The colonizing fungus as a food provider

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Chapter 17

The colonizing fungus as a food provider

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INTRODUCTION

Filamentous fungi colonize food ingredients, penetrating into them, releasing a variety of enzymes, raising metabolites and reaction products and increasing their biomass. In many cases, this sequence of events is considered as spoilage (Pitt and Hocking, 1985) because of off-odours, unwanted discolourations, taste defects, and toxicity. Interestingly, however, mankind experienced that in certain situations, colonizing filamentous fungi (moulds in short) bring about desirable changes in foods, and termed them fermentations (Fukushima, 1985). Considering the chiefly aerobic metabolism of moulds, the usage of the term fermentations - indicating the anaerobic mechanism of energy generation - is incorrect by definition. Nevertheless the term fungal food fermentation is widely accepted in the sense of bioprocesses resulting in improvements of quality.

Whereas the origin of fungal food fermentation is the Orient, some specific processes have developed in Europe. Increasing international travel and trade have been the vehicle for the worldwide distribution of some of the derived products.

In principle, the birth of fungal food fermentations must have been from "spoilage"; from an ecological point-of-view it may thus be expected that the concept of "spoilage associations" (Dalgaard *et al.*, 2003) also applies to fungal food fermentations. This implies ecological niches consisting of suitable substrate, microbial competition, and favourable envi ronmental conditions. It therefore seems quite logical to encounter moulds with optimum growth temperature (T_{opt}) of 20-30 °C in the moderate climates, and those with T_{opt} of 30-40 °C in the humid tropics.

Table 1 presents an overview of selected filamentous fungi, the fermented foods in which they feature, their role in quality improvement, and some recent literature references. In the following sections these cases will be discussed in some detail, with a focus on the scientific questions of recent interest.

Zygomycetes

Among the Zygomycetes, mainly the order of Mucorales is of relevance for this chapter. Four genera, i.e., *Actinomucor*, *Amylomyces*, *Mucor*, and *Rhizopus* are of functional importance in a diversity of Oriental fungal fermented food products. The genus *Amylomyces* is considered by some (R. A. Samson, personal communication) as a domesticated form of *Rhizopus*. *Amylomyces rouxii* could not be discriminated from *Rhizopus oryzae* on the basis of 18S-28S rRNA because the amplified sequences were identical (Abe *et al*., 2004); *A. rouxii* could only be distinguished from *R. oryzae* because of its much higher number of chlamydospores in the aerial and substrate mycelium. On the other hand, the genotypes of *Actinomucor*, *Rhizopus* and *Mucor* were shown to be distinguishable as separate clusters (Han *et al*., 2004b).

Actinomucor elegans (Han *et al*., 2001) and *A. taiwanensis* (Chou *et al*., 1988) are used as pure culture starters in the manufacture of Chinese fu-ru or sufu (Figure 1a).

Table 1. Overview of filamentous fungi used in food fermentation

Zygomycetes	Species	Foods	Functionality	References
Actinomucor	*A. elegans, A. taiwanensis*	sufu, fu-ru (China)	texture, flavour	Chou *et al*., 1988; Han *et al*., 2001
Amylomyces	*A. rouxii*	ragi (Indonesia), marcha (India)	glucose release from starch	Tsuyoshi *et al*., 2005
Mucor	*M. circinelloides, M. rouxii, M. indicus*	ragi, marcha, tempe (Indonesia), pehtze (China)	enzymic transformation, flavour production	Pedraza-Reyes and Lopez-Romero, 1991; Agranoff and Markham, 1997
Rhizopus	*R. microsporus, R. oligosporus, R. oryzae*	koji (Japan), nuruk (Korea), chu (China), marcha, tempe	texture, enzymic transformations, vitamins	Ginting and Arcot, 2004; Nout and Kiers, 2005
Ascomycetes				
Monascus	*M. pilosus, M. purpureus, M. ruber*	angkak, red yeast rice (China)	colour, flavour, secondary metabolites	Juzlova *et al*., 1996; Akihisa *et al*., 2005
Neurospora	*N. sitophila, N. intermedia*	oncom (Indonesia)	texture, flavour, enzymic modifications	Beuchat, 1986
Deuteromycetes				
Aspergillus	*A. oryzae, A. sojae, A. niger*	soy sauce (East Asia)	carbohydrases, proteases, other lytic enzymes	Nout and Aidoo, 2002; Hanya and Nakadai, 2003
	A. glaucus, A. melleus, A. repens, A. candidus	katsuobushi (Japan)	enzymic transformation, flavour production	Campbell-Platt, 1987
Penicillium	*P. glaucum*	katsuobushi		
	P. camemberti	Camembert, Brie (France)	texture, flavour	Leclercq Perlat *et al*., 2004a
	P. nalgiovense	salami (Europe)	colour, flavour	Fink-Gremmels *et al*., 1988
	P. roqueforti	Roquefort, blue Stilton, Danablue (Europe)	colour, flavour	Gripon, 2003

The genus *Actinomucor* was described earlier (Benjamin and Hesseltine, 1957); recently *A. taiwanensis* was described as a separate species (Jong and Yuan, 1985; Chou *et al*., 1988).

The process of preparing sufu or fu-ru starts with the production of soymilk by soaking dehulled soybeans, grinding, sieving and cooking the watery extract. Next, a coagulation step is carried out, by adding salts or acid, in order to obtain a precipitate of mainly soy protein and enclosed lipids. This is collected and pressed to obtain sheets of tofu (soybean curd) of the required moisture content and firmness. After cutting the tofu into cubes (dices) these are inoculated with a suspension of mould spores. Incubation during a few days usually results in a luxuriant mycelial development giving the dices a fluffy appearance. These are now called pehtze, and after flattening the mycelium as a protective skin on the cubes, pehtze is submerged in a maturation mix and left during several months to develop into a flavoursome, soft, cheese-like product. The main functions of the maturation mix are preservation, flavouring and colouring.

Figure 1. Fungal fermented foods (a: sufu; b: men; c: tempe; d: oncom; e: soy sauce; f: Camembert; g: blue-veined cheese).

The preservation is achieved by a combination of salt and alcohol (rice wine may be used), whereas ang-kak (see below) and other ingredients impart specific flavour and colour to the product (Su, 1986; Han *et al*., 2001). The major function of the moulds in this process is the formation of the protective layer of mycelial biomass surrounding the pehtze cubes, but most importantly, to release several enzymes (Han *et al*., 2003a) that are responsible for the partial degradation of the protein (Lu *et al*., 1996), fibre and lipid fractions in pehtze during the maturation. This degradation results in a softening of the texture, solubilization of the dry matter and accumulation of flavour enhancing compounds, such as glycine (Ma *et al*., 2004) and glutamic acid (Liu and Chou, 1994; Han *et al*., 2004c). In view of the optimization of industrial sufu-making processes, the response of *A. elegans* to temperature, salt (Han *et al*., 2003b) and alcohol has been studied. The higher the salt and alcohol levels during the maturation, the slower the enzymatic reactions take place and thus the more maturation time and costs are involved. With the objective of accelerating the maturation, the salt and alcohol levels could be lowered. This is feasible to a level of about 10% alcohol (Chou and Hwan, 1994) in combination with 6% salt; at lower levels the product is susceptible to spoilage by lactic acid bacteria (Han *et al*., 2004a), as well as survival by pathogens (Shi and Fung, 2000) and enterotoxin formation by *Staphylococcus aureus* (Han *et al*., 2005).

Amylomyces rouxii is a rather peculiar mould, described by Ellis *et al*. (1976). It finds its importance as a functional component of Oriental traditional starters for alcoholic fermentations. Its main properties of technological importance are the production of amyloglucosidase (Wang *et al*., 1984), its ability to colonize uncooked rice dough, and its restricted sporulation. Oriental traditional starters for alcoholic fermentations (Hesseltine *et al*., 1988; Leistner, 1990) are often prepared by mixing powdered milled rice with water to a dough, with the addition of herbs and spices. The dough is portioned into small flattened balls or tablets (Figure 1b) which are dusted with powdered starter from an earlier batch. These inoculated tablets are kept in a warm room during some days where they also gradually dehydrate. The final product is a hard, dry tablet which can be conveniently packed and transported for marketing. The microflora of such starters — Indonesian ragi, Vietnamese men, Indian marcha and numerous others — has been investigated by several researchers (Hesseltine *et al*., 1988; Tamang and Sarkar, 1995; Tsuyoshi *et al*., 2005); in principle, three categories of microorganisms can be encountered, viz. starch-degrading fungi (mainly *A. rouxii*, but some starters contain amylolytic yeasts such as *Endomycopsis fibuliger*), alcohol-tolerant yeasts (*Saccharomyces cerevisiae* in particular) and non-functional contaminants such as lactic acid bacteria, *Bacillus* spp., etc. When used in rice wine preparation, rice (either glutinous or non-sticky rice) is soaked, cooked, and the cooled mass is dusted with powdered starter tablets (the level of inoculation needs to be experienced first and depends on the composition and viability of the microflora within the tablet). During incubation at a warm place, a significant liquefaction takes place which results from the degradation of the gelatinized starch. When, after a few days, it is considered that sufficient glucose has been produced to start the alcoholic fermentation, more water is added to submerge the moulded rice, turning the aerobic incubation into anaerobic conditions. This will inhibit the formation of mould biomass and favour yeast fermentation; the yeast being present from the start will ferment as soon as glucose is released, but the most effective alcohol accumulation occurs during this submerged fermentation phase. When the fermentation has stopped, the residual rice and yeast is left to sediment and the supernatant wine is decanted. According to local preference, the wine can be clarified further by filtration, and its shelf-life can be prolonged by fortification, i.e., adding some distilled (rice) alcohol (Kozaki and Uchimura, 1990; Rhee *et al*., 2003). At a small scale of production, hardly any control of the process is possible and therefore it is not suprising that the yields of glucose and ethanol from rice, as well as important traits such as colour, volatile flavour and taste are rather unpredictable. In traditional rice wine fermentation starters, bacteria — including low numbers (2.6-4.2 log cfu g^{-1}) of lactic acid bacteria — are also present. The fact that the pH of good quality rice wine usually is in the pH range 3.9-4.2 does not necessarily imply the functional contribution of lactic acid bacteria (LAB) to its quality. The pH range indicated above is also found in pure culture experiments with moulds and yeasts and results from the formation of lactic acid — for example by *A. rouxii* (Saito *et al*., 2004) — and other acidic co-metabolites (by the yeast). If the number of LAB would become higher, the quality of the wine is likely to suffer because of acidity (in poor quality wines we measure pH as low as 3.2). Based on the above, LAB should not be considered as functional flora in rice wine starters, but rather as potential spoilage microorganisms. At an industrial scale, most rice wines (Chinese Shaohing, Japanese Saké, Korean Yakju) are produced with *Aspergillus oryzae* (see below); from 1000 kg polished rice, 3000 litres Saké of 20% v/v ethanol are obtained, representing an almost 100% yield (Nout and Aidoo, 2002). The manufacture of rice wine starters and rice wine constitute two different businesses. There is an increasing demand for the development of defined starters that combine maximum saccharification and alcohol productivity, instead of the traditional tablets of unknown composition and activity.

Mucor spp. such as *M. circinelloides*, *M. indicus*, and *M. rouxii* are encountered in a diversity of fungal fermented food products of the Orient (Tamang and Sarkar, 1995; Agranoff and Markham, 1997; Han *et al*., 2004b), including starter tablets as well as tempe, a fermented soybean food (see below). *Mucor* spp. grow rapidly and release a range of enzymes including amyloglucosidase, lipases (Chou *et al*., 1988), proteases (Han *et al*., 2003a), and carbohydrases (Pedraza-Reyes and Lopez-Romero, 1991). Whereas these are valuable properties that may contribute to the evolution of fermented foods, it appears that in fermentations where *Rhizopus* or *Amylomyces* are present as well, *Mucor* spp. are not the prime movers of the fermentation. They may, however, contribute in other ways, such as formation of certain flavour compounds or fatty acids (Oxlade, 1990; Agranoff and Markham, 1997). In a comparison of *Amylomyces rouxii* and *Mucor circinelloides*, it was found that the latter accumulated glucose less efficiently from rice starch; this was not related to glucoamylase activity but rather to its profuse biomass formation (Dung, 2004).

Rhizopus spp. (Schipper and Stalpers, 1984) of importance in food fermentation are *R. microsporus* and *R. oryzae*. The latter is mesophilic, forms a variety of enzymes particularly starch degrading enzymes and is encountered in a diversity of amylolytic starters for alcoholic fermentations such as koji, nuruk, chu and murcha (Tamang *et al*., 1996; Nout and Aidoo, 2002; Shrestha *et al*., 2002); its glucoamylase gene has been brought to expression in *Saccharomyces cerevisiae* to facilitate the direct production of ethanol from raw maize starch (Shigechi *et al*., 2004). Whereas fungal starch degradation for winemaking is mainly practised in Asia, the use of *Rhizopus* spp. was described as well in a complicated process for making Parakari, an indigenous alcoholic beverage made from cassava in Guyana (Henkel, 2005). *Rhizopus* spp. can produce health-promoting unsaturated fatty acids such as gamma-linolenic acid (GLA) (Liu *et al*., 2004). *R. oryzae* is also used for soybean fermentations, e.g., in tempe manufacture. On the other hand, *R. microsporus* is thermophilic and prefers temperatures ranges from 30-40 °C. Within this species, varieties are distinguished of which *R. microsporus* var. *oligosporus* (in short: *R. oligosporus*) is best known in relation with the tempe fermentation. Tempe (Figure 1c) originates from Indonesia and is made from cooked seeds (soybeans, cereals or others) or food-processing by-products, by solid substrate fungal fermentation (Nout and Kiers, 2005). In the traditional tempe process, simple methods are employed for the inoculation of the cooked beans. In principle it is possible to use some previously made tempe as inoculum (Ko and Hesseltine, 1979); as tempe contains a considerable load of bacteria, the re-use of tempe as an inoculum incurs the risk of fermentation failure due to bacterial overgrowth. Therefore, professional tempe manufacturers use traditional mould spore concentrates (Samson, 1993). These are, for example, harvested from cooked rice that has been grown with a selected *R. oligosporus* culture, or grown on cooked soybeans between leaves of *Hibiscus tiliaceus* (the waru tree) (Nout *et al*., 1992). The latter type of starter is widely used, is made by specialized households, and can be purchased in the public markets in Indonesia. For a better control of the fermentation, pure culture spore preparations can be used. These are grown on, for example, cooked rice and stored as dehydrated powders. It was observed that a majority of the spores thus produced are in a state of exogenous dormancy. Using defined media, it was reported earlier (Medwid and Grant, 1984) that a carbon source (e.g., glucose) and nitrogen (amino acids) are necessary to initiate the formation of germ tubes. Recently, it was shown that in addition to glucose, alanine and phosphates contribute to the germ tube formation and further outgrowth of mycelial biomass (Thanh *et al*., 2005). Some of the interesting properties of *R. oligosporus* in relation to the tempe fermentation are directly linked to this biomass. The characteristic binding of the bean particles by the mycelium results in a considerable stiffness of the tempe cake. The strength of the mycelium can be measured by physical methods (Ariffin *et al*., 1994) and can be used as an index for fungal growth and quality of tempe. It has been estimated that 5.9% (dry weight basis) of tempe consists of fungal biomass (Sparringa and Owens, 1999). The production and metabolism of such considerable quantities of biomass may easily result in technological problems such as overheating and insufficient supply of oxygen. The traditional, empirical and labour-intensive, tray or bed solid-state fermentation functions well, provided that the depth of the bed and the temperature and ventilation of the environment are in balance. In larger-scale mechanized fermentations, heat and mass transfer can be controlled, especially in mixed fermentors. This has been shown convincingly in rotating drum fermentors (Oostra *et al*., 2000), cooled either by air or by spraying mist (Nagel *et al*., 2001), as well as in the agitated bed koji fermentors (Figure 2) used in Japan (Nout and Aidoo, 2002). In the case of tempe, this kind of fermentation implies a departure from the traditional brick-shaped final product, because agitated fermentation results in particulate fermentation products. Nevertheless, mechanized systems could be of interest in tempe fermentations, e.g., when producing novel nutrition ingredients such as tempe flour (Han *et al*., 1999). Another point of relevance for the acceptability of tempe concerns discolourations caused by enzymatic browning. Phenoloxidase activity, in particular from laccase, has been observed in several fungi such as *Agaricus bisporus* (Wiegant *et al*., 1992) and *Aspergillus oryzae* (Lertsiri *et al*., 2003), the activity of the latter fungus being associated with browning of fermented Thai soybean paste. Laccase has also been reported in *R. oligosporus* (McCue *et al*., 2004), but its role in tempe in relation to browning and quality acceptance has not yet been investigated. An aspect that has not yet attracted much attention is the volatile favour of tempe, in particular the "mushroomy" flavour of freshly fermented tempe. From mushroom research it has been shown that 1-octen-3-ol is one of the major volatiles responsible for the characteristic mushroom smell (Kubickova and Grosch, 1997). It would be of interest to investigate the behaviour of *Rhizopus* spp. in this respect and study the biochemical pathways, precursors and genes involved in key flavour compounds. *Rhizopus* biomass produces a diversity of carbohydrases such as polygalacturonase, endocellulase, xylanase, arabinase, beta-D-glucosidase, alpha-D-galactosidase, beta-D-xylosidase, alpha-L-arabinosidase, and alpha-D-glucosidase (Sarrette *et al*., 1992) that contribute to the degradation of dietary fibre (non-starch polysaccharides), which mainly consist of arabinogalactans, galactomannans, xylans and pectic substances (Fransen, 1999). This degradation is the cause of a gradual softening (De Reu *et al*., 1997) of the texture of the fermented product during storage. In addition, the action of proteases, particularly aspartic-(35 kD) and serine (33 kD) protease, each existing in different isoforms (Heskamp and Barz, 1998) causing enzymic protein degradation, results in a strongly improved digestibility of tempe (Kiers *et al*., 2003). Similar phenomena were observed in tempe made from chickpea (Reyes Moreno *et al*., 2004) and maize (Cuevas Rodriguez *et al*., 2004) with concomitant decreases of phytic acid and tannin levels. In addition, the tempe fermentation adds health benefits to the soybean by converting isoflavonoids such as genestein and daidzein into compounds with increased antioxidative capacity such as 3-hydroxyanthranilic acid (Jha *et al*., 1997; Matsuo *et al*., 1997; Berghofer *et al*., 1998). These have been associated with reductions of various types of degenerative diseases. The release of phenolic antioxidants by *R. oligosporus* from isoflavones in soybean (McCue and Shetty, 2003), pineapple waste (Correia *et al*., 2004b) and soy-guava waste (Correia *et al*., 2004a) was associated with the considerable β-glucosidase activity of the mould. It has been postulated that the presence of laccase could increase the formation of polymeric phenolics; the latter were shown to inhibit the growth of the peptic ulcer-associated *Helicobacter pylori* (McCue *et al*., 2004).

Several vitamins (Nout and Kiers, 2005), including folates, mainly 5-formyl-tetrahydrofolate (Ginting and Arcot, 2004), are synthesized during the tempe fermentation. Although a variety of desirable modifications are ascribed to *Rhizopus* spp., there is always a need to ascertain safety of the fermented foods obtained. It was reported that whereas *R. microsporus* can form rhizoxins and rhizonins, the strains of *R. oligosporus* and *R. chinensis* investigated did not produce any of these pharmaceutically active (rhizoxins) or highly toxic (rhizonins A and B) metabolites (Jennessen *et al*., 2005).

Ascomycetes

In relation with food fermentations, the most significant ascomycetes are the yeasts, especially *Saccharomyces cerevisiae*. There are, however, also a few filamentous fungi that are classified are Ascomycetes, and that have some very interesting properties which make their fermentation products quite appetizing!

The first example is the genus *Neurospora*, with *N. sitophila* and *N. intermedia*. These prefer temperatures of 25-35 °C and produce a very rapidly growing mycelium with large numbers of spores that are very easily detached and spread into the environment. Because of this property, they can spread in laboratory collections and cause havoc! They also can lead to spoilage in bakeries when they contaminate slicing machines. The fermented food of relevance here is oncom (ontjom) (Figure 1d), originating in West-Java and made from peanut press cake, a by-product of peanut oil pressing, by soaking one day, mixing with starchy ingredients such as cassava residues, steaming for about 1 hour, cooling and inoculating with some pre-grown fungal mycelium on the same material. The inoculated dough is shaped in flat rectangular boxes (moulds) obtaining brick-shaped pieces that are covered in banana leaves and incubated during a few days at ambient temperatures (25-30 °C) (Beuchat, 1986). Two types of oncom are distinguished, namely oncom hitam (black oncom) and oncom merah (red oncom) which contain different mycoflora. The yellow-red type contains mainly *Neurospora*, whereas the black oncom contains significant amounts of *Rhizopus* spp. Whereas the black colour of *Rhizopus* sporangiospores is caused by melanoids, several pigments including carotenoids, mainly β-carotene, form the basis of the orange-yellow colour of *Neurospora* (de Fabo *et al*., 1976).

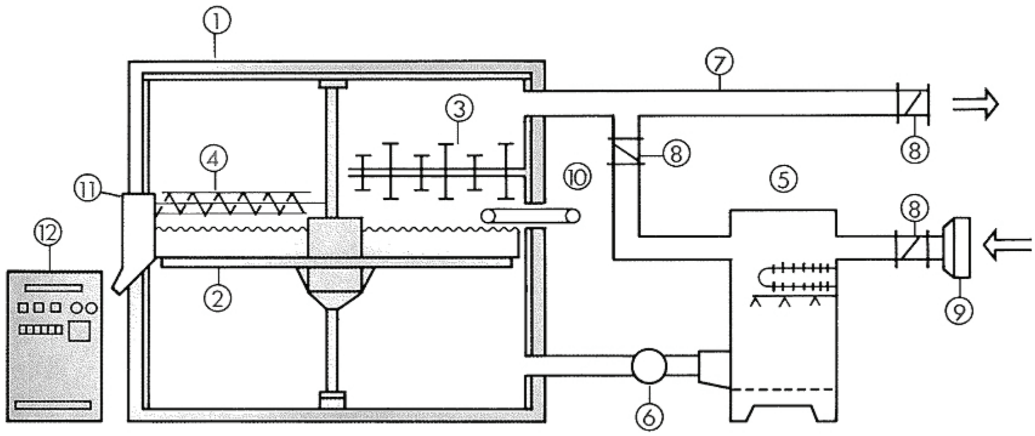

Figure 2. Schematic view of an agitated koji solid-state fermentor.

The flavour of oncom has been described as fruity and somewhat alcoholic; after frying, mince-meat or almond flavours were observed. The enzymic activities (proteases, lipases) contribute to a considerable increase in free fatty acids, and degradation of proteins. Although this does not result in improved protein efficiency ratios, the protein digestibility is improved (Beuchat, 1986) which is of importance for consumers with digestive disorders. Recently, experiments on "oncom-miso" made from soybeans and okara (soymilk extraction residue) demonstrated increased antioxidative and antimutagenic activity, associated with the enzymic release of isoflavone-aglycones (Matsuo, 2004). In contrast with the use of spore-based starters of *Rhizopus*, for example, tempe inoculation, starters for oncom are propagated and maintained by vegetative growth, in a kind of fed-batch solid-state fermentation. Through a moist mixture of peanut-presscake and cassava offal (fibrous residue of cassava starch extraction process), previously overgrown mixture is mixed and incubated. This product will constitute the starter for the next fermentation batch. Although very little controlled experimentation has been done on this fermentation it is presumed that the method of vegetative propagation is needed because the *Neurospora* spores either have limited viability when stored in a dehydrated form, or have a restricted germination ability.

Monascus (*M. ruber*, *M. pilosus* and *M. purpureus*) is of special interest because of its production of secondary metabolites (Figure 3). Traditionally this organism is used in the production of Chinese "red kojic rice," also referred to as "red-mould rice" and "red-yeast rice." Interestingly, this product has been known in the scientific literature as ang-kak or angka, but in mainland China this name is hardly known. Traditionally, polished rice is soaked overnight, cooked or steamed, cooled and inoculated with spores of *Monascus* spp. Solid-substrate fermentation during approximately one week allows the mould to grow and produce its pigments. The finished fermented product has an attractive red-purple colour and is used as a biocolouring for red sufu (fu-ru), distilled alcoholic beverages, and ceremonial products. Major azaphilone pigments include the orange pigments rubropunctatin and monascorubrin, purple pigments rubropunctamin and monascorubramin, and the yellow pigments ankaflavin and monascin (Pastrana *et al*., 1995; Akihisa *et al*., 2005). They are heat-stable over a wide pH-range, and thus of interest as "bio-colorants" in foods. In addition, several other secondary metabolites have been identified such as the furanoisophtalides xanthomonasin A and B, the amino acids (+) and (-) monascumic acid (Akihisa *et al*., 2005), monascusone A and B (Jongrungruangchok *et al*., 2004), monacolins (Juzlova *et al*., 1996) and γ-aminobutyric acid (GABA) (Wang *et al*., 2003). The flavour of red kojic rice is pleasant: the volatile metabolites (Juzlova *et al*., 1998) included alcohols, aldehydes, ketones, esters and terpenoid compounds. It was reported earlier (Peters *et al*., 1993) 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. Recently, major flavour compounds were identified as 3-methyl-1-butanol, ethanol, ethyl acetate, 2-methyl-1-propanol, ethyl butanoate and 3-methylbutyl acetate (Chung *et al*., 2004). Only after complete detoxification, saccharides were assimilated for fungal metabolism. These properties are of importance for controlled production of singular flavour components. Industrial production of the pigments might be more efficient in liquid submerged fermentations rather than in solid-state fermentations; it was shown that ratios of carbon and nitrogen in liquid media determine the production of specific pigments.

Of recent interest are the health promoting effects of angkak. It was reported (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. Cholesterol lowering ability (Liu *et al*., 2005) of red rice was ascribed to monacolin K (Wang *et al*., 2003).

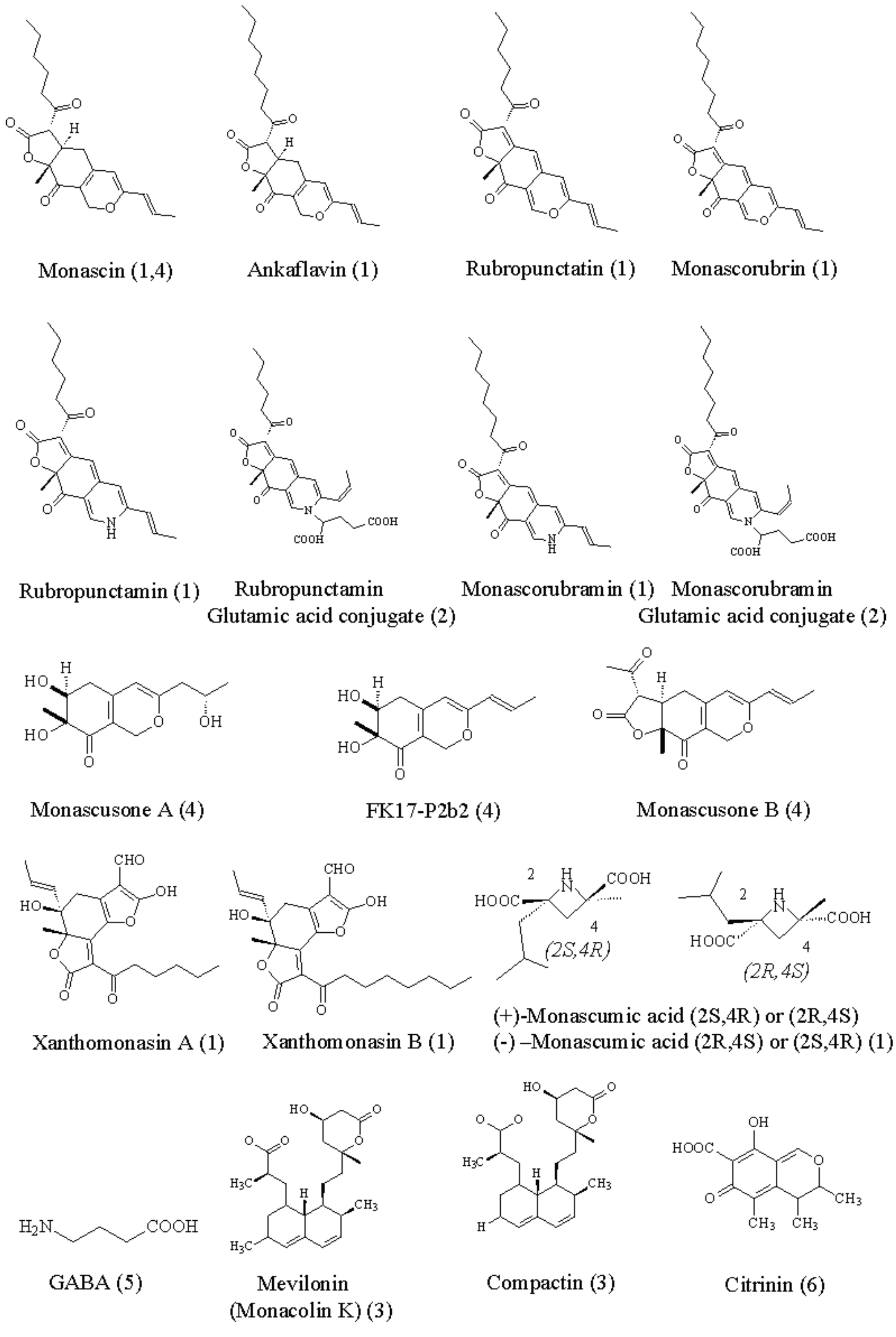

Figure 3. Secondary metabolites of *Monascus* spp. Compiled from literature references. 1: Akihisa *et al*. (2005); 2: Blanc *et al*. (1994); 3: Juzlova *et al*. (1996); 4: Jongrungruangchok *et al*. (2004); 5: Wang *et al*. (2003); 6: Liu *et al*. (2005).

Mevinolin (= monacolin K), compactin and derivates such as pravastatin, and simvastatin are inhibitors of 5-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase — a liver enzyme involved in cholesterol production — and can prevent hyperlipidemia (Juzlova *et al*., 1996; Yang *et al*., 2005); for human application a daily dose of 5 mg monacolin K has been recommended (Yang *et al*., 2005). Whereas animal tests with monacolin K did not reveal toxicity as such, a significant transient reduction of cardiac and liver ubiquinone (Coenzyme Q10) levels was reported (Yang *et al*., 2005) which may have negative effects in the long run if such products are taken as a regular part of the diet. GABA has a hypotensive effect (Wang *et al*., 2003), and red rice was reported to have anti-inflammatory effects, while it induced antigens (Akihisa *et al*., 2005) and decreased adipogenic transcription factors (Jeon *et al*., 2004). Monascusone A had no antimicrobial, or cytotoxic effects (Jongrungruangchok *et al*., 2004); the toxicology of red rice still needs further clarification. For example, it was observed that in certain fermentation conditions the mycotoxin citrinin may be produced. The risk of mycotoxin formation might seriously jeopardize the use of liquid fermentation-derived *Monascus* pigments as GRAS ingredients. Obviously it is of interest to analyze the traditional red kojic rice from China for the presence of citrinin; we analyzed a limited number of samples from Guang dong, Jiangsu, Hunan, Fujian and Beijing and did not find detectable quantities (detection limit 1 ppb) of citrinin (Han, 2003). Other investigations revealed low levels of citrinin in lipid extracts of red rice which had very low cytotoxic effects (Liu *et al*., 2005); levels of citrinin in experimental fermentations could be reduced under optimized fermentation conditions (Wang *et al*., 2003).

Deuteromycetes

Among the Deuteromycetes, the genera *Aspergillus* and *Penicillium* play a dualistic role in food technology. Some of the species used in age-old fermentation processes appear to be closely related to proficient producers of highly toxic and carcinogenic mycotoxins. No wonder why there is so much interest in the aspect of safety of *Aspergillus* and *Penicillium*–derived fermented food products.

Aspergillus oryzae and *A. sojae* are typical industrial moulds that have been used for centuries in the production of koji for the manufacture of soy sauce and miso (Wood, 1982). Whereas DNA fingerprints of *A. oryzae* isolates did not match those of *A. flavus*, *A. parasiticus* or *A. sojae* (Wicklow *et al*., 2002), all *A. sojae* strains had identical DNA fingerprints and were considered having originated from a common ancestral clonal population, a domesticated form of *A. parasiticus* (Wicklow *et al*., 2002). *A. sojae* is incapable of forming aflatoxins. AFLR (aflatoxin pathway-specific regulatory gene) was found to be impaired in its ability to activate transcription of aflatoxin biosynthetic genes, as well as being unable to interact with AFLJ (co-activator gene) (Chang, 2004). In traditional products such as Chinese and Japanese koji and soy sauce (Blesa *et al*., 2004), and Korean Meju, Doenjang and fermented barley (Yang *et al*., 2004) the mycotoxins ochratoxin and aflatoxins, respectively, could not be detected. Nevertheless, in some Meju and barley samples the presence of aflatoxigenic moulds were detected using multiplex PCR targeted towards 3 genes involved in aflatoxin biosynthesis (Yang *et al*., 2004). This indicates that even though non-starter aflatoxigenic strains may be present as chance contaminants, there is little chance that these will produce aflatoxins in the fermented product. This may be caused by the food environment, or by microbial competition (Ehrlich *et al*., 1985; Nout, 1989). Koji is made by soaking soy beans, *Glycine max,* in water, boiling and draining and mixing with ground or crushed roasted wheat. The mixture is placed on trays and mixed with *A. 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 (Nout and Aidoo, 2002). Its major function is comparable to that of barley malt in brewing technology, i.e., it is a rich source of lytic enzymes. In the production of soy sauce (Figure

1e), the koji is mixed with salt brine (23% w/v) in a ratio of 1:1.5 to make the salt mash or moromi, which undergoes lactic acid bacterial and yeast fermentations for at least one year at ambient temperatures during which colour and flavour develop resulting in quality soy sauce (Nout and Aidoo, 2002). During this process, the carbohydrases degrade wheat starch into fermentable sugars, and proteolytic enzymes degrade soy protein into peptides and other non-protein nitrogenous compounds, such as glutamic acid. Although the moulds are the prime movers in the conversion of soy sauce, other microorganisms, particularly osmotolerant yeasts (*Zygosaccharomyces rouxii*) and halotolerant lactic acid bacteria (*Tetragenococcus halophila)*, are involved. The combination of mixed alcoholic and lactic acid fermentations results in a highly complex mixture of taste and flavour compounds. During the final step of manufacture, the filtered sauce is pasteurized. A number of flavour compounds including alcohols, glycerol, esters, 4-hydroxy-5-methyl-3(3H)-furanone (HMMF), 4-hydroxy-2 (5)-ethyl-5(2)-methyl-3(2H)-furanone (HEMF) and 4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF), are formed. Of the furanones, HEMF produced by *Z. rouxii* and *Candida* spp. gives Japanese-type soy sauce its characteristic flavour (Hanya and Nakadai, 2003). This compound is also reported to have antitumor and antioxidative properties (Nagahara *et al*., 1992; Koga *et al*., 1998). The industrial scale production of koji is carried out in solid-state fermentations; as mentioned earlier in this chapter, solid-state fermentation presents technological challenges because of the specific behaviour of mycelial fungi. In solid-state, limitations to heat and mass transport rapidly result in gradients of temperature, gas-phase composition, nutrients, water and metabolite levels. Under conditions of decreased water activity, *A. oryzae* forms polyols such as erythritol, arabitol and mannitol as a survival strategy (Blomberg and Adler, 1992; Witteveen and Visser, 1995). Mycelial biomass growing on a surface was shown to consist of a compacted mat and a more open-structured aerial mycelium, the latter making an important contribution to the oxygen uptake and respiratory capacity of *A. oryzae* (Rahardjo *et al*., 2002). A proteomics approach was used to demonstrate that, compared with submerged (liquid) fermentation, solid-state fermentation conditions result in stronger (enolase, amylase) or exclusive (zinc-finger transcription factor, glucoamylase) expression of genes for key enzymes (Te Biesebeke *et al*., 2002). These new findings support some of the earlier observations that fungi behave in a specifically different manner when grown on solid substrates.

Aspergillus glaucus, *A. melleus*, *A. repens*, and *A. candidus* have all been reported as functional mycoflora in the fermentation of fish in Japan. The product Katsuobushi is made from Bonito or skipjack tuna flesh (*Katsuwouno pelamis*) which is cut to strips, steamed, and left to dry in a barrel for about 3 weeks. During this period, mould fermentation takes place; mould is scraped off and the fish oven-dried until hard. The presence of *A. ochraceus* (natural contaminant) may lead to mycotoxin contamination. Furthermore, the product contains high levels of histidine, which could be decarboxylated by contaminant flora and increase the risk of histamine poisoning (Campbell-Platt, 1987). This indicates that there is scope for improvement of the microbiological and chemical control of quality and safety of this type of product.

Compared with *Aspergillus*, the genus *Penicillium* is equally important for food and biotechnology. Several *Penicillium* spp. such as *P. italicum* and *P. expansum* cause extensive economic losses as toxigenic spoilage agents in the citrus fruit business. Others, such as *P. chrysogenum*, are widely exploited for their antibiotics production. Only a few species are used as food, particularly *P. camemberti*, *P. nalgiovense*, and the *P. roqueforti* group. *P. camemberti* is used in the manufacture of surface-ripened cheeses such as French Camembert and Brie (Figure 1f). This type of cheese is made by pasteurizing cows' milk, followed by addition of lactic acid bacteria starter (*Lactococcus lactis* and *Streptococcus cremoris*), rennet, and calcium chloride. After coagulation, the curd is cut, transferred to moulds, turned, rubbed with salt, and sprayed with mould spores at the surface of the young cheese. The mould fermentation takes place during 1-4 weeks at 10-

14 °C (Campbell-Platt, 1987). The microbiology of this type of product is complex: in the basic cheese, lactic acid bacteria are essential for flavour, lactose depletion and lactate production. Various yeasts and bacteria are involved in the maturation, along with *P. camemberti*. On the basis of pure culture experiments under aseptic cheesemaking conditions, it was observed that lactate serves as an important carbon source for the energy metabolism of *P. camemberti* (Adour *et al*., 2004). Whereas bacteria (*Brevibacterium linens*), yeasts (*Kluyveromyces lactis*) and other fungi (*Geotrichum candidum*) contribute to proteolysis, formation of esters (ethyl, butyl, and isoamylacetates) and other volatiles (3-methyl butanol, methyl-3-butanal, 2-octanone), *P. camemberti* has an exclusive contribution to the character of Camembert cheese (Leclercq Perlat *et al*., 2004a; Leclercq Perlat *et al*., 2004b). First, it is responsible for the mycelial surface growth. Second, it is the major proteolytic organism releasing ammonia that dominates the flavour and high pH (7.5) in late stages of maturation. Third, it produces volatiles such as styrene, 2-pentanone and 1-octen-3-ol (Husson *et al*., 2005).

P. nalgiovense is a white sporulating mould that is widely used as a surface growth on fermented meat products (traditional Salami sausages, country cured hams) (Fierro *et al*., 2004). The safety of this species could be improved if its potential to produce toxic secondary metabolites could be eliminated. There is an interest to develop genetic manipulation tools for "self-cloning," in which genes of a microorganism are cloned within the microorganism itself (Akada, 2002). Revised national guidelines for GM (genetically modified) food (Japan, April 2001) exempt self-cloned bakers' yeast from labeling or treatment as GM yeast (Akada, 2002). This is expected to facilitate the introduction of modified microorganisms on the consumer market (Fierro *et al*., 2004).

The *P. roqueforti* group can be differentiated into the three species *P. roqueforti*, *P. carneum* and *P. paneum*, using profiles of volatile metabolites (Karlshoj and Larsen, 2005). Whereas *P. roqueforti* spp. cause important economic losses by spoilage of bakery products and ensiled animal feeds, their prime feature is their colour and flavour production in "blue-veined cheeses" such as the French Roquefort, English Stilton, and Danish Danablue (Figure 1g). Roquefort is a blue-veined cheese, with strong flavour strong aroma and creamy consistency. It is prepared from ewe's milk, which is first coagulated with rennet followed by addition of *P. roqueforti* and addition of salt and storage for the maturation. Finally it is pierced just before leaving for the ripening in the cave of Roquefort.

During this maturation, both natural and provoked fermentation will take place. As Roquefort is made with raw milk, interesting natural fermentations of the milk by microflora like *Leuconosto*c or *Geotrichum* will take place, modifying the curd structure and facilitating the growth of the conidia of *P. roqueforti* that were added to the cheese.

Nowadays "Roquefort" is an AOC (Appellation d'Origine Côntrolée) and this name can only be used for a cheese made of milk coming exclusively from the south of France, transformed in a specific process, defined by the law, and ripened in the cave of the city of Roquefort. The maturation takes place in caves where the temperature is naturally regulated; however, this temperature should not be lower than -5 °C (specified by law) and must be lower than 37 °C (maximum temperature for growth of *P. roqueforti*). The holes made in the cheese will ensure a homogenous growth of the mould throughout the product (the strain needs oxygen for a good growth). After 15 days maturation in the cave, the cheese will be packed and transferred to a low temperature room where a slower maturation will continue for at least 3 months. In the blue cheese, *P. roqueforti* plays an important role in the degradation, especially by proteolysis and lipolysis (Gripon, 2003). Indeed in blue cheese, up to 10% of total amino acids are free amino acids and up to 20% of fatty acids are free fatty acids. The latter are particularly important because they will be transformed into methyl ketone, butyric and caproic acids which are responsible for the strong zesty flavour of the blue cheese.

The blue-green colour of the blue-veined cheese is provided by fungal melanins (Figure 4) in the conidia of *P. roqueforti*. These are synthesized by polyketide pathways (Wheeler and Klich, 1995), starting from malonate which is transformed into polyketides, dihydroxynaphtalene and finally into melanins.

Some *P. roqueforti* strains are able to produce mycotoxins (patulin, penicillic acid, PR toxin, roquefortine). During the past years, the risk of mycotoxin contamination of blue-veined cheeses has been the subject of investigations.

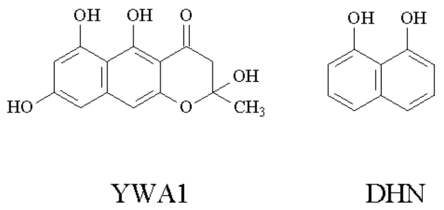

Figure 4. DHN (1,8-dihydroxynaphtalene) and YWA1, intermediates in the melanin biosynthesis of *Penicillium* spp.

Until now, only small traces of roquefortine have been found in cheese inoculated with toxigenic strains (Erdogan and Sert, 2004). It has been assumed that the conditions in cheese (nutrient composition, salt level, pH, etc.) are not favourable to mycotoxin production or stability.

FUTURE CHALLENGES

The ever-improving performance of molecular and analytical techniques offers opportunities to characterize existing food products and to support process innovations. In view of the protection of origin (AOC or certified origin of production) an unequivocal characterization of traditional fermented foods and their microflora will be required; this could be based on combinations of food compositional analysis and metabolite profiles, nucleic acid patterns such as obtained by DGGE and other methods. Innovative processes using non-traditional fermentation conditions — for example by immobilized cells, or in agitated solid-state fermentors — or using pure culture inoculation instead of multi-strain natural fermentations, may invoke changes in secondary metabolite production. In view of maintaining the character of the food, as well as safeguarding the safety of the consumer, the impact of novel processing should be investigated, understood and possibly controlled. Although the aspect of GMO (genetically modified organism) is still sensitive with the general public, safe techniques such as "self-cloning" could be helpful to obtain food-safe fungi that can be guaranteed as "mycotoxin-free." Finally, the fact that fungal fermentation technology is an important source of income in the Asian region raises the question how other regions of the world — particularly the less industrialized African countries — can benefit from this traditional know-how in the development of small- or medium-scale enterprise.

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