986), data in this chapter
Tsu (China): vinegar	Millet, rice, sorghum		Mycelial fungi, yeasts, acetic acid bacteria	Wang and Fang (1986)
Jiu (China): Chinese liquor	Sorghum	Daqu (wheat, barley, peas)	*Aspergillus*, *Mucor*, *Rhizopus*, *Monascus*, *Trichoderma*, *Absidia*, *Rhizomucor*, *Emericella*; *Saccharomyces*, *Candida*, *Pichia*, *Issatchenkia*	Xie et al. (2007), Zhang et al. (2007), Wang et al. (2008a)

Fig. 2.1. Soy bean tempe (tempe kedele) sold at a market of Malang, East Java, Indonesia

damage. At a larger scale or when labour costs are high, dry dehulling is more economic, despite the disadvantage of higher losses of soy beans resulting from the abrasion of the soy bean hulls.

In all cases, soaking is an essential step to increase the moisture content of the beans as to to render them edible and enable microbial activity during the fermentation, but also to extract naturally occurring antimicrobial substances (saponins) and bitter principals. For this reason, the soaking water must be discarded and the beans cooked in fresh water. Cooking times vary according to custom and depend on the equipment used. The warming-up and cooling-down may take considerable time; of essence is the actual cooking at approximately 100 °C, which should last 20–30 min. After cooking the hot water is discarded as soon as possible, and the very hot beans are spread out on trays to enable steaming-off of the beans. This evaporation

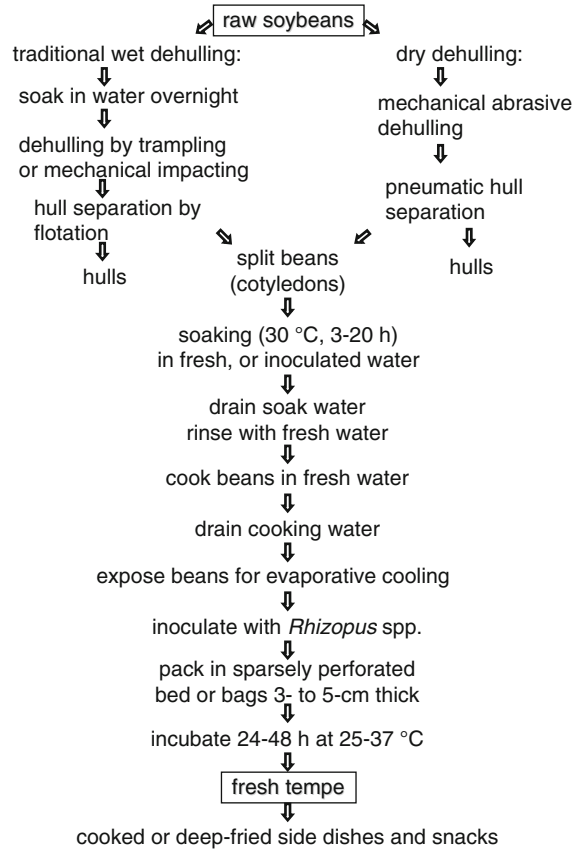

Fig. 2.2. Traditional manufacture of tempe

has two major effects: the removal of free water that could otherwise enhance microbial spoilage during the later stages of the process and a rapid cooling to about 20–25 °C within 10–15 min. The cooled beans are inoculated using tempe starter containing mainly sporangiospores of *Rhizopus oligosporus*, *R. oryzae* and sometimes *Mucor* spp. These are mixed homogeneously, followed by preparing layered beds or packages of approximately 3–5 cm thickness. An essential condition is that a limited supply of air can reach the beans. For this purpose, packagaing materials or bed covers are perforated in a more or less evenly distributed manner. Polythene sheet or bags are commonly used for commercial purposes; traditionally banana leaves or other plant material is used. Both give good quality products. Incubation for 1–2 days at ambient temperature (25–30 °C) is enough to allow spore germination and outgrowth of luxuriant mycelium binding the beans together to form a solid, sliceable cake of fresh tempe. Due to the restricted air supply, fungal spores are not or hardly produced and this results in an attractive creamy-white tempe colour with very little grey or black discolouration due to the pigmented sporangiospores. Fresh tempe is not eaten raw, but first cooked, e.g., in stews or fried in oil to give delicious crisps (tempe kripik). There are numerous ways to enjoy tempe (Shurtleff and Aoyagi 2001).

C. Fungi Involved and Their Relevant Properties

The major genus of importance for tempe making is *Rhizopus microsporus*, with varieties *microsporus*, *oligosporus*, *rhizopodiformis* and *chinensis* (Liou et al. 1990; Nout and Rombouts 1990). Zheng and Chen (1998) describe an additional variety *tuberosus*. Nout et al. (1992) investigated the leaves of the Indonesian Waru tree (*Hibicus tiliaceus)* of which the leaves are used as a carrier for tempe mould starter locally known as "usar". On leaves harvested in Indonesia, *R.oryzae* and *R. microsporus* var. *oligosporus* (further referred to as *R. oligosporus*) were found frequently besides a mixed flora of soil fungi; on leaves of the same *Hibiscus* spp. harvested in Africa and Europe the same soil fungi were found but no *Rhizopus* spp. This suggests that the widespread use of *Rhizopus* spp. in the manufacture of tempe results in its preponderance in the air spora. Most likely, *Hibiscus* leaves act as one of its natural reservoirs.

Of all sporangiospores present in starter concentrates used to initiate the fermentation, only a minor part may be viable, whereas significant numbers of spores have been sub-lethally injured. It was observed that well dried sporangiospores remain viable at ambient tropical temperatures for about three months, after which period they need to be activated. The germination of sporangiospores is strongly activated by amino acids such as L-alanine (Thanh et al. 2007).

The germination of *R. oligosporus* sporangiospores is influenced by temperature, presence of organic acids and fungal "self-inhibitors". De Reu et al. (1995) observed that 37 °C was optimum for germination and that acetic acid was inhibitory. The latter is of relevance as during the first soaking period, heterofermentative lactic acid bacteria may produce lactic and acetic acids.

Whereas it was shown earlier that *Rhizopus* spp. can grow at low (0.2%) oxygen concentrations, Lin and Wang (1991) observed that of 18 tested strains none was able to grow under absolute anaerobic conditions. They also observed that several strains were sensitive to oxygen toxicity (H_2O_2 or O_2^- or OH•), and that their growth was restored in the presence of catalase. Other environmental conditions affecting the formation of biomass are the temperature, CO_2 concentration and water activity (Sparringa et al. 2002). Monitoring of fungal growth and biomass formation on natural substrates can be achieved only by indirect means. Nout et al. (1997) provide an overview of techniques of indirect estimation of biomass. In tempe, Peñaloza et al. (1992) reported the use of on-line capacitance measurement; alternatively glucosamine is measured as a measure of cell wall chitin production. Sparringa and Owens (1999) proposed a conversion factor of 12 g dry biomass per gram glucosamine for *R. oligosporus.*

Rehms and Barz (1995) found that several tempe-forming *Rhizopus* spp. (*R. oligosporus*, *R. microsporus* var. *chinensis*, *R. oryzae*, *R. stolonifer*) were able to utilize the flatulence-associated oligosaccharide raffinose as the sole carbon and energy source. However, Graffham et al. (1995) also studied the nutritional requirements of mucoraceous mycelial fungi and observed that *Rhizopus* spp. could not use raffinose and stachyose or the mineral-complexing phytic acid as sole carbon and energy source. This was underlined by the finding of Ruiz-Téran and Owens (1999) who observed that, in bacteria-free tempe, levels of stachyose and raffinose were reduced during the tempe process as a result of leaching during soaking and cooking, but that the residual levels were not reduced further by the action of a strain of *R. oligosporus*. The fact that these substances are degraded nevertheless during the fermentation of "ordinary tempe" underlines the importance of mixed cultures of fungi as well as some of the accompanying bacterial species during the fungal fermentation.

The growth of *R. oligosporus* on a starchy model substrate was described by Mitchell et al. (1990) as a multiple-stage

process as follows: release of amylolytic enzymes by the mycelium, enzyme diffusion, starch hydrolysis by the enzymes, diffusion of glucose, uptake of glucose by mycelium. The mycelium penetrates into several layers of soy bean cells to approx 25% of the width of the cotyledon (Ko and Hesseltine 1979). This was recently confirmed by the finding that the penetration in 40 h was approx 2 mm; the mycelium moved in intercellular spaces and did not penetrate intact soy bean cells (Varzakas 1998). Several empirical methods were developed to measure the strength of the mycelial binding of the soy beans. Ariffin et al. (1994) developed an improved technique and reported that tempe incubated at 30 °C developed maximum strength after 30 h, after which the mycelium degenerated gradually.

Limitation of heat and mass transfer easily results in overheating of substrate beds in solid-substrate fermentations. Mixing during fermentation can be applied to improve heat and mass transfer and to obtain a more homogenous fermentation. Han et al. (1999) studied the effect of mechanical stress caused by intermittent rotation on the behaviour of *R. oligosporus* and *R. microsporus* on soy beans. They observed that biomass formation and enzyme production are negatively affected by mechanical stress, and that the sensitivity towards mechanical stress of the two strains tested was quite different.

As mentioned above, bacteria and yeasts accompany the mould fermentation. If lactic acid bacteria can dominate during soy bean soaking, the cooked beans will be slightly acid and this was shown to have important implications for the microbial composition of the final product (Mulyowidarso et al. 1990; Nout and Rombouts 1990). Wiesel et al. (1997) reported that mixed inocula of *R. oligosporus*, *R. oryzae*, *Citrobacter freundii* and *Brevibacterium epidermis* resulted in tempe covering the daily requirements of niacin, vitamin K, ergosterol, tocopherol, pyridoxine, riboflavin and biotin.

D. Biochemical Modifications and Implications for Health

Rhizopus spp. and the accompanying microflora consisting of bacteria and yeasts produce a range of enzymes that degrade proteins, lipids and carbohydrates of cooked soy beans. Proteases of *Rhizopus* were found to be mainly cell wall bound (Baumann and Bisping 1995). Heskamp and Barz (1998) studied proteases of nine strains of *R. oryzae*, *R. microsporus* var. *chinensis*, *R. stolonifer* and *R. oligosporus* and obtained various isoforms of aspartic (35 kDa) and serine (33 kDa) proteases. The proteolytic activity of *R. oligosporus* proved to be functional in cereals such as buckwheat, resulting in a lowering of allergenic proteins and an improvement of rheological behavior in noodle making (Handoyo et al. 2006).

Hering et al. (1990) found that during laboratory tempe fermentation the total level of crude lipids did not change much, but that the relative level of unsaturated fatty acids (especially oleic acid) increased. Sarrette et al. (1992) observed that as a function of time a range of polysaccharide degrading enzymes are formed by *R. oligosporus* growing in soy beans. The highest activities were measured after 20–30 h of fermentation; polygalacturonase, endocellulase, xylanase, arabinase, β-D-glucosidase and α-D-galactosidase were most prominent. De Reu et al. (1997) were able to correlate these polysaccharidase activities with softening of the soy beans and decreasing levels of non-starch polysaccharides.

Whereas some of the low-molecular-mass breakdown products will be metabolized by the microflora, the overall result of the microbial enzyme activity is a considerable degradation of polymeric substances in oligomeric and smaller units improving tempe digestibility process (Matsuo 1996; Kiers et al. 2000). This degradation is reflected in the spectacular increase of water-soluble dry matter and the in vitro accessibility (soluble matter that passes through dialysis tubing mimicking intestinal absorption). Table 2.3 shows the effect of *R. oligosporus* LU575 on absorbability with and without enzymic digestion. These

Table 2.3. In vitro digestibility of soy bean tempe fermented with a pure culture of *Rhizopus oligosporus* LU 575 (Kiers et al. 2000)

Fermentation period at 30 °C (h)	Accessibility (% dry matter)[a]		Fermentability (% dry matter)[b]
	Without digestion	With digestion[c]	
0	2	21	8
24	18	22	1
48	22	27	1

[a]Water-soluble matter passing through dialysis membrane (14 kDa cut-off).
[b]Based on production of gas and acetic acid by *Clostridium perfringens* ATCC12916 at 37 °C during 24 h.
[c]All at 37 °C in artificial saliva (pH 7) for 30 min, artifical gastric juice (pH 4) for 60 min, pancreatic solution (pH 6) for 30 min.

limited data indicate that tempe is of particular interest in patients suffering intestinal digestive deficiencies. The same table shows the fermentability (by anaerobic colon microflora) of undigested residues that would enter the colon in vivo. Residues of tempe reduced fermentability in the human gut and therefore tempe is more desirable to eat than cooked soy beans.

Other biochemical changes include the formation of vitamins (riboflavin, nicotinic acid, pantothenic acid, pyridoxin, folates, some biotin). It was observed that during the fungal fermentation, a fivefold increase of folates (mainly 5-formyl tetrahydrofolate and 10-formyl tetrahydrofolate) takes place in tempe probably as a result of de novo synthesis (Ginting and Arcot 2004). Cyanocobalamin is formed by bacteria in tempe, but it has not yet been established whether this can be utilized by humans. Several antinutritional factors are degraded during the process of tempe manufacture, mostly by leaching and thermal inactivation during soaking and cooking. However, phytic acid is degraded by fermentation (Eklund Jonsson et al. 2006) and this results in improved mineral bio-availability. For instance, Kasaoka et al. (1997) found in studies with iron-deficient rats that consumption of tempe achieved higher liver iron levels than unfermented cooked soy beans.

Of much interest is the modification of soy bean isoflavones by microbial activity, into substances with antioxidant and radical-scavenging activity that could have health-promoting effects. For instance, Klus and Barz (1998) demonstrated the formation of polyhydrolylated isoflavones from biochanin A and genistein by *Micrococcus* and *Arthrobacter* spp. isolated from tempe, and Matsuo et al. (1997) showed that 3-hydroxyanthranilic acid (HAA) is formed by fungal transformation of soy bean flavonoids. GABA-enriched tempe-like fermented soybean was produced by anaerobic fermentation with selected strains of *Rhizopus microsporus* (Aoki et al. 2003b); GABA (gamma-amino butyric acid) reduced hypertension in rat models over two months (Aoki et al. 2003a).

The anti-diarrhoeal effect of tempe that had been observed in child feeding in Indonesia, could also be established in piglets (Kiers et al. 2003). Piglets suffered less frequent and less severe weaning diarrhoea and had more efficient feed conversion when fed mixed feed containing soy bean tempe (Kiers et al. 2003). This could be ascribed to a reduction of fluid loss from the upper gut (Kiers et al. 2007), as well as a reduced adhesion of pathogenic *Escherichia coli* (ETEC) to the pig intestinal mucosa (Kiers et al. 2006). The bioactive component is water-soluble and has a molecular mass of >5 kDa (Kiers et al. 2007). It was established recently that this tempe related bio-activity also reduced the adhesion of ETEC to human intestinal cells of the Caco-2 type (Roubos-van den Hil et al. 2009).

Within the genus *Rhizopus*, the formation of anticancer drugs rhizoxins and toxic rhizonins have been described. From a range of *Rhizopus* species and strains grown on semi-synthetic and natural substrates, *R. oligosporus* and *R. chinensis* did not produce the secondary metabolites mentioned above. In contrast, *R. microsporus* produced rhizoxins and one strain produced rhizonins (Jennessen et al. 2005). It was found recently, however, that the production of rhizonin is not caused by the fungus (Partida Martinez et al. 2007) but by symbiont bacteria of the genus *Burkholderia* that are localized in the fungal cytosol.

E. Industrial Aspects

Whereas small-scale home production uses the traditional equipment and starters, several innovations should be mentioned because they have changed the scene of tempe making. For instance, wet bean dehulling is carried out mechanically using simple motor-driven concrete-disc impactors. For packaging fermentation beds, polythene sheet has displaced the banana leaves of old. Powdered starter concentrates are commercially available.

In Indonesia, the annual production of tempe was estimated at about 80000 t with 14% of the soy beans produced in Indonesia being used for tempe production (Yokotsuka and Sasaki 1998).

Recently, some interesting process lines and novel products were developed (Nout and Kiers 2005). Semi-continuous process lines of a capacity of 600 kg/day tempe are commercially used. Dry dehulled beans are soaked overnight in a hopper vessel and transported by belt conveyor through a boiling water bath in a period of 20 min. Next they are drained and dried and cooled under a fan; if needed the bean temperature can be adjusted using heating section. Inoculation takes place using a mechanical dispenser, and the inoculated beans are filled into perforated polyethylene tubing of 5 cm diameter. The incubation of these sausages takes place at 32 °C for 24 h, in a carefully designed room with forced ventilation. These products are pasteurized for improved shelf-life and microbiological hygiene (Fig. 2.3). In addition to the standard traditional tempe in sausage shape, a series of products are commercially available that can be easily recognized by west European consumers, for instance smoked tempe with sausage taste, various salads, burgers and meat loafs.

New products can be developed for dedicated markets such as the elderly and the diseased. For example, a trebling of the isoflavone level of tempe could be achieved by inclusion of 20% isoflavone-rich soy bean germ (Nakajima et al. 2005). Dopamine is used in the management of Parkinsons' disease. Faba beans are a good source of its precursor, laevo-dihydroxy phenylalanine (L-DOPA). It was observed that tempe-like fermentation of faba beans with *R. oligosporus* resulted in a doubling of the L-DOPA

Fig. 2.3. Packaged, pasteurized tempe (De Hobbit, Belgium)

Fig. 2.4. Red kojic rice (Angkak)

content in addition to a favourable increase of antioxidant polyphenols (Randhir et al. 2004).

III. Red Kojic Rice (Angkak)

A. The Product

Red kojic rice, also referred to as Angkak, Anka, Red Qu, Chinese red rice, or *Monascus* fermented rice (Fig. 2.4) is traditionally obtained by fermentation of cooked rice with the mycelial fungi *Monascus* spp., such as *M. purpureus*, *M. anka* or *M. ruber*. It has a specific aroma and purple-red colour and is used as a natural colorant in red spirit, red furu and red rice (Chiao 1986). Excellent reviews of the secondary metabolites of *Monascus* are given by Blanc et al. (1994), Juzlova et al. (1996) and Lin et al. (2008).

B. Traditional Manufacturing Process

Traditionally, polished rice is soaked overnight, cooked or steamed, cooled and inoculated with spores of *Monascus* spp. (Fig. 2.5). Solid-substrate fermentation

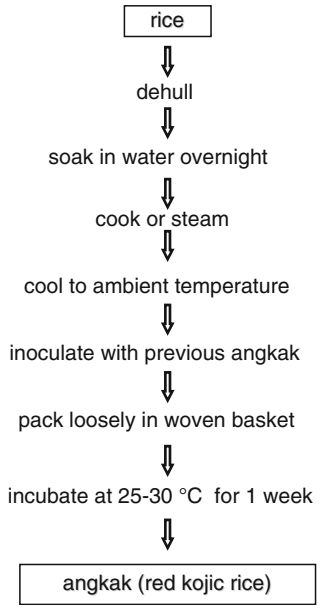

Fig. 2.5. Traditional manufacture of Angkak (red kojic rice)

during approximately one or two weeks allows the mould to grow and produce its secondary metabolites. Major pigments (Fig. 2.6) include the orange pigments rubropunctatin and monascorubrin, purple pigments rubropunctamin and monascorubramin and the yellow pigments ankaflavin and monascin (Pastrana et al. 1995). They are heat-stable and stable over a wide pH range, and thus they are of interest as "bio-colorants" in foods. Recently, using TOFMS, the additional yellow pigments xanthomonascin A, xanthomonascin B, monascopyridine A, monascopyridine B and yellow II were recorded. The nature and quantity of individual pigments produced are strain- and environment-dependent (Miyake et al. 2008).

C. Fungi Involved and Their Relevant Properties

Growth and pigment production in a liquid culture of *Monascus anka* MF 107 was distinguished into three phases by Fu et al. (1996), viz. a first phase dominated by mycelial growth and pH decrease from 5.5 to 4.6, a second phase of steady pigment production and pH increase from 4.6 to 8.4 and a third phase of gradual deterioration.

The role of the key chain elongation process by which the poly-beta-ketide carbon skeleton of the pigments is assembled was studied by Hong et al. (1995). Of several precursors tested, crotonic acid and sorbic acid enhanced

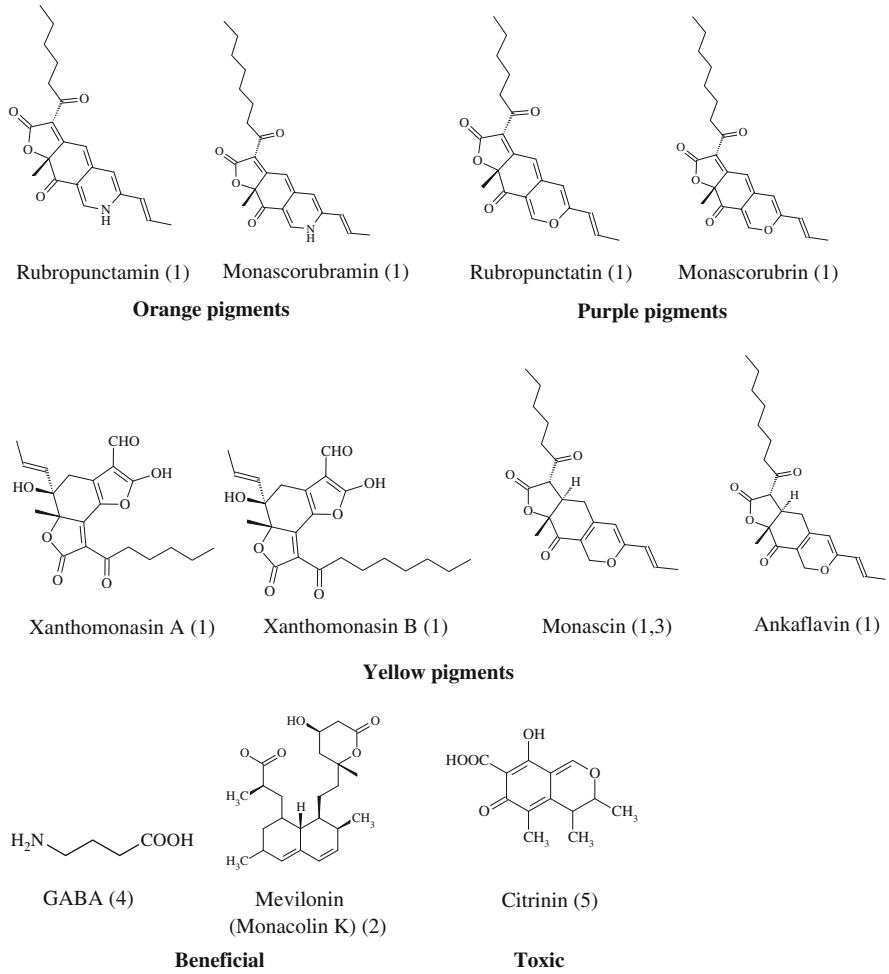

Fig. 2.6. Selected secondary metabolites of *Monascus* spp. in red kojic rice Compiled from literature references. *(1)* Akihisa et al. (2005), *(2)* Juzlova et al. (1996), *(3)* Jongrungruangchok et al. (2004), *(4)* Wang et al. (2003), *(5)* Liu et al. (2005)

pigment productivity, with sorbic acid being incorporated more efficiently. Ethyl esters of these acids as well as cinnamic acid had a stimulatory impact on the biosynthesis. Apparently, the biosynthesis routes are not highly specific, as new red pigments were produced when these poly-beta-ketide intermediates were used as precursors. The red/yellow ratio could be influenced by the ratio of carbon (glucose) and nitrogen (mono-sodium glutamate) sources (Pastrana et al. 1995).

The physiology of a strain of *Monascus anka* KCCM 11832 was studied in liquid culture by Kang and Jung (1995). Ohantaek and Mudgett (1992) studied *M. purpureus* ATCC 16365. Both groups observed that optimum environmental conditions for pigment production are different from those for mycelial growth. For pigment production, shaking of submerged cultures strongly stimulated pigment formation. In solid-state fermentation, 50% O_2 was optimum for pigment production. Optimum conditions for pigment production were 25 °C at pH 6 in a medium containing 2% rice, 0.05% peptone and 0.1% $MgSO_4$ during 7 days. Less peptone and higher temperatures resulted in more mycelium.

In addition to the pigments, *Monascus purpureus* also produces a typical angkak flavour. The volatile metabolites as reported by Juzlova et al. (1998) included alcohols, aldehydes, ketones, esters and terpenoid compounds. The highest flavour activity (concentration: threshold value) was ascribed to 2-heptanone, 2-nonanone, ethyl acetate, ethanol, 2-methyl-1-propanol and 3-methyl-1-butanol (Chung et al. 2004). In an earlier study, Peters et al. (1993) reported that in media containing saccharides (glucose) and fatty acids (octanoic acid), the relative toxicity of the fatty acid forced the mould into a detoxification process, oxidising octanoic acid to methyl ketones and secondary alcohols. Only after complete detoxification, saccharides were assimilated for fungal metabolism. These properties are of importance for controlled production of singular flavour components.

During recent years, much interest has been shown in the potential health benefit but also in the possible risks of angkak. Several studies investigated the effects of *Monascus* metabolites, such as in vitro and in vivo anti-inflammatory and hypo-allergenic (Akihisa et al. 2005), stimulation of bone formation (Gutierrez et al. 2006), colon cancer prevention (Hong et al. 2008), down-regulation of adipogenic transcription factors (Jeon et al. 2004) and prophylaxis of Alzheimer disease pathogenesis (Lee et al. 2008a). In particular, monacolin K (lovastatin) and gamma-amino butyric acid (GABA) Fig. 2.6 are of medical interest (Lin et al. 2008). It was reported by Wang et al. (1997) that during an 8-week trial in a group of 324 hyperlipidemia patients, a daily dose of 1.2 g angkak resulted in significant reductions of serum total cholesterol and low-density cholesterol.

D. Industrial Aspects

Traditional angkak is produced commercially at an industrial scale in China. Most angkak is produced as intermediate product, to be used for colouring sufu and wines. For example, the largest plant in Beijing produces 16000 t/year furu and 200 t/year angkak. The annual production of angkak for the whole of China is estimated at over 15000 t (B-Z. Han, personal communication). Because of the solid texture of the product, its manufacture is by solid-substrate fermentation. Lucas et al. (1993) described a swing bioreactor providing mild agitation. In this reactor *Monascus purpureus*-inoculated rice was fermented into angkak. The advantage of controlled reactors is that environmental conditions can be controlled. Optimum conditions for the angkak fermentation included 34% moisture content of the substrate (cooked rice) and a fermentation period of 7 days at 28.8 °C.

In addition, the use of angkak extracts or purified *Monascus* pigments as colouring agents has been patented for a variety of foods (Samson 1993a), including salami and sausages (Fabre et al. 1993). In the late 1970s it was observed that submerged fermentation was considerably faster and gave higher yields of pigments than solid-substrate fermentation. Consequently, for industrial pigment production, submerged fermentations appeared to be more appropriate (Chiao 1986). More recently, solid-liquid fed-batch cultures gave even higher pigment yields (Lee et al. 1995). Further, the use of liquid cultures with inorganic nitrogen sources was associated with the formation of bioactive orange pigments (monascorubrin and rubropunctatin) that had some embryotoxicity and teratogenicity (Martinkova et al. 1995). In solid-substrate rice fermentations or in liquid cultures with organic nitrogen sources, these orange pigments were converted into inactive compounds (amines).

Certain strains of *Monascus* spp. can produce the mycotoxin citrinin (Fig. 2.6). The levels of citrinin in controlled fermentations can be maintained at safe levels by optimizing culturing conditions and by using selected non-toxic strains. However, in many industrial operations, uncontrolled starter cultures are used for the fermentation. Some investigations of citrinin levels in industrially produced angkak revealed that 20% of Korean angkak exceeded the tolerated level of 50 g/kg (Kim et al. 2007); in retail samples from Taiwan lipid extracts had citrinin levels ranging from 280–6290 g/kg, and some sample extracts exerted cytotoxic effects on the human cell line HEK293 (Liu et al. 2005). Newly discovered monascopyridines C and D were associated with tumor formation (Knecht et al. 2006). These new findings indicate the need to control the quality of starters and process conditions in order to minimize the health risk due to toxic secondary metabolites (Trucksess and Scott 2008).

IV. Amylolytic Starters

A. The Product

Amylolytic starters are used to saccharify starchy and other high-molecular-weight carbohydrate material for fermentation processes. Ragi means inoculum (Cronk et al. 1977) and the name following ragi indicates the use of starter, thus ragi-tempe is an inoculum for tempe. There are several amylolytic ragi-type starter cultures available in the markets in most Asian countries and these include Indonesian ragi-tapé, ragi *peuyeum* (for cassava), Malaysian ragi-*tapai*, *jui-paing*, Thai *loopang* (grown on bran), Philippine *bubod levadura*, Chinese *levure chinoise*, Indian *bakhar* and Nepalese *murcha*. Figure 2.7 shows murcha sold at a market in Darjeeling, India. Amylolytic starter cakes have been used to substitute diastatic malt

Fig. 2.7. Murcha amylolytic starters sold at a market of Darjeeling, India

extract as a saccharifying agent in producing rice-based weaning food (Yusof et al. 1995). Takeuchi et al. (2006) purified and characterized an amylolytic enzyme from yeast, *Pichia burtonii*, which was isolated from murcha. The extracellular enzyme, a glycoprotein, showed strong amylolytic properties in presence of starch but its activity was inhibited by metal ions such as Cd^{2+}, Cu^{2+}, Hg^{2+}, Al^{3+} and Zn^{2+}.

Tane koji, an essential enzyme starter in the production of soy sauce, miso, saké and amazake (sweetened rice), is described in Section VI. Lin et al. (2006) reported on an enhanced antioxidative activity of soybeans fermented with GRAS filamentous fungi (*Aspergillus oryzae*, *A. sojae*, *A. awamori*, *Actinomucor taiwanesis*, *Rhizopus* spp.). They found that α-diphenyl-2-picryl-hydroxyl (DPPH) scavenging effects and the Fe^{2+}-chelating ability and reducing power of the fermented soybean were high. Soybeans fermented with *A. awamori* had at least a sixfold higher antioxidant capacity than the control, thus indicating a potential for developing a healthy food supplement. Lee et al. (2008b) reported similar results with blackbean koji.

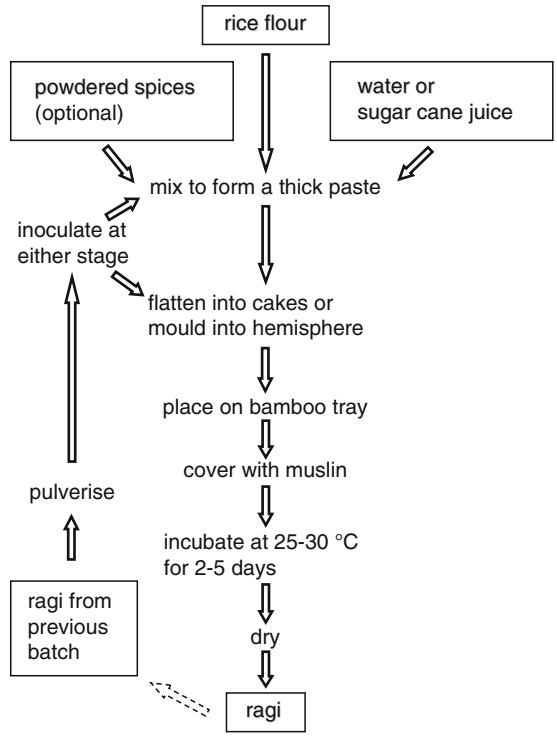

Fig. 2.8. Traditional manufacture of Ragi

B. Traditional Manufacturing Process

In the preparation of starter cake shown in Fig. 2.8, rice or wheat is ground and thoroughly mixed with spices. A mixture of garlic, pepper, rhizomes, onion and root is used in the preparation of the starter and producers regard their recipes as secret passed from generation to generation. The ratio of ground rice to mixed spices is about 14:1. Water is added to make a dough-like material which is shaped into small balls or cakes of about 4 cm in diameter and 1cm thick. Dry powdered ragi from previous batches is sprinkled over the cakes. The latter are then placed on a wooden bamboo tray, covered with a cloth and incubated at ambient temperature for 2–5 days, during which the dough is slightly raised and covered with fungal mycelia. Spices in the ingredients play a major role in preventing growth of undesirable micro-organisms. The cakes are air- or sun-dried and have a shelf-life of several months (Ko 1986; Cook and Campbell-Platt 1994; Saono et al. 1996).

In the Philippines, *bubod levadura* is made by several processes depending on the locality or region. In the Benquet process, glutinous rice is soaked, drained, ground and mixed with pureed ginger and wild roots and then inoculated with powdered bubod containing *Mucor* spp. and other amylase producers which are responsible for the early fermentation and then with yeasts which are responsible for the remainder of the fermentation. In the Bontoc process, no spices are added and the cakes are not usually inoculated, but in the Ifugao process, rice grains are roasted before soaking and spices are added and then inoculated with powdered bubod (Tanimura et al. 1978).

C. Fungi Involved, Their Relevant Properties and Implications for Health

Studies on the microflora of ragi and Chinese yeast cake date from Went and Prinsen Geerligs (1895). Several mycelial fungi and yeasts, with amylolytic activities, have been isolated from ragi cakes (Dwidjoseputro and Wolf 1970; Hesseltine et al. 1985). The principal moulds are *Amylomyces rouxii*, *Rhizopus* spp., *Mucor* spp., *Aspergillus* spp. and *Fusarium* spp. (Table 2.4). *Hansenula* spp., *Endomycopsis (Saccharomycopsis) fibuligera*, *Candida* spp. and *Saccharomyces cerevisiae* are the common yeasts in many starter cakes. Murcha or marcha is a traditional amylolytic starter commonly used in the Himalayan regions of India, Nepal and China. Tsuyoshi et al. (2005) isolated several yeast strains from murcha and the main species were *Saccharomyces bayanus*, *Candida glabrata*, *Pichia anomala*, *Saccharomycopsis fibuligera*, *Saccharomycopsis capsularis* and *Pichia burtonii*. Some of the species

Table 2.4. Amylolytic moulds isolated from Malaysian and Indonesian tempe, tempe-ragi, tapé and ragi-tapé. Functional moulds are indicated in **bold**. Adapted from Merican and Yeoh (1989) and Steinkraus (1996)

Product	Country/origin	Fungal species
Tempe	Malaysia/Kajang, Selangor	**Rhizopus** spp., *Aspergillus* spp., *Penicillium* spp.
Tempe	Malaysia/Kuala Lumpur, Selangor	**Rhizopus** spp., **Mucor** spp.
Tempe	Malaysia/Bangi, Selangor	**Rhizopus** spp., *Aspergillus* spp.
Tempe	Indonesia/jakarta	**Mucor javanicus**, *A. niger*, *Fusarium* spp.
Ragi-tempe	Indonesia/Surakarta	**R. oryzae**, **R. stolonifer**
Ragi-tempe	Indonesia/Malang	**R. oryzae**, **R. arrhizus**, **R. oligosporus**, **Mucor rouxii**
Tapé and ragi-tapé	Indonesia	**Rhizopus** spp., **R. oryzae**, **Mucor rouxii**, **M. javanicus**, **M. circinelloides**, *Fusarium* spp., **Aspergillus oryzae**, **Amylomyces rouxii**

showed high amylolytic activity. Sujaya et al. (2004) used five different types of ragi tapé starters for the production of Balinese rice wine, brem, and isolated 51 yeast strains, with *Saccharomyces cerevisiae* accounting for 69% of the total yeast isolates.

Hesseltine et al. (1988) studied 41 amylolytic oriental fermentation starters from seven Asian countries and found that they contained three genera of *Mucorales* (*Rhizopus*, *Mucor*, *Amylomyces*). Every sample contained at least one Mucoraceous mould and one yeast. Later, they studied nearly 100 amylolytic yeast strains isolated from ragi and other starters and found the predominant yeasts were *Endomycopsis (Saccharomycopsis) fibuligera* and, to a lesser extent, *Saccharomycopsis malanga* (Hesseltine and Kurtzman 1990). Kozaki and Uchimura (1990) also isolated three types of moulds (two *Mucor* spp., one *Rhizopus* spp), two types of yeast (*Saccharomyces cerevisiae*) and a mycelial yeast, *Saccharomycopsis fibuligera* from Philippine bubod and tapuy. Of 41 yeast strains isolated from Indonesian ragi and tapé (Indonesian sweetened rice), 19 had amylolytic activity, none were proteolytic and all the moulds were amylolytic (Saono et al. 1996). The exact mycoflora of ragi varies with location and the particular food for which the starter is to be used.

Certain genera of yeasts and moulds are known to play a leading role in the fermentation of rice wine and sweetened rice. Based on their abilities to produce high amylolytic activities (α-amylase and/or amyloglucosidase), a number of strains of *A. rouxii*, *Rhizopus* spp. and *Endomycopsis* spp. were selected and a combination of *A. rouxii* and *E. fibuligera* for instance was shown to produce a good quality tapé. Fungal amylases are in demand in industrial food processes and are preferred over other microbial sources due to more accepted GRAS status (Gupta et al. 2003). However amylolytic lactic acid bacteria have also been used in a simple novel fermentation process for the preparation of high-energy-density fermented gruels for young children (Nguyen et al. 2007).

Amylomyces rouxii is an important constituent of starter cultures used in the production of fermented foods in the Far East, Southeast Asia and the Indian subcontinent. The filamentous fungus is closely related to certain strains of *R. oryzae* producing lactic acid. According to Ribes et al. (2000), *R. oryzae* occasionally causes the human disease, mucormycosis; it is also a possible pathogen in industrial crops (Phytopathological Society of Japan 2000). Saito et al. (2004) compared *A. rouxii* and *R. oryzae* in lactic fermentation of potato pulp and other agricultural by-products into food materials. They concluded that *A. rouxii* was preferable to *R. oryzae* for use in fermenting those substrates as food materials.

D. Industrial Aspects

Starter inocula are generally produced by household or village manufacturers who use closely guarded recipes. Because of the difficulty in controlling microbial activities of mixed cultures contained in starter cakes, pure cultures of the selected strains of *Rhizopus* spp. (mainly *R. oryzae*) have been used for industrial production of enzyme starters, in particular, for the distillery industry (Lotong 1998).

Mass production of spores of *Rhizopus* spp. on rice adhering to inside surfaces of flasks is applied in the fermentation industry in Asian countries, in particular, Taiwan and Thailand. The process involves steeping ordinary or glutinous rice for 3–6 h and transferring to a flask or bottle. The container is swirled to spread the grains inside its surface and it is plugged with cotton and steamed. After cooling it is inoculated with spore suspension and incubated at 35 °C for 4–5 days after which large quantities of spores are produced.

In commercial runs in Thailand, enzyme preparations are made by grinding glutinous rice into coarse powder, followed by mixing with rice bran. The mixture is steamed and after cooling, mixed with spore suspension and spread onto wooden bamboo trays. The inoculated material is incubated at ambient temperature for 36–40 h after which the koji cakes are sun-dried and ground into a powder. In some distilleries, glutinous rice may not be used as it has been reported that less amylolytic enzyme, amyloglucosidase, is produced in substrate containing glutinous rice (Lotong 1998).

V. Furu (Sufu)

A. The Product

Sufu, or tou-fu-ru (Wang and Fang 1986) is an ancient Chinese cheese-like product with a creamy consistency (Fig. 2.9). Sufu is made from soy beans and is an easily digested and nutritious protein food (Su 1986). It is a popular side-dish, e.g., with breakfast rice. There are several varieties of colours ranging from grey to dark red (obtained with angkak) and flavours ranging from sweet and bland to strong and offensive. A comprehensive overview of the different categories of furu, their flavor characteristics and industrial manufacture was given by Han et al. (2001b).

B. Traditional Manufacturing Process

Furu or sufu is obtained by a three-stage process (Fig. 2.10). During the first stage, soy bean curd (tou-fu or tofu) is obtained by extraction of soymilk from soy beans followed by precipitation of the soy protein by acid or by added calcium salts. The precipitate is pressed to blocks of tofu. The second stage is a fungal solid-substrate fermentation. Cubes of tofu are heated at 100 °C for 10–15 min to reduce the moisture content to about 70%, firming-up its consistency and pasteurizing the cubes before they are inoculated (naturally by covering them with straw, or by using pure culture starters) with mycelial fungi such as *Actinomucor elegans*, *A. taiwanensis*, *Mucor hiemalis*, *M. silvaticus*, *M. praini, M. subtillissimus*, or *Rhizopus chinensis*. After 2–7 days at 12–25 °C depending on strains used (Fukushima 1985) the mycelium has covered the tofu cubes. The product is now referred to as pehtze. During the third stage, an enzymatic ripening takes place. The pehtze is submersed in brine and kept in closed jars for 2–4 months. During this period the fungal (mainly proteolytic) mycelia-bound enzymes are released (Wang and Hesseltine 1970) and, while diffusing into the pehtze, the texture softens and flavour is developed. The composition of the brine strongly influences the ripening process, as well as the flavour of the final sufu. Brines exclusively containing approx. 12% NaCl combined with the use of mixed fungal–bacterial pehtze inocula result in the most offensive flavours. Mixed brines consisting of approx. 10% NaCl and 10% rice wine ethanol give more neutral flavours. In Japan, very bland flavours are preferred. The Japanese product “tofuyo” shows a similarity

Fig. 2.9. Furu (Sufu)

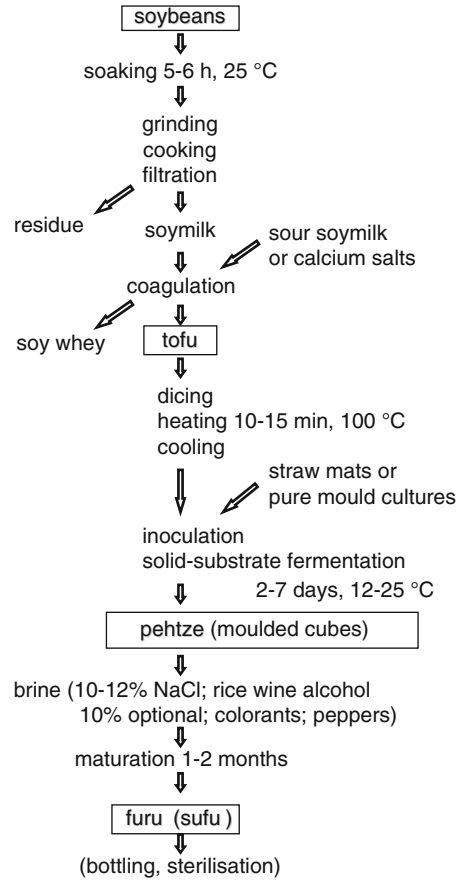

Fig. 2.10. Traditional manufacture of Furu (Sufu)

to sufu because it is obtained by enzymatic ripening of tofu (Yasuda and Kobayashi 1989). The difference however, is that the required enzymes are obtained from angkak rather than from the typical sufu mycelial fungi mentioned earlier. Using various additive ingredients (angkak, soy mash, rice wine, hot peppers) a variety of sufu colours and fragrances can be achieved (Wang and Hesseltine 1970). Finally, sufu is bottled with brine and heated to achieve commercial sterility. The predominant groups of micro-organisms in furu are Gram-positive spore-forming bacteria and lactic acid bacteria in low-salt sufu, whereas some sufu contained significant levels of *Bacillus cereus* and *Clostridium perfringens* (Han et al. 2004b). From a range of commercial sufu from all over China, it was observed that the most likely spoilage microflora are lactic acid bacteria such as *Lactobacillus casei* (Han et al. 2001a). Although most products were safe from the microbiological point of view and no viable *Staphylococcus aureus* were encountered, some contained detectable levels of staphylococcal enterotoxin A (Han et al. 2001a). A sampling plan was proposed to monitor the safety of this type of product. Criteria included tolerable levels of Enterobacteriaceae, *B. cereus*, *C. perfringens*, and *S. aureus* (Han et al. 2001a).

C. Fungi Involved and Their Relevant Properties

The fungal genera involved (*Actinomucor*, *Mucor*, *Rhizopus*) all belong to the Mucoraceae. Predominating mycelia fungi from sufu of China and neighbouring Vietnam are *Actinomucor repens*, *Actinomucor taiwanensis*, *Mucor circinelloides*, *Mucor hiemalis*, *Mucor racemosus*, and *Rhizopus microsporus* var. *microsporus* (Han et al. 2004a).

The temperature range for growth of these species is 20–30 °C. During the summer season in Beijing, the ambient temperatures exceed this range. It was found that the tempe fungus, *Rhizopus oligosporus*, is better suited to higher temperatures and provides a very similar modification of the pehtze. Optimum relative humidity for pehtze development was found to range over 95–97% (Han et al. 2003a). Incubation temperatures also influence the modification of isoflavones and the activity of β-glucosidase by *Actinomucor elegans* (Yin et al. 2005). At 26 °C, more beneficial isoflavone aglycons were formed than at 32 °C. It would be worthwhile to investigate the temperature-dependent isoflavone modification by *R. oligosporus*. Optimum conditions for growth of *A. taiwanensis* on tofu were 25–30 °C at 97% relative humidity when inoculated on tofu of 65% moisture content. Under these conditions, a maximum production of protease, α-amylase, α-galactosidase and lipase enzymes was reported by Chou et al. (1988). A glutaminase from *A. taiwanensis* was purified and studied (Lu et al. 1996). Its pH and temperature optima were 8.0 and 45 °C, and it was stable at ≤35 °C and pH 6.0–8.0. The enzyme still exhibited 50% of its maximum activity in the presence of 10% w/v NaCl, demonstrating its potential impact during the ripening stage.

The fungal proteases are not extracellular, but mycelium-bound. The salty brine provides the ionic strength needed to release them into the solution, enabling their diffusion into the soy bean curd (tofu). Their effect is the degradation of the soy bean protein into peptides, free amino acids and other non-protein nitrogenous substances. Table 2.5 illustrates the gradual degradation as a function of brine composition and mould species involved in pehtze fermentation.

The degradation of soy bean protein was enhanced in the absence of ethanol during the ripening (Chou and Hwan 1994). No intact proteins could be detected in ripe tofuru (sufu), and the resulting peptides had molecular mass <10 kDa. Free amino acids included tyrosine and considerable levels of hydrophobic amino acids (Rao et al. 1996). High amounts of glutamic acid and leucine were reported by Chou and Hwan (1994). In view of the bitter-tasting

Table 2.5. Biochemical changes taking place during Sufu maturation (data selected from Chou and Hwan (1994)

Maturation time at 25 °C (days)	*Actinomucor taiwanensis*[a]				*Actinomucor elegans*			
	S[b]		S+A[c]		S		S+A	
	ANR[d]	FFA[e]	ANR	FFA	ANR	FFA	ANR	FFA
0	5	45	5	45	5	30	5	30
15	9	70	6	80	11	110	9	80
30	15	120	8	110	17	190	8	190
45	18	180	9	80	18	220	9	180
60	20	160	11	70	23	240	15	170
75	27	170	13	75	23	300	18	100

[a]Mycelial fungi used for pehtze fermentation.
[b]Brine: 12% w/v NaCl.
[c]brine: 12% w/v NaCl + 10% w/v ethanol.
[d]Amino nitrogen ratio = (amino N/total N) × 100%.
[e]Free fatty acids (mg/g lipid).

hydrophobic amino acids, it will be of interest to investigate the significance of their presence in sufu for its taste. The modification of pehtze by degradation of protein and lipids is affected by the salt levels in the maturation brine. Lowering the salt level from the regular 14% to 8% or even 5%, shows more rapid and complete degradation of protein and lipid fractions (Han et al. 2003b) and this also includes the softening of the pehtze texture. The major amino acids present in the fraction of water-soluble nitrogen compounds are, in descending order: glutamic acid, leucine, aspartic acid, alanine, phenylalanine and lysine (Han et al. 2004c). Considering the flavor-enhancing activity of glutamic acid and glutamate, the addition of furu to dishes obviously strengthens the meaty flavour.

The degradation of soy protein during furu maturation results in the formation of bio-active peptides that have a beneficial effect by inhibiting angiotensin converting enzyme (ACE), being antithrombotic and having surface tension and antioxidant properties (Gibbs et al. 2004). It was reported that peptidases of the sufu mould *Actinomucor elegans* result in a debittering of certain hydrophobic peptides in soy protein hydrolysates (Li et al. 2008). It may be inferred that such beneficial effects also take place in the maturation of furu. Some lipase activity is produced that releases fatty acids from the soy bean oil (Table 2.5). The presence of ethanol during maturation was associated with lower levels of free fatty acids in matured sufu (Chou and Hwan 1994). The fatty acids react with the wine alcohol to produce esters that add to the fruity flavour (Campbell-Platt 1987). The complex flavour of sufu was reported to contain 22 esters, 18 alcohols, seven ketones, three aldehydes, pyrazines, two phenols and other volatile compounds by Hwan and Chou (1999). Maturation in the presence of ethanol (rice wine) resulted in higher levels of volatiles.

D. Industrial Aspects

Sufu is produced at an industrial scale. The production of tou-fu is highly mechanized. In China alone, annual production is estimated at about 300000 t (B.-Z. Han, personal communication). Pehtze fermentations are carried out using tray fermentations in incubation chambers, and maturation in large vessels.

The control of maturation, flavour development and consistency are closely guarded secrets. Obviously, combinations of salt, wine and additives as well as humidity and temperature control are key factors.

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 published data are available on these issues, except a study on the halophilic flora of sufu. During the brining stage, halophilic lactic acid bacteria, notably *Tetragenococcus halophila* (previously *Pediococcus halophilus*) are found in a majority of brands of sufu (Pao 1995). In view of the microbiological safety of sufu, mixed brines containing 10% NaCl and 10% ethanol were preferred for the protection against disease-associated bacteria such as *Staphylococcus aureus*. Salt concentrations used traditionally in the maturation brine (about 20%) are high, and have the function to safeguard the product against spoilage. It is of commercial interest to reduce salt levels because maturation can proceed faster as mentioned earlier. In addition, there is no harm in reducing salt intake by the average consumer. How far could salt levels be lowered, while still maintaining a wholesome and safe product? It was observed that lactic acid bacteria spoilage may occur when 10% salt (in brine) is used. At levels $\leq$18% in the brine, staphylococcal enterotoxins were produced during challenge experiments with *Staphylococcus aureus* (Han et al. 2005). Micro-organisms possessing lysine decarboxylase such as *Bacillus subtilis* can form mildly toxic biogenic amines, e.g., histamine (Kung et al. 2007). Although most tested sufu had safe levels of biogenic amines, some contained 160 ppm histamine which is three times the tolerable limit. The salt levels of the investigated sufu's ranged from 6% to 12% (in the product). It is not known which salt level was present in the histamine-containing sample, but considering the above data a suggested safe minimum level would be $\geq$8% salt in the product.

VI. Soy Sauce

A. The Product

Soy sauce is a light to dark brown liquid with a meat-like salty flavour used in cooking and as a table condiment (Fig. 2.11). Traditionally made in

Fig. 2.11. Soy sauce

China, Japan, Korea, Thailand, Philippines, Indonesia, Malaysia and Singapore, soy sauce is now also produced in Europe and both North and South America. It is of very ancient lineage and today the production of soy sauce includes every level of sophistication from domestic or village-scale, to advanced controlled production systems of a very high quality product. There are two specific fermentation procedures involved in soy sauce production, namely aerobic koji fermentation involving the use of *Aspergillus oryzae* or *A. sojae* and an anaerobic moromi or salt mash which undergoes lactic acid bacteria and yeast (*Saccharomyces rouxii*) fermentations.

B. Traditional Manufacturing Process

Traditionally soy beans, *Glycine max*, are soaked in water, boiled and drained and mixed with ground or crushed roasted wheat. The mixture is placed on trays and mixed with *Aspergillus oryzae* or *A. sojae* (tane-koji) and allowed to ferment at about 30 °C for 5 days to form koji. The principal function of the mould is the elaboration and release of a range of hydrolytic enzymes, including amylases, proteases, cellulases, invertases, as well as lipolytic enzymes. The koji is mixed with salt brine (23% w/v) in a ratio of 1.0:1.5 to make the salt mash or moromi, which undergoes lactic acid bacteria and yeast fermentations for at least one year at ambient temperatures during which colour and flavour develop resulting in quality soy sauce (Fig. 2.12). Under moromi fermentation conditions, the

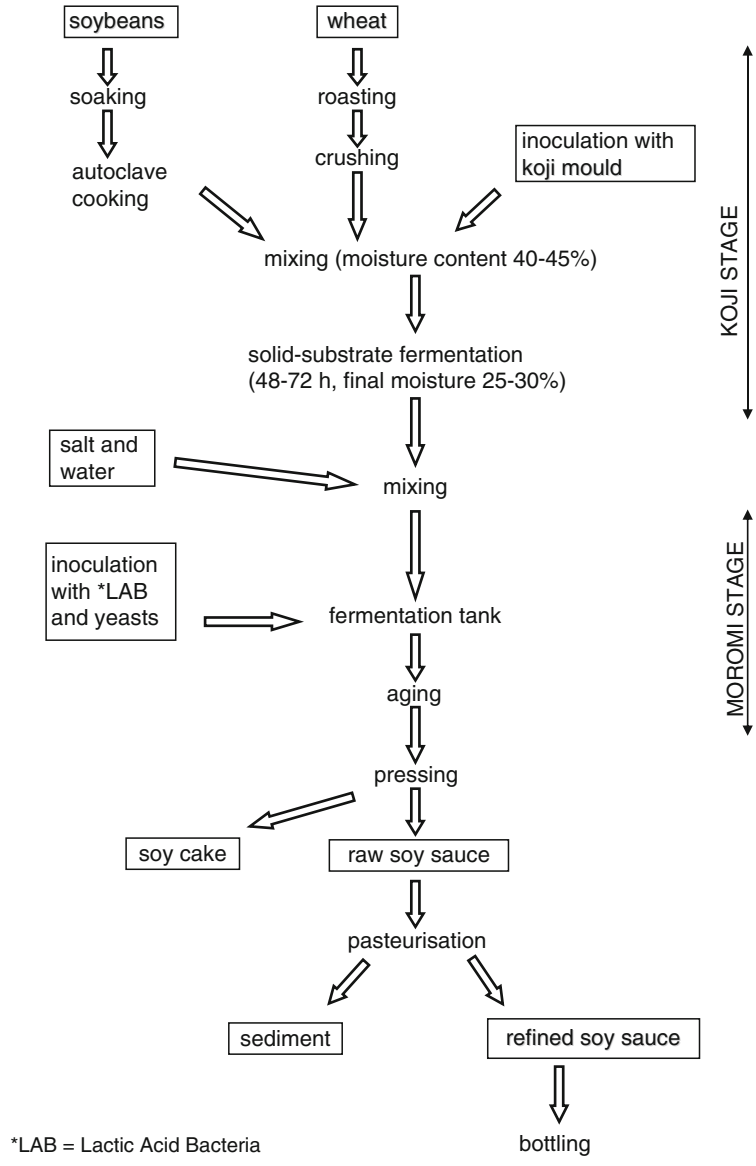

Fig. 2.12. Traditional manufacture of soy sauce

koji mould is rapidly destroyed but its extracellular enzymes continue to hydrolyse substrates, albeit slowly, in the saline environment. The strong brine creates a favourable condition for a few desirable organisms. At first *Tetragenococcus halophila* grows and produces lactic acid which lowers the pH to 5.5 or less and then acid-tolerant dominant yeast, notably *Zygosaccharomyces rouxii* grows and produces about 3% alcohol and several compounds which add characteristic aroma to soy sauce. The fermented moromi is then filtered, siphoned, drained or decanted. The raw soy sauce is then boiled or pasteurized – a process which produces not only stability, flavour and colour in the final product but also clarity, inactivation of residual enzymes and resistance to spoilage yeasts.

C. Fungi Involved, Their Relevant Properties and Implications for Health

The most important function of the koji mould in soy sauce fermentation is the production of extracellular and exocellular enzymes, in particular, carbohydrase and protease complexes. The yellow-green Aspergilli (koji moulds) used in Asian soy bean fermentations have long been a subject of much debate amongst fungal taxonomists. In *Aspergillus* classification (Table 2.6), the koji moulds belong to the Section Flavi Gains (= *Aspergillus flavus* group Raper and Fennell; Samson 1993b; Samson et al. 2004). The true koji moulds comprise *Aspergillus oryzae* (Raper and Fennell 1965), *Aspergillus sojae* (Murakami et al. 1982) and *Aspergillus tamarii* Kita (Raper and Fennell 1965), the distinction between them being based on conidial head colour, growth at 37 °C and dimensions of conidiophores, vesicles and conidia. The selection of strains of *Aspergillus* used in soy sauce fermentation in Japan and the Far East is based, among others, on ability to sporulate for the preparation of seed starter, colour and flavour of the final product, enzyme production, inability to produce toxins and length of stalk.

The two main groups of enzymes produced by *A. oryzae* during koji fermentation are carbohydrases (α-amylases, amyloglucosidase, maltase, sucrase, pectinase, β-galactosidase, cellulase, hemicellulase, pentosan-degrading enzymes) and proteinases, although lipase activity has also been reported (Aidoo et al. 1994; Chou and Rwan 1995). These major enzymes hydrolyse carbohydrates and proteins to sugars and amino acids and low-molecular-weight peptides respectively. These soluble products are essential for the moromi fermentation.

Zygosaccharomyces rouxii is the dominant moromi yeast which grows to produce 3% alcohol and several compounds which add characteristic aromas to soy sauce, although other yeasts such as *Candida versatilis* and *C. etchellsii* produce phenolic compounds, 4-ethylguaiacol and 4-ethylphenol, which contribute to the soy sauce aroma. A review on the diversity and functionality of yeasts in soy sauce and other yeast fermented Asian foods has been published (Aidoo et al. 2006).

The discovery of aflatoxins in the 1960s led to an extensive examination of koji moulds for toxin production. Although no aflatoxins have been demonstrated in *A. oryzae, A. sojae* or *A. tamarii*, it has been reported that all can produce other mycotoxins such as aspergillic acid, cyclopiazonic acid, kojic acid under specific environmental conditions (Table 2.7; Frisvad 1986; Trucksess et al. 1987). Aflatoxigenic fungi do not appear to occur in regions with a mean temperature below 16 °C and this therefore may explain why traditional Japanese fermented foods such as soy sauce, miso (fermented soybean paste), sake, katsuo-bushi (dried bonito) and others do not contain aflatoxins (Tanaka 2002). Although other mycotoxins including sterigmatocysin (precursor of aflatoxins) and ochratotoxin A could not be detected, the effects of other mycotoxins associated with fermented foods and herbal plant foods on human epithelial cell lines have been reported (Manabe 2001; Calvert et al. 2005; Mohd Fuat et al. 2006).

Table 2.6. Accepted *Aspergillus* species Section Flavi (Samson 1993b)

Species	Author
A. avenaceus	Smith
A. clavato-flavus	Raper and Fennell
A. flavus	Link
A. leporis	
A. nomius	Kurtzman et al.
A. oryzae (Ah16)	Cohn
A. parasiticus	Speare
A. sojae	Sakaguchi and Yamada
A. subolivaceus	Raper and Fennell
A. tamarii	Kita (= *A. flavo-furcatis* Batista and Maia)
A. zonatus	(Kwon and Fennell) Raper and Fennell

Table 2.7. Toxins reported to be produced by koji moulds (Frisvad 1986; Trucksess et al. 1987)

Mould	Mycotoxin
Aspergillus oryzae	Cyclopiazonic acid, kojic acid, maltoryzine, β-nitropropionic acid
A. sojae	Aspergillic acid, kojic acid
A. tamarii	Kojic acid

Kataoka (2005) reported that flavones from Japanese-style soy sauce had beneficial health properties and also antibacterial activity against the most common pathogenic organisms. In spite of its high salt content, soy sauce in the diet could protect against cardiovascular diseases (CVD) because of the levels of antioxidants. McVeigh et al. (2006) reported that soy proteins, regardless of isoflavone content, were responsible for cardiovascular benefits. Ørgaard and Jensen (2008) have published a review of the most recent research findings on the potential benefits of soy isoflavones on obesity in humans. Murooka and Yamshita (2008) also reviewed the health benefits of traditional fermented products, particularly shoyu, miso, tempe, natto and black rice vinegar, produced and sold in Japan.

D. Industrial Aspects

About 4×10^6 t of soybeans are used annually in Japan and about 1×10^6 t go into food consumption. In 1964 there were over 4000 producers of soy sauce or shoyu as local industry in Japan. Now, there are about 1600 soy sauce producers, the majority belonging to the Japan Federation of Soy Sauce Manufacturers Cooperatives (JFSSMC). Annual soy sauce consumption in Japan is 920 000 kl and it is estimated that United States consumption of Japanese-style soy sauce is about 130 000 kl. There are four Japanese-style soy sauce breweries in the US and 13 in Asia, Europe and elsewhere, with a cumulative annual production except Japan of 210 000 kl, which is increasing yearly (JFSSMC 2009). According to JFSSMC, the consumption of soy sauce in Japan has dropped in recent years due mainly to decreasing population and attendant ageing society. In spite of this, Japan's exports of soy sauce to 60 countries have increased in recent years. One of the major producers of shoyu, Kikkoman, started producing genuine fermented shoyu in the United States. Kikkoman also produces shoyu in Singapore, Brazil, Taiwan and the Netherlands, and further shoyu plants are planned in, e.g., California (Yokotsuka and Sasaki 1998).

The large-scale industrial production of soy sauce involves five main unit operations, viz. the preparation of the raw materials, the koji process, mash or moromi production, pressing and finally refining (Fig. 2.12). The koji process for making enzymes needed in soy bean fermentation traditionally uses wooden trays which are stacked vertically with about 10 cm gaps separating them, and each tray is filled to a depth of about 5–8 cm (Yokotsuka 1983; Aidoo et al. 1984).

In the koji fermentation, it is important to: (1) maximise enzyme production, (2) prevent denaturation of the enzymes, (3) avoid presence of undesirable microorganisms and (4) minimise the utilisation of nutrients by the koji moulds. The mechanical bioreactor types of koji manufacture have been almost exclusively developed in Japan and the three major features of these bioreactors are the batch and continuous types, the rotary drum and the surface-flow system of aeration (Yokotsuka 1983; Fukushima 1998; Yokotsuka and Sasaki 1998). Package-type rotary koji-making equipment is shown in Figs. 2.13 and 2.14. These koji bioreactors now involve automated inculation of substrate, controlled mass transfer, automated heaping and turning of the fermenting mass and automated harvest of the finished koji. These industrial koji bioreactors enable excellent control of temperature and humidity (two

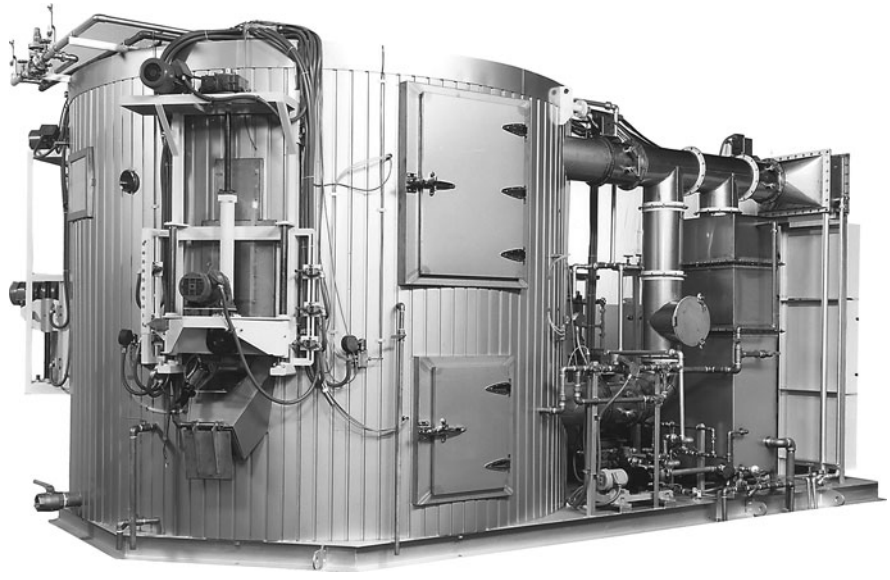

Fig. 2.13. Rotary type automatic koji making equipment. Reproduced with permission from Fujiwara Techno-Art Co. Ltd., Japan

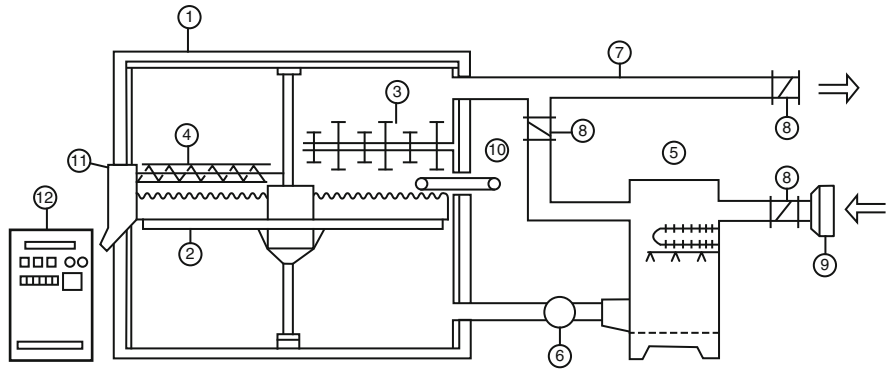

Fig. 2.14. Cross section, rotary type automatic koji making equipment. Reproduced with permission from Fujiwara Techno-Art Co. Ltd., Japan. 1. Koji room, 2. Culture bed, 3. Turning machine, 4. Heaping and discharging machine, 5. Air conditioner, 6. Blower, 7. Air duct, 8. Air damper, 9. Plate fin heater, 10. Koji material inlet, 11. Koji product outlet, 12. Control panel

important environmental conditions for mould growth), effective mass transfer and removal of inhibitory gases and volatiles and further ensure regular and complete mixing. In essence, this is the most advanced example of a solid-substrate fermentation worldwide.

The submerged moromi fermentation is carried out in wood, concrete or steel tanks with a capacity of 10–30 m^3 and, in Taiwan, the traditional ceramic pots and wooden tanks are now replaced with epoxy resin-coated cement tanks with a capacity of over 30 t. The moromi is separated by a pressing machine into raw soy sauce and cake. This process takes 2–3 days and although such mechanised systems add extra cost, the lower pressures and longer filtration times result in a loss of flavour. The raw soy sauce is then cooled, blended according to user demand, pasteurised at 70–80 °C and finally bottled.

In 1991 Kikkoman reported on the application of new biotechnology to soy sauce fermentation whereby immobilised cells in a bioreactor were used for continuous production of soy sauce of good quality. The technique involved the use of a broth culture of *A. oryzae* to hydrolyse a liquified soy bean–wheat mixture and the liquid was passed through a series of bioreactors containing immobilised enzyme, glutaminase, from *Candida famata*, immobilised cells of *Tetragenococcus halophila*, *Zygosaccharomyces rouxii* and *C. versatilis* (Hamada et al. 1991).

The use of automated machinery for soy sauce production was initiated by major Japanese shoyu manufacturers. Now the equipment has wider industrial application in the production of miso, saké, amazake, beer malt, antibiotics and other enzyme preparations.

VII. Wines

A. The Products

In principle, the term “wine” is used for products of alcoholic fermentation of fruit juices containing readily fermentable mono- and disaccharides. In this chapter however, we follow the common usage of the term wine to include Oriental rice wines and derived products. These are produced from the hydrolytic breakdown of cereal starches and other polysaccharides. In all cases, the breakdown of the carbohydrate source is primarily due to amylolytic enzymes elaborated by mycelial fungi. The wine ranges from simple Thai rice wine to highly sophisticated Japanese saké (Fig. 2.15). Rice and/or cereal wines are produced at both cottage and commercial scale in Japan,

Fig. 2.15. Saké

China, Korea, Thailand, Philippines and Burma (Campbell-Platt 1987; Cook and Campbell-Platt 1994). Wines may be distilled to obtain a liquor or spirit, for instance the famous Indonesian brem bali, an alcoholic liquor produced in Bali from the liquid portions of tapé ketan.

B. Traditional Manufacturing Process

Saké is a pale yellow rice wine of Japanese origin with an alcohol content of 15–16% or higher (Fig. 2.16). Rice, *Oryza sativa*, is polished to remove protein, lipids and minerals which are in excess in the bran and germ, washed, steeped in water and steamed for 30–60 min, then cooled. A starter or *tane-koji* is then made by culturing *Aspergillus oryzae* on rice at 28–30 °C for 5–6 days. The rice is mixed with starter and yeast *moto* or *ragi* starter and water to form the main mash or moromi. The main fermentation is carried out in open tanks starting at a temperature of about 12 °C and increasing to temperatures not exceeding 18 °C. After 3 weeks of fermentation, the mash is pressed out, allowed to settle, filtered, pasteurised at 55–65 °C, blended, diluted with water and bottled.

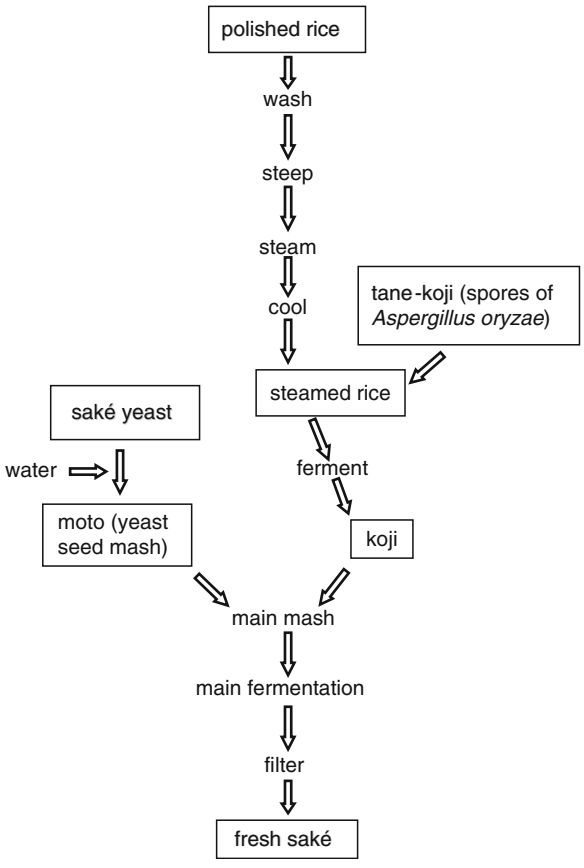

Fig. 2.16. Traditional manufacture of Saké

Malaysian rice wine or *tapai* is lighter in colour ranging from red to pink. It is made from cooked gelatinised rice and red pulverised ragi (yeast cake or jui-piang) and fermented for up to 30 days at 25 °C.

Yakju and *takju* are Korean alcoholic beverages originally made from rice, but are now made from wheat, barley, corn or millet. The starter or *nuruk* is prepared by inoculating *Aspergillus usamii* on moist wheat for up to two months. The mycoflora of nuruk includes *Rhizopus*, *A. niger* and yeasts. Nuruk serves as a source of amylolytic enzymes to saccharify starch, followed by conversion of the sugars to ethanol. In the traditional yakju process, steamed, cooled rice is mixed with nuruk and yeast inoculum is added. Takju is made by diluting fresh yakju liquor prior to filtration. Kim et al. (2004) studied the effects of yakju on human and mouse cancer cell lines and concluded that the Korean rice wine had strong anti-cancer effects as a results of certain constituents in the wine. In the Philippines, *tapuy* (Igorot ethnic group) is an acidic, but sweet alcoholic rice wine and is known by other names such as *binubudan* (Ifugao), *binuburan* (Ilocano), or *purad* (Tagalog). The Thai rice wine is a cloudy yellow liquid made from glutinous rice and, in India, *madhu*, *jnard* and *ruhi* are social drinks made from rice and produced in the Nagaland and in the eastern hill regions.

C. Fungi Involved and Their Relevant Properties

Although the starters used for hydrolysis of rice and other cereals in the production of wine are generally complex mixtures of essential and non-essential micro-organisms, the major amylolytic enzyme producers range from the mycelial fungi *Aspergillus*, *Mucor* and *Rhizopus* to *Amylomyces rouxii* (Vo et al. 1993; Park et al. 1995; Basuki et al. 1996; Steinkraus 1996).

The fungi which are involved in the production of rice wine are given in Table 2.8. Selected strains of *A. oryzae* are used in the preparation of tane-koji in the manufacture of saké. The mould produces α-amylase (liquefying amylase) and amyloglucosidase (saccharifying amylase) to hydrolyse starches to dextrin, maltose and glucose and produces acid and alkaline proteases to hydrolyse proteins to peptides and amino acids. Other essential moulds in the production of rice wine include *A. usamii* and *Amylomyces rouxii*. Moulds belonging to the genera *Mucor* and *Rhizopus* are usually the main enzyme producers for the production of rice wines in India. The main yeasts which ferment saccharified rice starch to alcohol are *Endomycopsis burtonii*, *E. fibuliger*, *Saccharomyces cerevisiae* and *Candida lactosa*, although *Endomycopsis* (*Saccharmycopsis*) *fibuligera* produces amylolytic enzymes

Table 2.8. Fungi used in the production of Asian rice wines

Country	Wine	Yeasts and moulds
China	Shaoxing	*Aspergillus oryzae*, *Rhizopus* spp., *Saccharomyces cerevisiae*
Japan	Saké	*A. oryzae*, *S. saké*, *Hansenula anomola*
Korea	Yakju, Takju	*A. oryzae*, *A. sojae*, *Rhizopus* spp., *S. cerevisiae*, *H. anomala*, *H. subpelliculosa*, *Torulopsis saké*, *T. inconspicua*, *Pichia polymorpha*
Thailand	Sato, Ou	*Mucor* spp., *Rhizopus* spp., *Candida* spp., *Saccharomyces* spp.
Indonesia	Arak, Brem Bali (distilled liquors)	*Mucor* spp., *Rhizopus* spp., *Candida* spp., *Saccharomyces* spp.
Philippines	Tapuy	*Endomycopsis fibuliger*, *Rhodotorula glutinis*, *Debaromyces hansenii*, *Candida parapsilosis*, *Trichosporon fennicum*
Malaysia	Tapai	*Amylomyces rouxii*, *Rhizopus* spp., *Endomycopsis* spp.
India	Ruhi, Madhu, Jnard	*Mucor*, *Rhizopus*

as well (Reiser and Gasperik 1995; Yip et al. 1997; Brimer et al. 1998).

Other yeast species, *Hansenula*, *Pichia* and *Torulopsis* have also been isolated from rice wine. Esters, fusel oils, acids and other compounds which contribute to flavour are also produced. Isoamyl acetate is an important flavour component in sake brewing. However various yeast mutants with improved fermentative activity and precusor of inhibitors of genes responsible for synthesis of flavour components have been reported. Studies showed that production of flavour compunds in saké brewing could be improved with the mutant strain resulting in 1.4-fold increase in isoamyl acetate. Mutant strains with enhanced fermentative activity could improve occasional 'stuck' or high ethanol fermentations which often result in low-quality saké (Watanabe 2002; Hirooka et al. 2005).

D. Industrial Aspects

The commercial production of saké in Japan employs a highly sophisticated technology in contrast to the indigenous production of rice wine in other parts of Asia. In the manufacture of saké, starch saccharification is achieved during production of koji with highly automated koji bioreactors (see Soy sauce koji). Fugiwara Techno-Art Company (Japan), a leading manufacturer of koji making and brewing equipment, has developed the sky-type continuous rice steamers which have become popular because of the quality of rice produced, low operating cost and ease of operation.

On a commercial scale, the main fermentation mash or moromi containing koji, steamed rice and water is fermented with moto in 10–20 m^3 capacity tanks each containing from 1.5–10.0 t of rice. After fermentation, which usually takes about three weeks, the mash is pressed and the saké is allowed to settle for up to 10 days. One tonne of polished rice yields about 3000 l of sake (20% ethanol, v/v) and 200–250 kg of moromi filter cake, *sake-kasu* which is used to make pickles and soups. In Korea, there are over 3000 breweries for the production of yakju and takju, with annual production in excess of 8700 t and 1 600000 t, respectively.

Saké yeast is known for its high alcohol yielding capacity. In Japan, one of the leading research institutes in saké brewing, Gekkeikan, has produced a 'super yeast', capable of fermenting pretreated cellulosic material to alcohol (Gekkeikan 2008). Super yeast was created by integrating koji mould genes that produce cellulolytic enzymes into saké yeast using cell surface engineering with the enzymes being densely displayed on the surface of the yeast. Gekkeikan hopes that such technology developed for the production of bioethanol in the Japanese sake brewing would contribute to the resolution of environmental issues.

VIII. Chinese Liquor

A. The Product

Chinese liquor or "jiu" is a collective name for a wide variety of strong alcoholic liquors, obtained by distillation of cooked sorghum that has undergone alcoholic fermentation. The starter used for the alcoholic fermentation is called "qu". In many cases, the qu is brick-shaped and because of the large size of the bricks (they can weigh 1–2 kg each), the starter is called "big qu", in Chinese "daqu". Chinese liquor is very important in Chinese culture and has been described since historic times. It plays an important role during festivities, hospitality and business events. Its strength can be around 50% v/v alcohol, although nowadays the standard tends towards the internationally usual 38–40% v/v alcohol. The flavor of Chinese liquor varies according to manufacturer, from very mild to sauce- and strong-flavoured.

B. Traditional Manufacturing Process

Figure 2.17 shows the principle of the traditional manufacturing process. There are many specific variations that are applied in commercial production; many specific details are kept confidential for competitive reasons. In principle, the process consists of the following stages.

1. Production of "daqu" starter. The ingredients wheat, barley and peas are ground, mixed and the mixture made into a stiff dough with water. The dough is filled into brick-shaped molds and pressed to obtain enough cohesion to allow the bricks to retain their shape. The bricks are stacked a few layers high in an incubator room where temperature and humidity can be controlled. During a period of several weeks, the bricks are incubated according to a defined time–temperature profile. During this period, the bricks are colonised by successive populations of fungi and bacteria. The microflora is complex and undefined. However, the strict control of ingredients formulation and processing conditions results in "typical" daqu types, which are distinguished by their colour (exterior as well as interior) and flavor. After completion of the incubation stage, the now hardened daqu bricks are stored under protective roofs for periods up to 12 months so that enough starter is available throughout the year.
2. Alcoholic fermentation. *Sorghum* grains (mostly red sorghum) are ground, moistened with some water and left overnight to allow a homogeneous distribution of moisture. The moist sorghum meal is then steam-cooked in order to gelatinize the sorghum starch. Prior to cooking the sorghum had been mixed with rice bran in order to avoid excessive stickiness of the steamed mixture. The steamed mixture is cooled to inoculation temperature and a considerable quantity (10–20% of total weight) of powdered daqu is mixed into the mixture. The inoculated sorghum is transported to the incubation room where it is filled into airtight vessels and allowed to ferment during a period of 2–3 weeks, depending on local conditions and preferences. During this period the daqu micro-organisms and their enzymes will act upon the compounds from sorghum, daqu and rice bran. Because of the high inoculation level, it is fair to state that the daqu itself also forms part of the substrate.
3. Distillation and maturation. After fermentation the vessel is emptied and its contents are transferred to a steam distiller. Here, all volatiles are steam-distilled and collected in a water-cooled condenser. The raw distillate is standardized to the desired alcoholic strength and transferred to earthenware maturation jars of 500–1000 l, and will remain there for maturation. This may take several months to years, and should result in a harmonious and stable flavor. Finally, the single or blended matured liquor is bottled in glass or traditional earthenware jars.

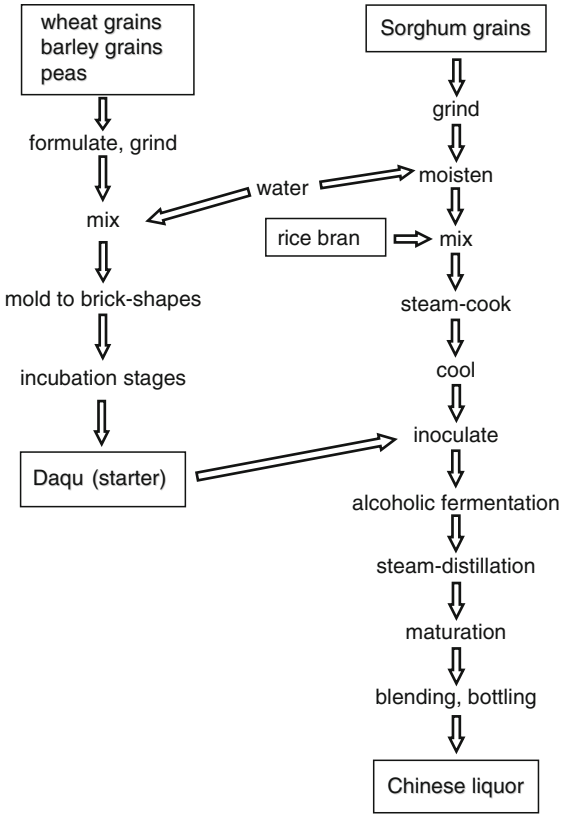

Fig. 2.17. Traditional manufacture of Chinese liquor. Outline of principle only.

C. Fungi Involved and Their Relevant Properties

The microbiology of this process should be distinguished in the microbial succession during the production of daqu and the process of alcoholic fermentation. Daqu is colonized by a complex mixed microflora of fungi and bacteria. Among the filamentous fungi, *Aspergillus*, *Mucor*, *Rhizopus*, *Monascus*, and *Trichoderma* spp. were encountered in Moutai daqu (Wang et al. 2008a), and *Absidia*, *Rhizopus*, *Rhizomucor*, *Aspergillus* and *Emericella* spp. in Shaoxing qu (Xie et al. 2007). Predominating yeasts were identified as *Saccharomyces*, *Hansenula*, *Candida*, *Pichia*, and *Torulaspora* spp. in Moutai daqu (Wang et al. 2008a) and *Saccharomyces*, *Candida*, *Clavispora*, and *Pichia* in Shaoxing qu (Xie et al. 2007). Predominating bacteria were described as *Bacillus*, *Acetobacter*, *Lactobacillus* and *Clostridium* spp. (Wang et al. 2008b). There is a recent research development in daqu microbiology because scientific data on its composition are still quite scarce. Nevertheless, such data are essential to further development of well controlled and

defined products. Concerning the functionality of the groups of micro-organisms, it has been proposed that bacteria are important sources of proteases and amylases to digest starch and to provide butyric acid for fermentation and flavour development; yeasts are responsible for the alcoholic fermentation, and production of volatile ester flavours; and moulds would contribute to degradation of polymeric substrates, formation of esters and other volatiles.

The alcoholic fermentation could be expected to be dominated by yeasts. Indeed, *Issatchenkia* and *Saccharomyces* spp. have been encountered (Zhang et al. 2007). After one week of fermentation, *Lactobacillus acetotolerans* (Zhang et al. 2005; Wang et al. 2008b) was reported as a predominant organism. Other bacterial species were reported and these may play a distinctive role in typical flavour formation. It was suggested that *Bacillus*, *Bacteroides* and *Clostridia* may contribute to strong aromas whereas *Bacillus*, Flavobacteria and Gammaprotobacteria would give rise to sauce-flavoured liquor (Wang et al. 2008b).

The flavours in Chinese liquors comprise a wide range of volatiles such as the fruity and floral ethyl and butyl esters of butanoic, pentanoic, hexanoic and octanoic acids, the sweaty hexanoic acid and the nutty or roasted notes of pyrazines such as 2,5-di-methyl-3-ethylpyrazine (Fan and Qian 2005, 2006a). A range of 27 alkyl and acetylpyrazines was identified (Fan et al. 2007); clove, smoky and goaty flavour notes were ascribed to 4-ethylguaiacol, 4-methylguaiacol and 4-ethylphenol (Fan and Qian 2006b).

D. Industrial Aspects

The annual production of Chinese liquors was estimated at 5×10^6 t in 2007. This quantity is based on data from some large-scale companies; in addition, there is an unknown number of smaller-scale semi-artisanal workshops where liquors are produced. Very little is known about the processes carried out, the micro-organisms involved and the composition of the liquors produced. In view of the importance of these products, it may be expected that further research will yield meaningful data to enable industrial development of safe and unique products.

IX. Conclusions

Fungal food fermentations are practised in nearly all the continents, but those originating in Asia are of very ancient lineage and also present the greatest variety of products. Some of the traditional production processes developed from low and intermediate biotechnology into highly sophisticated and automated systems.

Examples of some well known fungal fermented foods are presented in this chapter and traditional manufacturing processes, biochemical changes, the essential fungi involved in the fermentation process and industrialisation, process control and innovations are also presented. However, it is recognised that there are still numerous lesser-known or less developed fermented products in the continent that are not covered. One of the biggest hurdles in transfer of technology is the transfer and utilisation of information from the laboratory research base to pilot and commercial scale and the Oriental fermented food industry has been reasonably (or highly!) successful in this respect.

Problems, advantages and future developments associated with fungal fermented foods may be summarised as follows:

- Some of the constraints of fungal food fermentations are: (1) optimisation of the fermentation process; the majority of these processes are based on solid substrate fermentations (SSF) and one of the problems with SSF is limited heat and mass transfer, (2) the types of organisms are usually limited to those that can grow at reduced moisture levels, (3) using monitoring devices to determine moisture, pH, etc. becomes a problem and (4) the spore inoculum needed may be quite large.
- There is a greater need to monitor possible production of toxic fungal metabolites, in particular, mycotoxins which may be formed during fermentation.
- The advantages of fungal food fermentations include: (1) bioenrichment of food through a diversity of aromas, flavours and texture, (2) bioenrichment of foods with vitamins, proteins, amino acids and essential fatty acids, (3) preservation of food through production of alcohols, acids and esters, (4) production of food colours, (5) improved digestibility of food, (6) production of edible fungal biomass and single-cell protein.
- The fermentation systems are usually simple, requiring less space in relation to yield of product because less water is used and the substrate is concentrated. The desired product may be

readily extracted by addition of solvent. Thus, neutraceuticals and novel compounds may be produced from fermented foods by commercial companies. Also the ingredients are relatively simple, for instance whole grain with water sufficient to moisten the substrate.

Future developments should focus on improving process control, the use of immobilised systems and/or enzymes and use of genetically modified organisms to maximise productivity without health risks.

Extensive research and developmental studies in isolation and/or selection of desirable fungi and carefully controlled fermentation processes of some Asian fungal fermented foods have led to products of high quality, improved digestibility, extended shelf life, maximum utilisation of raw materials, exclusion and/or reduction of fungal toxins and improved nutritional values. A plethora of published scientific data on Asian fungal food fermentations, viz. natural or spontaneous fermentation (e.g., idli), starter-mediated single-stage fermentation (e.g., tempe kedele) and multi-stage fermentation (e.g., soy sauce), is a testimony of the extent to which research and development have been devoted to such industry. It is noteworthy that industrialisation of the traditional methods used in the production of fungal fermented foods has led to improved and highly acceptable products without change in flavour, texture, colour, aroma or fragrance of the products. However there are still many fungal fermentation processes in Asia that require improvement in substrate preparation and utilisation, process control, product yield and hygiene standards, particularly at a small to medium or cottage scale.

We are of the opinion that the introduction of some of these fungal fermented foods (tempe, soy sauce, saké) into foreign markets, particularly in Europe, and in both North and South America is essential as demand for healthy foods, naturally fermented products, protein-rich meat substitutes, exotic foods of plant origin, is increasing world-wide.

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