Microbial dynamics during barley tempeh fermentation

Xin-Mei Feng (冯新梅)

Faculty of Natural Resources and Agricultural Sciences
Department of Microbiology
Uppsala

Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2006

Acta Universitatis Agriculturae Sueciae
2006: 59

ISSN 1652-6880
ISBN 91-576-7108-7

Tryck: SLU Service/Repro, Uppsala 2006

Abstract

Feng, X.M., 2006. *Microbial dynamics during barley tempeh fermentation. Doctoral dissertation*.
ISSN 1652-6880, IBSN 91-576-7108-7

Tempeh is a traditional staple food in Indonesia mainly made from soybeans. Barley tempeh has been developed by adapting the soybean tempeh process. During soybean tempeh fermentation, the filamentous fungus *Rhizopus oligosporus* is dominant. However, other fungi and bacteria also occur, which may influence tempeh quality or even constitute a health risk. Introduction of food-grade lactic acid bacteria (LAB) and yeasts to tempeh fermentation may enhance tempeh nutritional and hygienic quality.

The abilities of LAB and yeasts to grow together with *R. oligosporus* during barley tempeh fermentation and their possible effects on tempeh quality were studied. The LAB *Lactobacillus plantarum* and *L. fermentum* and the yeasts *Saccharomyces cerevisiae, Pichia anomala and Kluyveromyces lactis* could grow during tempeh fermentation and the yeasts even during cold storage. LAB and yeasts did not negatively affect growth of *R. oligosporus* at an inoculation level of 10^4 cfu/g, respectively, but did so at higher inoculation levels. Yeasts slightly increased the ergosterol contents of final products, but did not have any obvious effects on amino acid, phytate and vitamin contents.

The most abundant volatile compounds produced by *R. oligosporus* were ethanol, 2-methyl-1-butanol and 3-methyl-1-butanol on malt extract agar and barley, and 2-butanone on soybeans. The mushroom odor compounds, 3-octanone and 1-octen-3-ol, were only present in soybean and soybean tempeh. Different strains of *R. oligosporus* produced similar volatile profiles in similar substrates. LAB did not influence the volatile production by *R. oligosporus*.

Mould growth was quantified by measurement of ergosterol content, hyphal length determination, image analysis and real-time PCR. Ergosterol content and hyphal length were applicable for the quantification of *R. oligosporus* when co-inoculated with LAB. Image analysis was developed based on changes of colour, structure and number of grains of barley tempeh. The image data correlated with the ergosterol contents and could be potentially used for online analysis of tempeh fermentation in industrial scale as no destructive sampling is required. A DNA extraction method that leaves barley grain particles almost intact was developed by adding extraction buffer with glass beads to frozen tempeh samples. Real-time PCR using species-specific primers was proven a fast and selective method for quantification of both *R. oligosporus* and yeasts during barley tempeh fermentation.

Key words: *Rhizopus oligosporus*, yeast, lactic acid bacteria (LAB), ergosterol, hyphal length, biomass, real-time PCR, volatile, image analysis

Author's address: Xin Mei Feng, Department of Microbiology, Swedish University of Agricultural Sciences, Box 7025, SE-75007 Uppsala, Sweden. Email: xinmei.feng@mikrob.slu.se

Contents

Appendix

Papers I-IV

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. **Feng, X. M**., Eriksson, A.R.B., Schnürer, J., 2005. Growth of lactic acid bacteria and *Rhizopus oligosporus* during barley tempeh fermentation. *International Journal of Food Microbiology* 104 (3), 249-256.

II. **Feng, X. M.,** Larsen, T. O., and Schnürer, J., 2006. Production of volatile compounds by *Rhizopus oligosporus* during soybean and barley tempeh fermentation. *International Journal of Food Microbiology*, doi:10.1016/j.ijfoodmicro.2006.06.025.

III. **Feng, X.M**., Passoth, V., Eklund-Jonsson, C., Alminger, M. and Schnürer, J., 2006. *Rhizopus oligosporus* and yeast co-cultivation during barley tempeh fermentation - Nutritional impact and real-time PCR quantification of fungal growth dynamics. *Food Microbiology,* doi:10.1016/j.fm.2006.06.007.

IV. **Feng, X. M.,** Olsson, J., Swanberg, M., Schnürer, J and Rönnow, D., 2006. Image analysis for supervising the barley tempeh fermentation process. (Submitted).

Papers **I**, **II** and **III** are reprinted with permission from the respective publishers.

My contribution to the papers included in this thesis has been as follows:

I. Participated in the planning of the study. Performed the laboratory work, data evaluation and contributed to the writing of the manuscript.

II. Took part in the planning of the project. Performed the laboratory work, most of the data analysis and manuscript writing.

III. Participated in the planning of the project. Performed the laboratory work, most of the data analysis and manuscript writing.

IV. Attended in the planning of the project. Performed the laboratory work, major part of the manuscript writing and part of the data analysis. The image data analysis methods and subsequent image data analysis were developed by Daniel Rönnow.

Introduction

Microbial food fermentation and world food supply

According to the USA Census Bureau's world population clock, the world population is 6.5 billion in 2006, and will reach 9 billion people in the year 2050. An increased food supply is needed to feed this growing population. To meet the demand, legumes, cereal grains and even some agricultural residues that are presently fed to animals and recovered in the form of milk, eggs, broilers, pork and beef, must be converted to human food. In terms of protein generation, meat production on average requires 11 times more energy than plant production (Pimentel & Pimentel, 2003). Thus, directly converting plant materials to human food would reduce the energy consumed by the agricultural sector. Moreover, increased consumption of vegetarian foods, such as legumes and grains, would lower food costs and promote better health (Marquart *et al.*, 2003). Even in developed western countries, more and more people are adopting vegetarian life styles or increasing the proportion of vegetables in their diet.

Plant-based foodstuffs are potentially rich in nutrients. However, the presence of anti-nutrients such as protease inhibitors and phytate, reduces the digestibility or bioavailability of proteins and minerals (Nout & Rombouts, 1990; Gibson *et al.*, 2006), and the presence of flatulence producing compounds, in particular in beans, may cause adverse effects in the gastrointestinal tract. Furthermore, it can be difficult to store plant-based foods due to spoilage or contamination by fungi that produce mycotoxins (Filtenborg *et al.*, 1996).

Microbial fermentation is considered as one of the oldest and most economical methods for food production and preservation (Buckenhüskes, 2001). The fermentation process may: 1) increase the digestibility and bioavailability of proteins, carbohydrates, lipids and minerals; 2) enhance the nutritional value such as vitamin content; 3) shorten the cooking time (e.g. soybean tempeh fermentation decreases the cooking time dramatically, from 5 to 6 h for raw soybeans to 4 to 10 min for soybean tempeh) (Steinkraus *et al.*, 1983); 4) convert animal feed into food fit for humans, such as oncom (produced from peanut press cakes with either the tempeh mould *Rhizopus oligosporus* or *Neurospora intermedia*), or tempeh Bongkrek (coconut press cakes fermented with the tempeh mould); and 5) increase the microbial safety (Steinkraus *et al.*, 1983). Therefore, microbial fermented vegetable foods are very important and staple components in the diets of millions of people, especially in developing countries where meat products are in poor supply.

Microbial food fermentation was practiced in ancient times without any knowledge of microorganisms (Beuchat, 2001). However, with modern technology, functional microorganisms in food fermentation have been identified. Food fermentation can be divided into three categories according to the types of dominant microorganisms: 1) bacteria dominate (e. g. in yoghurt, sauerkraut and natto); 2) yeasts dominate (e. g. in bread, beer and wine); 3) moulds dominate, for

example in blue and white cheeses in the Western countries, and tempeh, soy sauce, miso, sufu, Hamanatto, An-kaka, lao-chao, ogi, Sierra rice, Bongkrek, ontjom (oncom) and kenima in the Asian countries (Hachmeister & Fung, 1993), as well as papakari in Guyana in South America (Henkel, 2005). With the discovery of the fermentation microorganisms, industrial production processes were developed that simulated the traditional fermentation process. This industrialisation makes traditional food fermentations more popular and enhance their spread to new consumer groups (Beuchat, 2001).

Tempeh is one of such traditional fermented foods that has received attention from all over the world (Nout & Rombouts, 1990), and different countries make tempeh products from locally available substrates.

What is tempeh?

Tempeh production (especially soybean tempeh) has been extensively reviewed (Ko Swan & Hesseltine, 1979; Steinkraus *et al.*, 1983; Nout & Rombouts, 1990; Hachmeister & Fung, 1993; Astuti *et al.*, 2000; Nout & Kiers, 2005). In general, fresh tempeh of good quality is defined as a compact and sliceable mass of cooked particles of raw materials covered, penetrated and held together by dense non-sporulated mycelium of *Rhizopus* spp. (Nout & Rombouts, 1990). Tempeh is considered as the collective name for various plant materials fermented with the fungi belonging to the genus *Rhizopus*. The most important characteristics of tempeh fermentation are that the key microorganism belongs to the genus *Rhizopus* and that the final products are mycelial-knitted compact cakes (Nout & Kiers, 2005). The major desirable aspects of tempeh are its attractive flavour and texture, certain nutritional properties, and the reduced cooking time compared with the raw materials (Shurtleff & Aoyagi, 2001). Any available plant material can be used as a substrate, providing it can support the growth of *Rhizopus* spp. and it is suitable for human consumption after fermentation. Recently, tempeh flour has also been produced by drying and milling tempeh (Cuevas-Rodriguez *et al.*, 2004; Reyes-Moreno *et al.*, 2004). The nutritional improvement by fermentation is important for tempeh flour production, but the tempeh structure itself is not.

Origin of tempeh

Tempeh, also called ‘tempe kedele’ in Indonesia, is traditionally made from soybeans (Steinkraus *et al.*, 1983). Tempeh originated in central and east Java (Indonesia) at the beginning of the 18th century, and is now Indonesia’s most popular soy-protein food (Astuti *et al.*, 2000). Tempeh-like products have also been produced in China, such as soybean koji (Shurtleff & Aoyagi, 2001) or Douchi (Li-Te *et al.*, 2003; Li-Te *et al.*, 2004). Douchi (Fig.1) is made from black or yellow beans fermented by *Mucor* spp., *Aspergillus* spp., or *Rhizopus oligosporus*. Douchi (Chinese product), natto (Japanese product, soybeans fermented with *Bacillus subtilis*) and tempeh (Indonesia product, soybeans fermented with *R. oligosporus*) might all have the same origin (Astuti *et al.*, 2000; Li-Te *et al.*, 2003). During the years 1928-1931 William Morse of the USDA

observed a tempeh-like product in Beijing (Shurtleff & Aoyagi, 2001). In several places in China people still make a tempeh-like product, called 'Mei Dou Za' (Fig. 2), from tofu residues by spontaneous fermentation for about three days. It is a white mycelium knitted cake similar to okara tempeh (an insoluble residue of tofu or soymilk manufacture) (Matsuo, 1990; O'Toole, 1999).

Fig. 1. Douchi made from black bean (Photo: Xin-Mei Feng).

The basic process of tempeh production

Although different substrates can be used to produce tempeh, the basic fermentation process is similar for all substrates. It includes soaking, dehulling (if necessary), boiling and fermenting. The main differences between different substrates used in tempeh fermentation are the selection of optimal pre-treatments (e.g. when cereals are used it is sometimes necessary to modify the surface of the grain by cutting, cracking or pearling to obtain good growth of the mold), optimal soaking, boiling and incubating time, and optimal fermentation strain and inoculation level of *R. oligosporus*.

Substrates used for tempeh production

Traditionally, tempeh was made from soybean. Yellow-seeded soybeans are usually preferred as raw material (Sharma & Sarbhoy, 1984), but many different substrates can be used to produce tempeh (Table 1). Some substrates can only be processed to obtain high quality tempeh by combining them with soybeans (Wang *et al.*, 1968; Mugula, 1992; Shurtleff & Aoyagi, 2001) or can only be used to produce protein-rich tempeh flour (Cuevas-Rodriguez *et al.*, 2004). To my knowledge, most of the substrates listed in table 1 have only been tested under laboratory conditions, and have not reached the stage of industrial production.

Fig. 2. Production of Mei Dou Za (a. residue from tofu production, b. formed residue, c. ready for natural fermentation, d. freshly fermented (about 3 days fermentation), e. mature Mei Dou Za, f. sliced and dried Mei Dou Za) (Photos taken in Qian Jiang, Hubei Province, P. R. China in 2006).

Table 1. *Substrates have been used for tempeh fermentation*

Raw materials	Indonesian name	References
(1) Legumes		
Soybeans (yellow cultivars) (*Glycine max*)	Tempe kedele (kedelai)	(Nout *et al.*, 1987c; Varzakas, 1998)
Black gram (*Phaseolus mungo*)		(Jha & Verma, 1980)
Broad bean, bakla, horse bean, field bean (*Vicia faba*)		(David & Jitendra, 1981; Ashenafi & Busse, 1991e; Shurtleff & Aoyagi, 2001)
Chick pea (*Cicer arietinum*)		(Ashenafi & Busse, 1991d; Ashenafi & Busse, 1991e; Paredeslopez *et al.*, 1991; Reyes-Moreno *et al.*, 2004)
Common bean or red kidney bean (*Phaseolus vulgaris*)		(Paredes Lopez *et al.*, 1990; Kalavi *et al.*, 1996; Rodríguez-Bürger *et al.*, 1998)
Cow pea (*Vigna unguiculata*)		(Kiers *et al.*, 2000)
Horse (wild) tamarind (*Leucaena ensiformis*)	T. lamtoro	(Ko Swan & Hesseltine, 1979)
Jack bean (*Canavalia ensiformis*)	T. koro pedang	(Shurtleff & Aoyagi, 2001)
Lalab bean (*Lablab purpureus*)		(Shurtleff & Aoyagi, 2001)
Lima bean (*Phaseolus lunatus*)	T. koro	(Ko Swan & Hesseltine, 1979)
Mungbean or green gram (*Vigna radiata*)		(Shurtleff & Aoyagi, 2001)
Pigeon pea or red gram (*Cajanus cajan*)	T. gude	(Nout & Rombouts, 1990)
Sesban bean (*Sesbania grandiflora*)		(Nout & Rombouts, 1990)
Sweet lupine (*Lupinus albus*)		(Chango *et al.*, 1993; Fudiyansyah *et al.*, 1995)
Velvet bean (*Mucuna pruriens*)	T. benguk	(Ko Swan & Hesseltine, 1979)
Winged bean (*Psophocarpus tetragonolobus*)	T. kecipir	(Homma *et al.*, 1983)
Yellow pea (*Pisum sativum*)	T. kecipir	(Nout & Rombouts, 1990)
Bambara groundnut (*Vigna subterranea*)		(Amadi *et al.*, 1999)
African yambean (*Sphenostylis stenocarpa Hams*)		(Njoku *et al.*, 1991)

Table 1. *Continued*

Raw materials	Indonesian name	References
(2) Cereals		
Barley (*Hordeum vulgare*)		(Nout & Rombouts, 1990; Hachmeister & Fung, 1993; Berg *et al.*, 2001)
Wheat (*Triticum vulgare*)		(Hesseltine & Wang, 1980; Nout & Rombouts, 1990; Hachmeister & Fung, 1993)
Oats		(Nowak, 1992)
Quinoa		(Matsuo, 2006)
(3) Mixture of legumes with nonlegumes		
Cassava fibres, soybean hulls and soybeans	T. menjes	(Nout & Rombouts, 1990)
Finger millet with various legumes		(Mugula & Lyimo, 1999)
Sesame and soybean		(Shurtleff & Aoyagi, 2001)
Rice and black beans		(Rodríguez-Bürger *et al.*, 1998)
Maize and soybean		(Nout & Kiers, 2005)
Sorghum and common bean		(Nout & Kiers, 2005)
Sunflower and soybeans		(Vaidehi & Rathnamani, 1990)
(4) Press cake (by-products)		
Soybean residue from soy milk preparation or okara	T. gembus T. okara	(Ko Swan & Hesseltine, 1979; Matsuo, 1990)
Coconut residue from local coconut-oil pressing (Cocos nucifera)	T. bongkrek(Kelapa) T. enthoe T. tjenggereng	(Ko Swan & Hesseltine, 1979)
Groundnut presscake (*Arachis hypogaea*)	T. bungkil kacang	(Gandjar, 1981)
Rapeseed meal		(Rozan *et al.*, 1996)
(5) Other plant materials		
Rubber-seed		(Shurtleff & Aoyagi, 2001)
Apricot seeds		(Tuncel *et al.*, 1990)
Maize		(Cuevas-Rodriguez *et al.*, 2004)

Barley for tempeh production

Barley is the second most abundant cereal grain after wheat grown in Northern Europe. In Sweden, the average yearly production was about 1.7 million tons during the period 1993 to 2005. This was used in the following way: 62.3% for animal feeds, 9.9 % for beer production and 0.5 % for direct human consumption, with the remainder being exported (EuroStat data, supplied by Christina Wikberger, Swedish Board of Agriculture, Statistics Division). Thus, only 10.4 % of barley is used for human consumption in Sweden. From 1993 to 2005, there has been a small increase in the amount of barley used for direct human consumption, from 0.2 to 0.5 %. Since this is still a small fraction of the total barley production, there is a great potential to convert more barley into human foods. Barley tempeh could be one such alternative.

Characteristics of barley

The gross composition of barley varies notably between varieties, consisting of 49-66% starch, 14-28% dietary fibre and 9-22% crude protein (Oscarsson *et al.*, 1996). Consumption of barley products may elicit lower metabolic responses (i.e. lower blood glucose and insulin responses) and induce a greater feeling of satiety compared with white wheat bread (Granfeldt *et al.*, 1994) and could therefore decrease the risk for type 2 diabetes (McKeown, 2004). Consumption of barley products may also reduce total serum lipids and LDL-cholesterol in subjects with elevated serum cholesterol levels, and thus, reduce the risk of cardiovascular disease (Truswell, 2002; Behall *et al.*, 2004b; Behall *et al.*, 2004a). Some of these positive effects are probably due to the β–glucan content and the resulting viscosity (Wood, 2004). In addition, various botanical and structural characteristics of starchy foods have also been found to modify the post-prandial glucose and insulin responses in humans (Liljeberg *et al.*, 1992; Järvi *et al.*, 1995; Juntunen *et al.*, 2002). Whole grain barley is also rich in phytochemicals and other components that may provide additional health effects (Slavin *et al.*, 1997; Anderson, 2003). Barley contains large quantities of phytate (Eklund-Jonsson *et al.*, 2006), which is the major storage form of phosphorus (Reddy *et al.*, 1982). Phytate negatively affects the bioavailability of essential minerals by forming insoluble mineral-phytate complexes and thereby inhibits absorption (Hallberg *et al.*, 1989; Sandström & Sandberg, 1992; Bohn *et al.*, 2004). Malting is a non-fermentation method that reduces the phytate content of barley. A malting procedure that maintains a high nutritional value with a well preserved β–glucan content and extensively degradation of phytate (50-88% reduction) has been developed by steeping barley with lactic acid at a high temperature (48°C), followed by germination (Rimsten *et al.*, 2002). Recent studies have shown that the phytate content of whole grain barley can be reduced by 97% during a modified tempeh fermentation process with *R. oligosporus* (Eklund-Jonsson *et al.*, 2006).

Barley tempeh production

Barley tempeh has been fermented through a process similar to soybean tempeh (Hesseltine *et al.*, 1963; Hesseltine *et al.*, 1967). It was found that *Rhizopus* spp. with highly active proteolytic and lipolytic enzymes, but little or no amylase activity, were suitable for producing tempeh from cereal grains (wheat, barley, oat, rye and rice). This early attempt to produce barley tempeh used dehulled and cracked barley (Hesseltine *et al.*, 1967). However, commercial barley tempeh has not yet been produced.

Previously, a patented barley tempeh procedure has been developed by fermentating whole pearled barley kernels (Gourmet korn) with selected strains of *R. oligosporus* (Berg *et al.*, 2001). The fermentation process has recently been modified and applied on a new barley genotype (Karmose) with a high amylose and β-glucan content. The modified process has been found to strongly reduce the phytate content while preserving minerals (Eklund-Jonsson *et al.*, 2006) and also lower the glycemic index of barley tempeh (Alminger & Eklund-Jonsson, manuscript in prep).

Comparison of barley tempeh with soybean tempeh

Although the basic fermentation process is similar, there are still several differences between the barley and soybean tempeh processes (Fig. 3). *R. oligosporus* strains, which can produce soybean tempeh of good quality, do not always produce barley tempeh of good quality (Berg *et al.*, 2001). Barley is pearled before soaking, while soybean is dehulled before or after soaking. For barley tempeh production, the times for soaking, boiling or incubating are shorter than for soybean tempeh (**II**). Perforated containers were required to produce good quality soybean tempeh (**IV**). In contrast, petridishes loosely packaged in plastic bags were used to produce good quality barley tempeh (**I**). During fermentation the pH value of barley tempeh only increased slightly from pH 4.6 to pH 4.9 (**I** and **III**) (Fig. 4). In contrast, the pH of soybean tempeh significantly increases from pH 5 to pH 7 due to the release of ammonia in the final product (Sparringa & Owens, 1999a) (Fig. 4). An increased concentration of free ammonia might kill the tempeh fungus, leading to a reduced quality of the final product (Steinkraus *et al.*, 1983).

Foods made from tempeh

Soybean tempeh can be sold fresh, refrigerated (Fig. 5), frozen or even distributed in cans. This fermented product is remarkably versatile and can be served in hundreds of different Western or Asian-style recipes, such as tempeh burgers and sushi (Fig. 6); tempeh, lettuce and tomato sandwiches; tempeh chops topped with apple sauce; crisp slices or cubes added to salads, soups, pizza toppings, stir-fried rice, casseroles, sauces or tacos. The most popular and simplest way of serving is shallow-fried or deep–fried tempeh (Shurtleff & Aoyagi, 2001). Tempeh has also been processed into powder and used as an additive to infant weaning food

(Egounlety *et al.*, 2002), ice-cream (JuQin *et al.*, 2001), biscuit (Matsuo, 2006) or ready-to-prepare food mixes (Vaidehi *et al.*, 1996). In China, soybean tempeh is milled and mixed with meat to make sausages in order to reduce the use of meat, but still maintain the protein quality (Jujin *et al.*, 2000). Several dishes have also been prepared from barley tempeh (Fig. 7).

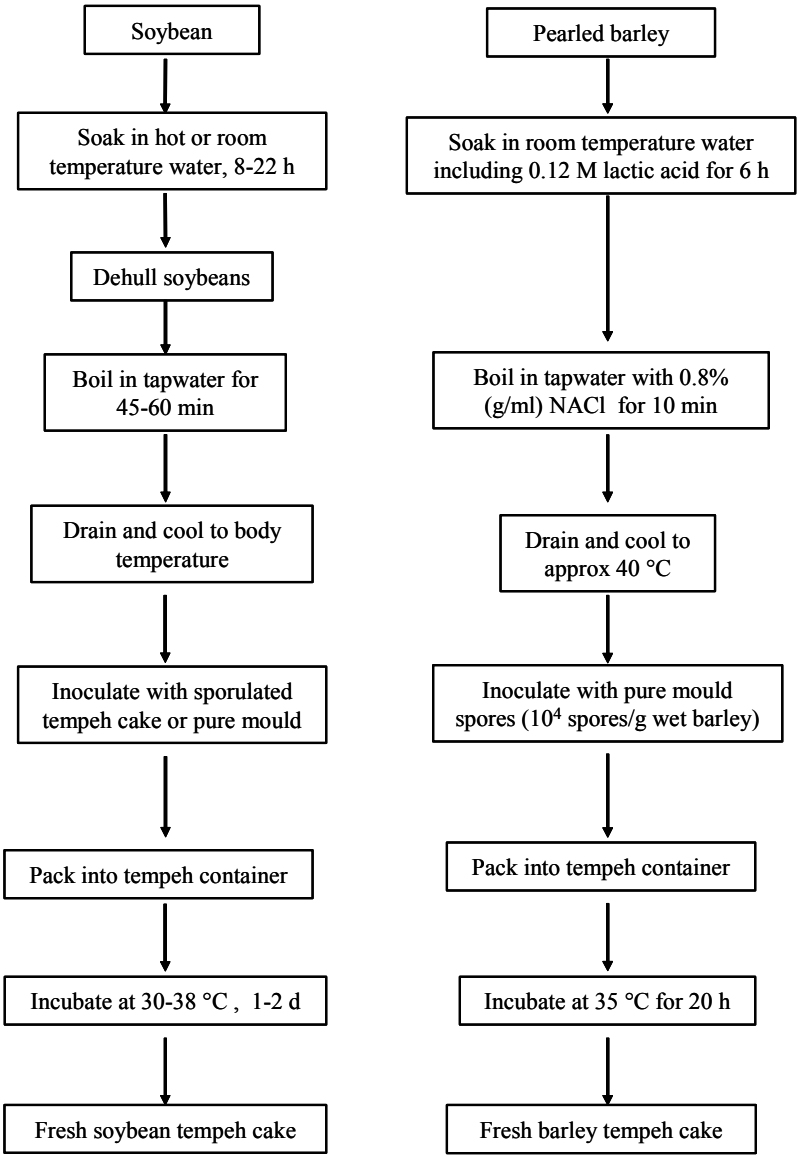

Fig. 3. Production processes of soybean tempeh (modified from Hachmeister & Fung, 1993; Nout & Kiers, 2005) and barley tempeh (modified from Berg *et al.*, 2001; **I**).

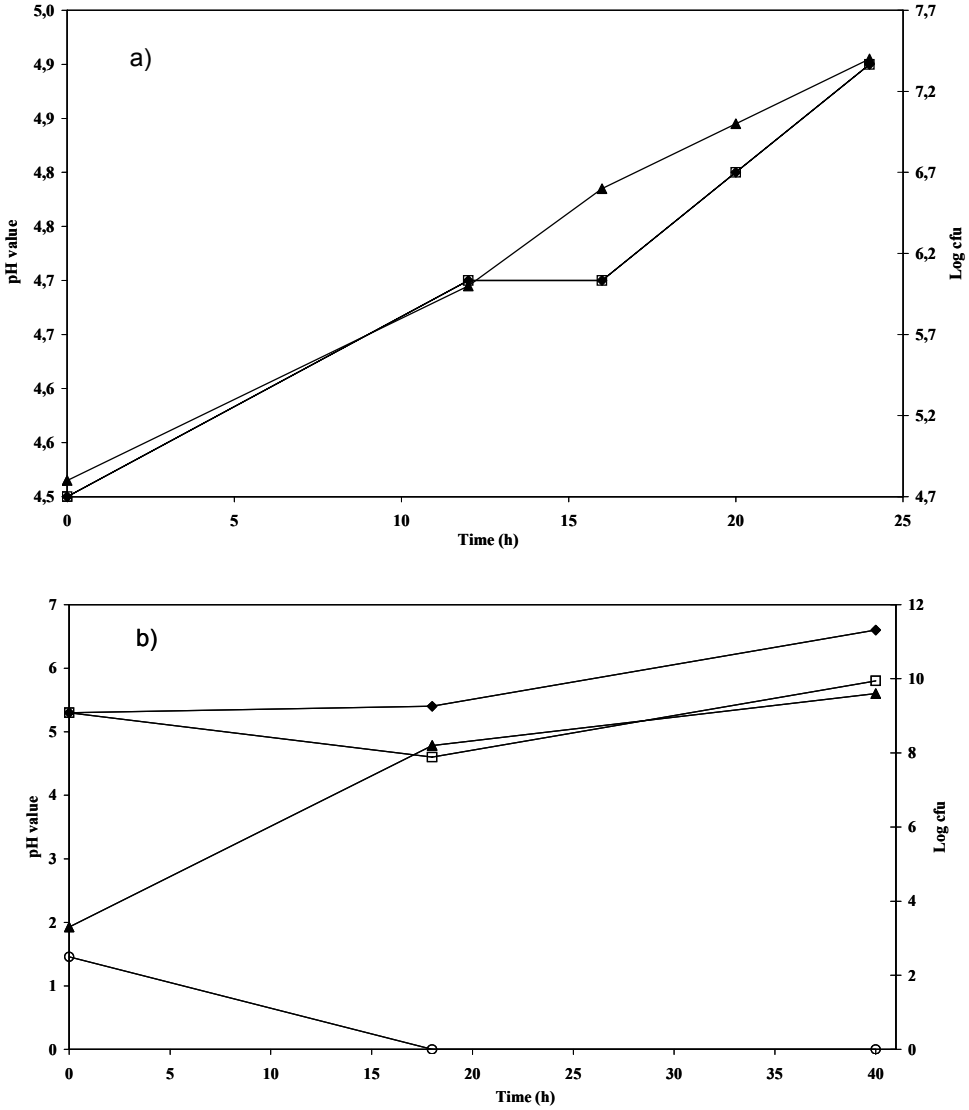

Fig. 4. Comparison of pH values and microbial growth in barley tempeh (**a**, data from **I**) and soybean tempeh (**b**, data from Ashenafi, 1991); pH values of barley or soybean tempeh fermented by *R. oligosporus alone* (♦), or together with *Lactobacillus plantarum* (in barley tempeh) or together with *L. plantarum* and *Listeria monocytogenes* (in soybean tempeh) (□); and the growth (log cfu) of *L. plantarum* (▲) and *L. monocytogenes* (○).

Fig. 5. Cold storage of soybean tempeh sold in a supermarket in the Netherlands (Photo: Xin-Mei Feng).

Fig. 6. Tempeh burger and Sushi sold in the Netherlands (Photo: Xin-Mei Feng).

Fig. 7. Barley tempeh dishes (Photo: Olligon AB).

Aims of this thesis

Soybean tempeh fermentation has been extensively documented (Steinkraus *et al.*, 1983; Nout & Rombouts, 1990; Nout & Kiers, 2005), but considerably less is known about barley tempeh. For instance, in soybean tempeh it was found that the presence of other microorganisms such as yeasts and lactic acid bacteria (LAB) could enhance the nutritional value and hygienic safety of soybean tempeh fermented by *R. oligosporus* (Suparmo, 1989; Ashenafi & Busse, 1991b; Nout, 1995; Wiesel *et al.*, 1997). In this thesis, I have investigated whether these microorganisms could grow in barley tempeh without reducing the growth of *R. oligosporus*. In order to determine the growth of microorganisms, several quantification methods were developed and evaluated. The effects of yeasts and LAB on the nutritional values of barley tempeh were also surveyed.

The specific goals of this thesis were to

- Investigate the growth abilities of lactic acid bacteria (LAB) in barley tempeh and their effects on *R. oligosporus* growth (**I**);
- Characterise volatile profiles produced by several *R. oligosporus* strains during barley or soybean tempeh fermentation or on malt extract agar (MEA) plates, and evaluate the effect of *Lactobacillus plantarum* on the volatile profile produced by *R. oligosporus* in barley tempeh (**II**);
- Investigate the growth abilities of yeasts in tempeh and their effect on *R. oligosporus* growth and nutritional quality (**III**);
- Develop and evaluate methods to determine the growth of *R. oligosporus* when co-cultivated with LAB (**I** and **IV**) and yeasts (**I** and **III**).

Microbial aspects of tempeh

Microbial diversity in tempeh fermentation

Traditional tempeh is the result of a mixed culture fermentation by a diverse group of microorganisms including moulds, yeasts, lactic acid bacteria and different gram-negative bacteria (Steinkraus *et al.*, 1983; Nout, 1995). *Rhizopus oligosporus* is the dominant tempeh fungus (Sharma & Sarbhoy, 1984), although some other moulds, such as *R. oryzae* and *Mucor* spp, may also contribute to the flavour, texture or nutritive value (Wiesel *et al.*, 1997).

Lactic acid bacteria may contribute to the microbial safety (Nout *et al.*, 1987a; Ashenafi & Busse, 1991b). Vitamin B_{12} production by bacteria, such as *Citrobacter freundii* or *Klebsiella pneumoniae* (Liem *et al.*, 1977; Okada *et al.*, 1985a; Suparmo, 1989; Keuth & Bisping, 1993; Keuth & Bisping, 1994; Wiesel *et al.*, 1997), has received special attention. However, these two species are both potentially pathogenic (Badger *et al.*, 1999; Struve & Krogfelt, 2004). Recently, also the probiotic *Lactobacillus reuteri* was reported to produce vitamin B_{12} (Taranto *et al.*, 2003).

Yeasts are frequently detected in tempeh, but their role is still unknown (Samson *et al.*, 1987). For industrialised tempeh production in the western world, the presence of unknown or potentially pathogenic microorganisms is not acceptable. There is therefore a need to investigate the ability of food-grade microorganisms to grow during tempeh fermentation and thus to exclude unwanted microorganisms and also to enhance nutritional quality.

The tempeh fungus *Rhizopus oligosporus*

Tempeh has been produced in Indonesia for many centuries. However, it was the Dutch scientist Prinsen Geerligs, who in 1895 identified the tempeh mould for the first time (Shurtleff & Aoyagi, 2001). Many different moulds are found in tempeh, but species within the zygomycete genus *Rhizopus* dominate (Steinkraus *et al.*, 1983). *Rhizopus* (Mucorales, Mucoraceae, Zygomycota) includes three species groups: *R. oryzae* group, *R. stolonifer* group, and *R. microsporus* group (Schipper & Stalpers, 1984), with species from the latter group dominating in tempeh.

Among them, *R. oligosporus* (Fig. 8) is the most preferred species in tempeh fermentation (Sharma & Sarbhoy, 1984), due to properties such as rapid growth at high temperature (30-42°C), an inability to ferment sucrose, high proteolytic and lipolytic activities and production of strong antioxidants (Steinkraus *et al.*, 1983). *R. oligosporus* is considered as a domesticated form of *R. microsporus* (Samson, 1985), which can produce toxic secondary metabolites such as rhizoxin, and rhizonins A and B (Jennessen *et al.*, 2005). Rhizoxin can inhibit mitosis and thus the cell cycle, and have been suggested as a potential anti-tumour agent (Tsuruo *et al.*, 1986; Takahashi *et al.*, 1987). Rhizoxin also causes rice seedling blight disease (Goh *et al.*, 1978), which was recently reported to be produced by an endosymbiotic bacterium (*Burkholderia* spp.) within *Rhizopus* spp. (Partida-Martinez & Hertweck, 2005). Rhizonins A and B have a strong hepatotoxic activity (Wilson *et al.*, 1984). However, *R. oligosporus* does not produce any of these metabolites in different laboratory and natural substrates, not even under prolonged incubation conditions conducive to the formation of these metabolites (Jennessen *et al.*, 2005), nor during barley tempeh fermentation (Feng, X-M and Nielsen, K. F., unpublished). *R. oligosporus* might have lost the rhizoxin-producing endosymbiotic bacteria and the ability to produce rhizonins during centuries of domestic cultivation in rich substrates such as soybeans.

Due to the great similarity in morphology between *R. oligosporus* and *R. microsporus*, additional differential characteristics are required to make sure that the correct strains are used for tempeh fermentation. The most recent study based on sporangiospore shape, size and ornamentation patterns under low-temperature scanning electron microscopy (SEM) demonstrated that the *R. microsporus* group includes three subgroups: 1) *R. rhizopodiformis*, *R. chinensis*, *R. azygosporus* and *R. oligosporus*, 2) *R. caespitosus*, *R. schipperae* and *R. homothallicus*, 3) *R. microsporus* (Jennessen *et al*, manuscript). *R. oligosporus* has the greatest number of large size irregular spores within the *R. microsporus* group and a proportion of more than 10 % irregular spores can be used as a marker to distinguish *R.*

oligosporus from other strains within the *R. microsporus* group (Jennessen *et al*, manuscript).

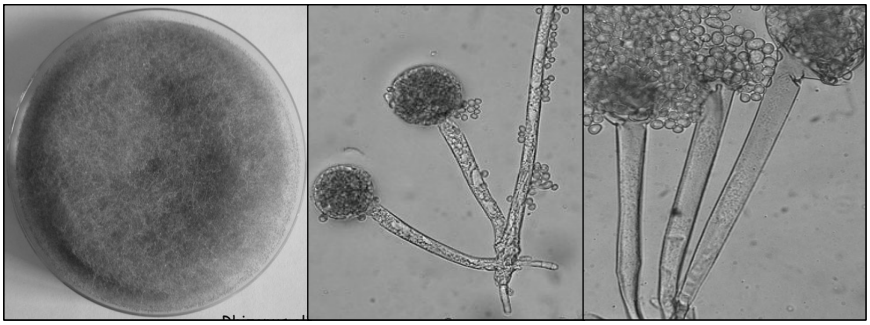

Fig. 8. Morphology of *R. oligosporus* (Photo: Inger Ohlsson).

Enzymes produced by *R. oligosporus*

During tempeh fermentation, the substrate is degraded by *R. oligosporus* enzymes, such as carbohydratases (e.g. polygalacturonase, endocellulase, xylanase, arabinanase and small quantities of α-D-galactosidase, β-B-galactosidase, β-D-xylosidase, α-L-arabinofuranosidase and α-D-glucosidase), lipases, proteases and phytases (Nout & Rombouts, 1990). In contrast, Rehms and Barz (1995) found that *R. oligosporus* did not produce α-galactosidase and consequently can not degrade flatulence-causing compounds such as stachyose and raffinose. Using the micro-enzyme API ZYM system (API Laboratory Products Ltd.), it was found that most *Rhizopus* spp. produced alkaline phosphatase, esterase C4, lipase C8 and C14, leucine aminopeptidase, valine aminopeptidase, acid phosphatase, phosphoamidase, β-glucosidase, N-acetyl-β-glucosaminidase and a-mannosidase (Jennessen, J., unpublished). α-galactosidase was not produced by any of the 10 *R. oligosporus* strains tested (Jennessen, J., unpublished). Among these enzymes, alkaline and acid phosphatases can degrade the anti-nutrient phytic acid. Phytic acid degrading enzymes can be repressed by high phosphate concentration and high pH values, and can also be affected by substrate composition (Andlid *et al.*, 2004). Substrate surface status can also influence the degradation of phytate (Eklund-Jonsson *et al.*, 2006).

Anti-microbial effects of *R. oligosporus*

R. oligosporus can inhibit the growth and aflatoxin B_1 accumulation of *Aspergillus flavus* and *A. parasiticus* (Nout, 1989). *R. oligosporus* has been reported to produce 4 to 5 anti-bacterial compounds during soybean tempeh fermentation (Anon, 1969; Wang *et al.*, 1969; Nowak & Steinkraus, 1988). The fungus also produces phenolic compounds that inhibit the growth of pathogenic bacteria such as *Helicobacter pylori* (Berghofer *et al.*, 1998; McCue *et al.*, 2003; Correia *et al.*,

2004a; Correia *et al.*, 2004b; McCue *et al.*, 2004; Vattem *et al.*, 2004). An antibacterial protein has been purified from *R. oligosporus*, with activities against *Bacillus* spp. (especially against *Bacillus subtilis*), *Staphylococcus aureus* and *Streptococcus cremoris* (Kobayasi *et al.*, 1992). *R. oligosporus* can also produce certain compounds that interfere with the adhesion of *E. coli* to small intestinal brush-border membranes (Kiers *et al.*, 2002).

Application of *R. oligosporus* in other fields

Due to the wide range of enzymes produced, the ability to grow rapidly at high temperature and the production of anti-microbial compounds as above-mentioned, *R. oligosporus* has recently received attention in other fields of applied microbiology, such as sufu production (Han *et al.*, 2003), agro-industrial solid waste treatment (Christen *et al.*, 2000), the production of phenolic compounds from agricultural wastes (McCue *et al.*, 2003; Correia *et al.*, 2004b; Vattem *et al.*, 2004) and bioremediation of heavy metals (Beolchini *et al.*, 2003; Othman & Amin, 2003; Yu *et al.*, 2003; Bishnoi & Garima, 2005).

Effects of inoculum concentration of *R. oligosporus* on tempeh fermentation

The inoculation levels of *R. oligosporus* strongly influenced tempeh fermentation. When *R. oligosporus* was inoculated at approximately 10^4 spores/g moist substrate, a barley tempeh cake with dense mycelial growth was obtained after 20 h (**IV**). With inoculation at approximately 10^2 spores/g moist barley, the fungus grew more slowly and a barley tempeh cake with dense mycelial growth was not obtained until after 28 to 32 h (**IV**). This slow growth may increase the risk of contamination with pathogens. When *R. oligosporus* was inoculated at approximately 10^6 spores/g moist barley, the time for obtaining dense mycelial growth was shortened to 15 to 20 h (**IV**). However, the growth was uneven (**IV**), probably due to oxygen limitation in the center. Similar results have also been reported by Nout and Kiers (2005) for soybean tempeh fermentation.

Effects of lactic acid bacteria on tempeh fermentation

There are no reports of food poisoning caused by consuming traditional soybean tempeh (Ko Swan & Hesseltine, 1979). Nout & Rombouts (1990) suggested that this safety is due to: 1) inherent properties of *Rhizopus* spp., 2) presence of lactic acid bacteria (LAB), 3) incubation under micro-aerobic conditions, and 4) the customary heating prior to consumption.

Table 2. *Growth of potential pathogenic bacteria and their inhibition by lactic acid bacteria (LAB) during tempeh fermentation*

Tempeh substrates	**Initial substrate pH**	**Pathogenic bacteria**	**Growth of pathogenic bacteria (log cfu/g) without LAB**	**Growth of pathogenic bacteria in the presence of LAB (at inoculation level log cfu/g)**	**Reference**
Soybean	4.9	*B. cereus*	5 to <2.7	*B. cereus* can't grow	(Nout *et al.*, 1987a)
Soybean	5.7-6.0	*B. cereus*	5 to 8	Can not be inhibited, but tempeh is acceptable after the inoculation of *L. plantarum*, *L. casei* spp. *alactosus* and *L. fermentum*	(Nout *et al.*, 1987a)
Horsebean, pea, chickpea, soybean	unacidified	*B. cereus*	2 to 8 in horsebean, 6 to 7 in others,	Can not be inhibited in horsebean, but markedly decreased in other substrates after the inoculation of *L. plantarum* (6.5)	(Ashenafi & Busse, 1991b)
Horsebean, pea, chickpea, soybean	acidified	*B. cereus*	2 to >7 (horsebean, pea), 5 to 6 (chickpea, soybean)	Decrease after the inoculation of *L. plantarum* (3.5)	(Ashenafi & Busse, 1991b)
Soybean	4.9	*S. infantis*, *E. aerogenes, E. coli*	2 to 7-9	Completely inhibited after the inoculation of *L. plantarum* (6.2)	(Ashenafi & Busse, 1989)
Pea, chickpea	7.1(tempeh)	*Micrococcus sp.*, *Bacillus sp.*	4 (natural occurred) to >8 in storage tempeh at 4 °C	Inhibited after the inoculation of *L. plantarum* (>6) during cold storage	(Ashenafi & Busse, 1991a)
Horsebean, pea, chickpea, soybean	Around 5.0	*L. monocytogenes*	2.5-2.7—4.5-6.2	*L. plantarum* (6.3) slightly reduces it in horse bean, completely inhibits it in other substrates	(Ashenafi, 1991)

Table 2. *Continued*

Tempeh substrates	**Initial substrate pH**	**Pathogenic bacteria**	**Growth of pathogenic bacterial (log cfu/g) without LAB**	**Growth of pathogenic bacterial with the presence of LAB (at inoculation level log cfu/g)**	**Reference**
Horsebean, pea, chickpea	unacidified	*S. infantis, E. coli*	2.3 - >6.5 (*S. infantis*); 2.3 - >6.5 (*E. coli*)	Inhibited to some extent after the inoculation of *L. plantarum* (6.3)	(Ashenafi & Busse, 1991c)
Horsebean, peas, chickpea	acidified	*S. infantis, E. coli*	2.3 - >6.0 (*S. infantis*); 2.3 - >6.0 (*E. coli*)	Strongly inhibited after the inoculation of *L. plantarum* (3.3)	(Ashenafi & Busse, 1991c)
Horsebean, pea, chickpea, soybean,	unacidified	*S. aureus*	2-3 – 8 (others), 4.7 (chickpea)	Markedly decreased in horsebean (4.3), pea (5.8) and in chickpea (3.2), completely inhibited in soybean after the inoculation of *L. plantarum* (>6)	(Ashenafi & Busse, 1992)
Horsebean, pea, chickpea, soybean,	acidified	*S. aureus*	<7 (horsebean, pea and soybean), <5 (chickpea)	Completely inhibited in soybean, reduce to 4 in others after the inoculation of *L. plantarum* (2-3)	(Ashenafi & Busse, 1992)

Notice: B. cereus=Bacillus cereus, L. plantaru=Lactobacillus plantarum, S. infantis=Salmonella infantis, E. aerogenes=Enterobacter aerogenes, E. coli=Escherichia coli, S. aureus=Staphylococcus aureus, L. monocytogenes=Listeria monocytogenes

The LAB are important during two process stages in legume tempeh fermentation: 1) soaking, where they acidify soaking water during natural soaking of beans to reduce the growth of potential pathogenic bacteria (Nout *et al.*, 1987c); 2) fermentation, where the growth of LAB limits the natural increase in pH during tempeh fermentation, which in turn inhibits the growth of a number of pathogenic bacteria (Table 2). The inhibitory effects of LAB on potential pathogens were found to depend on the substrates used and the initial substrates' pH values (Ashenafi & Busse, 1991c) (Table 2). Generally, higher initial substrates' pH values require larger inoculations of LAB to inhibit the growth of pathogenic bacteria.

The soaking time is shorter (3-6 h) in the barley tempeh process than for soybean tempeh production (6-24 h). This would not allow substantial growth of LAB and therefore, lactic acid was used instead of LAB strains during barley soaking to reduce the pH and inhibit the growth of potential pathogens. The growth of LAB

during barley tempeh fermentation has not been reported before. We investigated the growth of several LAB strains during barley tempeh fermentation. Five investigated strains of *Lactococcus lactis* and one strain of *Pedicoccus pentosaceus* did not grow during barley tempeh fermentation (Table 3), while *L. reuteri* grew very slowly (**I**). In contrast, *L. plantarum* and *L. fermentum* grew faster (**I**). *L. plantarum* showed especially strong growth, even at inoculations of less than 2 log cfu/ g moist barley (Fig. 9). This indicates the importance of selecting competitive species or strains for co-fermentation of tempeh.

The growth of LAB in barley tempeh did not reduce the pH (**I**) as it did in legume tempeh (Ashenafi & Busse, 1991d) (Fig. 4). This may be due to the fact that acid-soaked barley originally had a low pH value and a substantial buffering capacity. Many pathogenic bacteria are found to be difficult to grow under the the conditions prevailing during barley tempeh fermentation (Swanberg *et al*, unpublished 2000) (Fig. 10). This might be due to the low pH and to competition from *R. oligosporus*.

Table 3. *Growth of Lactococcus lactis and Pedicoccus pentosaceus during barley tempeh fermentation (Feng, unpublished, n=1)*

Species	**Strain No.**	**Characteristics**	**Growth (log cfu/g)**	
			0 h	23 h
P. pentosaceus[a]	fBB61		5.5	4.1
L. lactis[b]	SR3.52	Nisin producer from silage	4.3	3.1
L. lactis	SR3.53	Nisin producer from silage	6.3	5.7
L. lactis	SR 3.54	Nisin producer from silage	4.0	2.8
L. lactis	ATCC 11454	Nisin producer from cheese starter	4.6	<2
L. lactis	CNRZ 481	Lacticin producer from cheese starter	3.7	4.0

[a]: *P. pentosaceus=Pedicoccus pentosaceus;* [b]: *L. lactis=Lactococcus lactis*

LAB inoculated at 4 log cfu/g moist barley did not affect the growth of *R. oligosporus* (**I** and **II**), whereas larger inoculations did (Feng, unpublished). It is important to determine the degree of inoculation required when introducing LAB to tempeh. This will depend on the specific LAB strain, the substrate used and the germination ability of *R. oligosporus* spores.

The effects of LAB co-inoculation with *R. oligosporus* on the nutritional value of soybean or barley tempeh have not been investigated. However, some studies have been reported on the effects of LAB fermentation alone on the composition of barley fibres. β–glucan, total and soluble dietary fibre, but not starch, were

reduced after 16 h fermentation of barley whole-grain flours with *Lactobacillus* spp. (Skrede *et al.*, 2003). Chicken fed with this fermented barley whole-grain flour attained higher body weights compared with those fed on unfermented one. The contents of β–glucans and insoluble fibres, but not soluble fibre, were also reduced in barley fibre concentrates fermented with LAB (Lambo *et al.*, 2005).

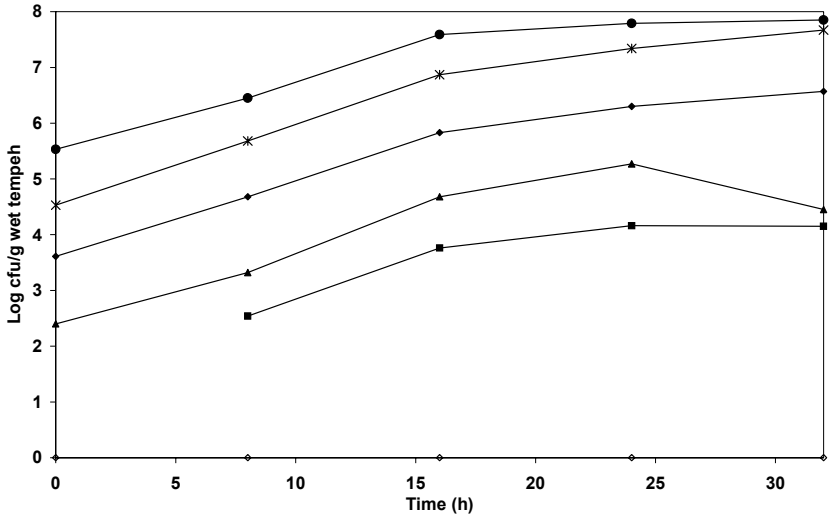

Fig. 9. Growth of *Lactobacillus plantarum* at different inoculation levels (log cfu/g moist tempeh) during barley tempeh fermentation (■: initial level below detection limit of 10^2 cfu/g) (Feng, unpublished, n=1).

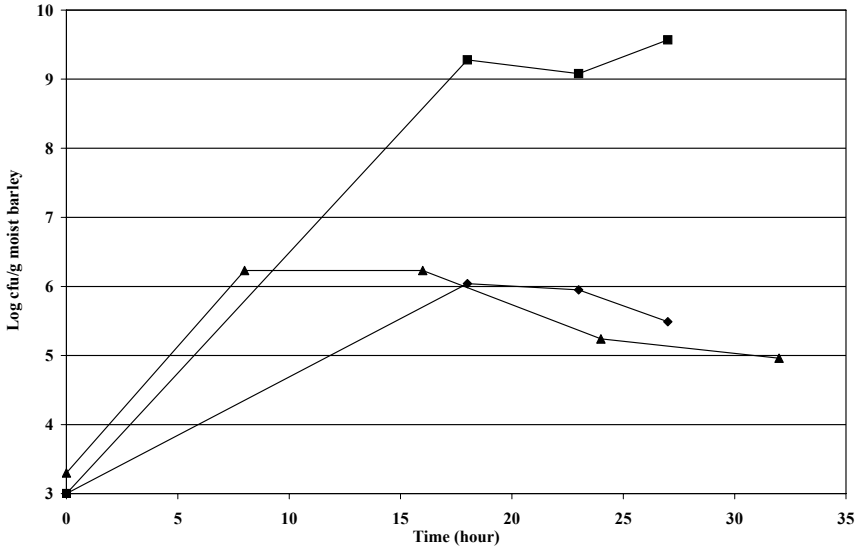

Fig. 10. Growth of *Bacillus subtilis* alone (■) or co-inoculated with *R. oligosporus* in barley (▲ and ♦ from two individual experiments) (Feng, unpublished, n=1).

LAB can produce organoleptic compounds in yoghurt (Stien *et al.*, 1999) and sourdough bread (Damiani *et al.*, 1996), folate (Sanna *et al.*, 2005; Kariluoto *et al.*, 2006), low-calorie polyols such as mannitol that are used to reduce the sugar content (Wisselink *et al.*, 2002) and other products such as sugar polymers, sweeteners and aromatic compounds (Leroy & de Vuyst, 2004). Furthermore, LAB can remove raffinose, stachyose, and verbascose from soybean (Scalabrini *et al.*, 1998; Leroy & de Vuyst, 2004), and proteinase inhibitors from legumes and cereals to prevent maldigestion (Holzapfel, 2002). LAB can also degrade phytic acid and tannins from cereals and legumes to increase mineral bioavailability (Sharma & Kapoor, 1996; Holzapfel, 2002), and degrade natural toxins such as cyanogenic glucosides from cassava (Kimaryo *et al.*, 2000; Holzapfel, 2002). Some LAB are considered as probiotics (Merk *et al.*, 2005; Shimosato *et al.*, 2006), and both viable and non-viable forms showed efficacy in shortening the duration of diarrhea (Ouwehand & Salminen, 1998). They can also inhibit the growth of fungi (Schnürer & Magnusson, 2005) and thereby reduce the production of mycotoxins, or reduce damage of mycotoxins to humans by binding them to their cell walls (Pierides *et al.*, 2000; Mokoena *et al.*, 2005; Shetty & Jespersen, 2006). Therefore, the growth of LAB in tempeh may improve both the nutritional value and safety of barley tempeh.

Effects of yeasts on tempeh fermentation

Yeasts are considered to be the oldest domesticated microorganisms, and have been used to produce alcoholic beverages and leaven bread dough for millennia (Walker, 1999). Certain yeasts have probiotic potential (van der Aa Kuhle *et al.*, 2005), prevent the growth of moulds in storage (Druvefors, 2004), inhibit the production of mycotoxins (Petersson *et al.*, 1998), or bind mycotoxins to the cell wall and consequently reduce the risk of occurrence of mycotoxins in foods (Yiannikouris *et al.*, 2003; Yiannikouris *et al.*, 2004; Shetty & Jespersen, 2006). The ability to bind mycotoxins is considered to be related to the β–D-glucan content in the yeast cell wall (Yiannikouris *et al.*, 2004). Yeasts can also produce the vitamin folate (Sanna *et al.*, 2005; Kariluoto *et al.*, 2006). They interact with LAB in many food products such as kefir (Yuksekdag *et al.*, 2004), sourdough (Gobbetti *et al.*, 1994b; Gobbetti *et al.*, 1994a; Damiani *et al.*, 1996; Gobbetti, 1998; Kariluoto *et al.*, 2006) and beer (Vaughan *et al.*, 2005).

Yeasts have been detected in commercial (Samson *et al.*, 1987) and traditional tempeh products (Steinkraus *et al.*, 1983). The most frequently detected yeasts belong to the genus *Trichosporon* (Table 4). Samson *et al* (1987) claim that none of the yeast species detected in tempeh are typical for this product, and that the flora does not seem to be specific for a particular commodity. *Trichosporon beigelii* is even a potential pathogen (Gonzalez *et al.*, 2001). This suggests a need to investigate the role of yeasts in tempeh fermentation and to introduce food-grade yeasts into tempeh. However, very few reports about yeasts in tempeh have been published so far. *Saccharomyces dairensis* has been isolated from soybean soaking water. When this isolate was re-inoculated into soaking water, it neither reduced the pH of soaking water, nor affected the quality of the tempeh (Nout *et*

al., 1987c). We introduced yeasts to barley tempeh at 4 log cfu/g moist barley and found that most of the investigated yeast strains, with the exception of *Rhodotorula glutinis*, could grow well during barley tempeh fermentation (**III**). The growth of yeasts could also increase the content of ergosterol (Provitamin D). However, only marginal effects on the amino acid and vitamin composition of the tempeh were found (**III**). As with lactic acid bacteria, if the yeast inoculation levels were too high, an inhibition of the growth of the tempeh fungus was observed (**III** and fig. 16). Thus, the amount of inoculated yeast must be carefully adjusted.

Based on the characteristics of food-grade yeasts, one can assume that the growth of yeast in barley tempeh may not only increase the ergosterol content, but may also play other important roles, such as excluding unwanted bacteria, yeasts and moulds, or producing vitamins.

Potential human health risks of tempeh

No cases of food poisoning have ever been reported after consuming tempeh (Ko Swan & Hesseltine, 1979). However, there is still a risk due to the potential growth of moulds and pathogenic bacteria (Table 2), and the production of mycotoxins and bacterial toxins. Pathogenic bacteria do not grow well in acidified substrates and even if they can grow, they are normally controlled by LAB (Table 2). The only bacterial toxin reported in tempeh hitherto is produced by *Pseudomonas cocovenenans* in tempeh bongkrek made from coconut grits or presscake (Ko Swan & Kelholt, 1981; Steinkraus *et al.*, 1983). Some fungal taxa closely related to *R. oligosporus* produce toxins (Jennessen *et al.*, 2005), and misidentification could potentially lead to the use of a toxin-producing strain for tempeh fermentation.

Inoculated *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli* did not grow during barley tempeh fermentation (Swanberg, unpublished), which might be due to the low pH under well-controlled fermentation conditions, as well as to competition from the fast growing *R. oligosporus*.

Table 4. *Yeasts observed in tempeh*

Species (number of isolates)	Origin	Reference
Trichosporon beigelii (34) *Clavispora (Candida) lusitaniae* (17) *Candida altosa* (13) *Candida intermedia* (11) *Yarrowia lipolytica* (7) *Lodderomyces elongisporus* (6) *Rhodotorula mucilaginosa* (6) *Candida sake* (3) *Hansenula fabián* (3) *Candida tropicales* (2) *Candida parapsilosis* (2) *Pichia membranaefaciens* (2) *Rhodotorula rubra* (2) *Candida rugosa* (1) *Candida curvata* (1) *Hansenula anomala* (1)	The Netherlands	(Samson *et al.*, 1987)
Trichosporon spp.	Malaysia	(Steinkraus *et al.*, 1983)
Trichosporon pullulans	Indonesia	(Steinkraus *et al.*, 1983)

Methods for quantitative determination of microorganisms during tempeh fermentation

From microbiological investigations of tempeh, we know that traditional tempeh fermentation is a mixed-culture fermentation with *R. oligosporus* as the dominant species. LAB and yeasts might improve the safety and nutritional value. Specific quantification methods are required to investigate the interrelationships and effects of different microorganisms during barley tempeh fermentation. Tempeh fermentation is a solid-substrate fermentation process (SSF) (Fig. 11), which makes quantification more difficult than with liquid substrates. Different quantification methods, such as determination of colony forming units, ergosterol content, hyphal length, image analysis and real-time PCR, were used in this thesis.

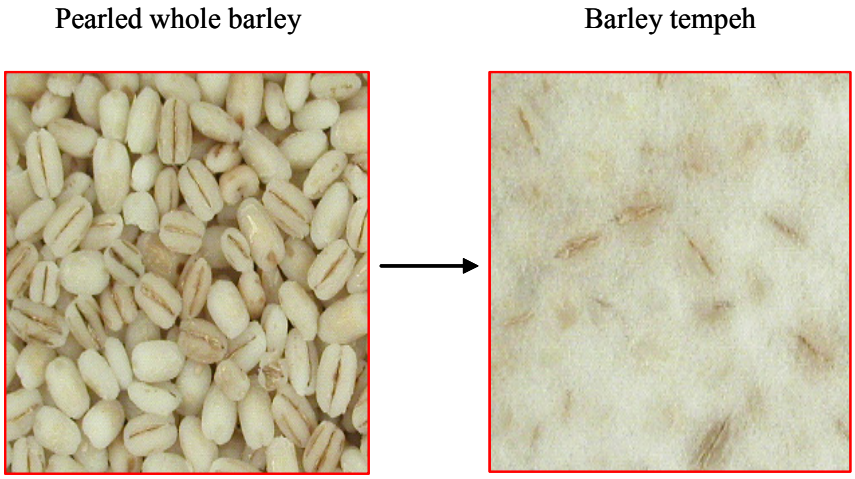

Fig. 11. Solid state fermentation of barley tempeh.

Determination of colony forming units

Bacteria and yeasts can be quantified in SSF by determining colony forming units (cfu) on appropriate agar substrates (**I** and **III**). However, the number of colony forming units of filamentous fungi generally represents more sporulation than fungal mycelial growth (Schnürer, 1993). Thus, other methods might be more suitable for determining the growth of *R. oligosporus* during barley tempeh fermentation.

Indirect methods for fungal quantification—hyphal length and ergosterol content

Quantification of mould biomass in SSF remains difficult. A direct gravimetric biomass determination would provide the most accurate estimate, but this is almost impossible due to the difficulty in separating fungal biomass from the substrate (Harris & Kell, 1985). Therefore, many indirect methods have been used to determine fungal biomass in SSF, such as CO_2 evolution (Steinkraus *et al.*, 1983), determination of hyphal lengths (**I;** Olson, 1950; Schnürer, 1993), ergosterol (**I**; Seitz *et al.*, 1979; Nout *et al.*, 1987b; Schnürer, 1993), chitin (Matcham *et al.*, 1985), or glucosamine contents (Sparringa & Owens, 1999b), or glucoamylase activity (Mitchell *et al.*, 1991b). During tempeh fermentation, the fungal mycelium knits the substrate particles together. Therefore, mycelial cohesive strength has also been used as an indicator of fungal biomass (Blakeman *et al.*, 1988). Determination of ergosterol content is the most commonly used method. Ergosterol is a provitamin of vitamin D, and is thus also an indicator of the nutritional value of food (**III**). The drawback with this method is that the ergosterol content of the mycelium is affected by environmental conditions (Nout *et al.*, 1987b). Moreover, ergosterol is present in both yeasts and moulds (Pasanen

et al., 1999), so it is impossible to differentiate yeast and mould growth by ergosterol content. Hyphal length has earlier been used to quantify filamentous algae (Olson, 1950), and later to determine fungal biomass in soil (Bååth & Söderström, 1980; Schnürer *et al.*, 1985) and in soybean tempeh (Nout *et al.*, 1987b). Hyphal length was strongly correlated with ergosterol content (**I**). However, this method is prone to large experimental errors and is also time-consuming (Stahl *et al.*, 1995; **I**).

Real-time PCR quantification

In real-time PCR, PCR amplification is linked to a detection process of the PCR products. Thus, amplification and product detection are performed at the same time, which makes it possible to follow the reaction kinetics. Several PCR products detection methods have been developed, including labelled oligonucleotide probes (oligoprobes) and fluorescent dyes that bind to dsDNA and enhance their fluorescence while binding (Mackay, 2004). Real-time PCR quantification has been used to detect and quantify white-rot fungi in wood (Eikenes *et al.*, 2005), fungal growth in soil (Filion *et al.*, 2003), aflatoxin-producing fungi in plant-based foods (Mayer *et al.*, 2003), contamination of bacteria, yeasts and moulds in milk (Vaitilingom *et al.*, 1998), and *Lactobacillus* spp. in the cheese-making process (Grattepanche *et al.*, 2005). It is proven a fast and selective method. Individual quantification of yeast and *R. oligosporus* in co-cultivation tempeh is almost impossible to perform by determination of colony forming units or ergosterol contents, since colony forming unit is less applicable for *R. oligosporus* and ergosterol is produced by both yeast and *R. oligosporus*. Real-time PCR could here provide the best alternative.

The main parameters of real-time PCR quantification are the C_T values and the amplification efficiency. The C_T value is defined as the cycle number at which the fluorescence surpasses the noise threshold. The amplification efficiencies (E) of the respective DNA amplicons were empirically determined from the amplification of serial dilutions of the respective target DNA according to Wilhelm and Pingoud (2003): E= 10 [-1/ slope], where the slope was calculated from the standard curve of DNA concentrations vs. the C_T values (Fig. 12). The determined E-values were 1.95 for the *R. oligosporus chs1* gene, 1.90 for the *S. cerevisiae PDA1* gene and 1.93 for the amp^R gene, i. e. close to theoretical maxium of 2.0.

The SYBR green dye I binds to dsDNA. When the initial concentration of DNA is larger, fewer PCR amplification cycles are needed to surpass the noise threshold, i.e. the C_T value is lower (Fig. 13).

The extraction of DNA is the key step in real-time PCR quantification of mixed-culture fermentation of barley tempeh. Most of the DNA extraction methods for plant-derived materials include a step of grinding samples in liquid nitrogen (Leal-Klevezas & Martinez-Soriano, 2001; Sharma *et al.*, 2003). This can be assumed to be difficult with large barley particles. We have developed an alternative method of DNA extraction. This method leaves the barley grain particles almost intact

since most of the fungal mycelia (Varzakas, 1998) and yeasts grow at the surface of substrates particles. Tempeh samples were frozen. An extraction buffer with glass beads was added to the frozen samples and extraction was performed by combining a mechanical and a heat-based cellolysis and homogenization (**III**). The extracted DNA was run with respective primers for real-time PCR (Fig. 14). An external standard DNA was run with the sample DNA at the same time in order to standardize the DNA extraction process. The final results were compared with the external standard DNA according to the formula:

$$R = \frac{E_{Standard}^{CT(Standard)}}{E_{Sample}^{CT(Sample)}}$$

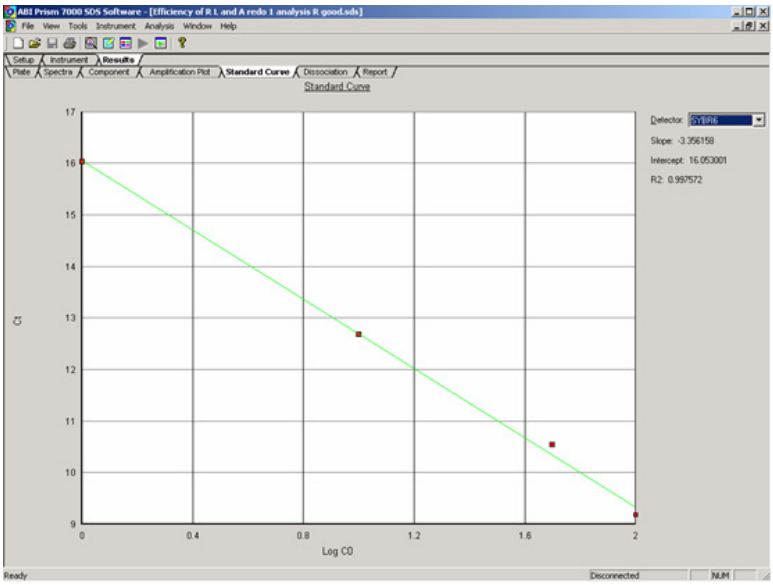

Fig. 12. Standard curve (C_T values plotted vs. log of initial DNA copy number) for determination of the amplification efficiency of the *Rhizopus oligosporus* target sequence (direct TaqMan software output).

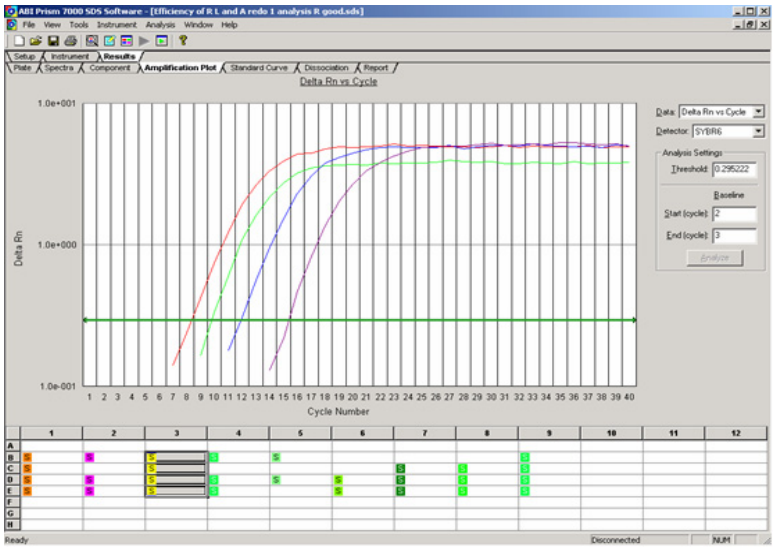

Fig. 13. Log of normalized reporter fluorescence (Delta Rn) plotted vs PCR cycle number for different concentrations of *R. oligosprus* target sequence. The noise threshold is shown by the green line (direct TaqMan software output).

The new method efficiently extracted DNA from the mixed *S. cerevisiae* and *R. oligosporus* cultures. A slightly lower efficiency of DNA extraction for *S. cerevisiae* was observed after 20 h fermentation (**III** and fig. 15), possibly due to the change of the yeast cell wall during the stationary phase (Walker, 1999).

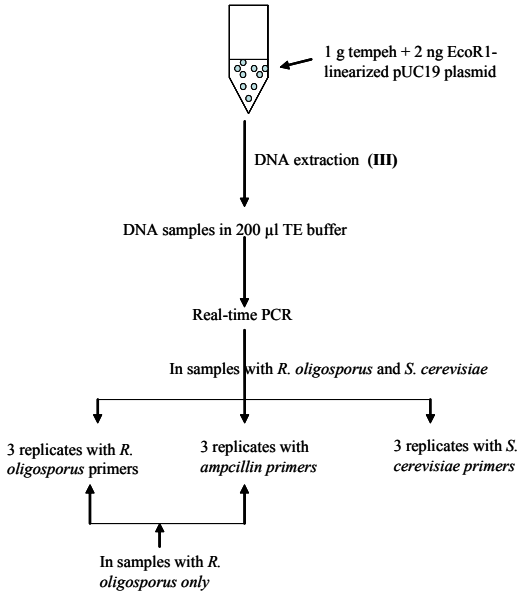

Fig. 14. Flow sheet of real-time PCR amplification of target sequence DNA during barley tempeh fermentation with *R. oligosporus* and *S. cerevisiae*.

We found that real-time PCR with specific primers could selectively quantify the growth of *S. cerevisiae* and *R. oligosporus* during barley tempeh fermentation (**III**). The real-time PCR results for the yeast were highly correlated with the corresponding log cfu data ($r=0.98$, $n=9$, $P<0.001$). The correlation between real-time PCR data and ergosterol contents for *R. oligosporus* was slightly lower ($r=0.83$, $n=5$, $p<0.1$).

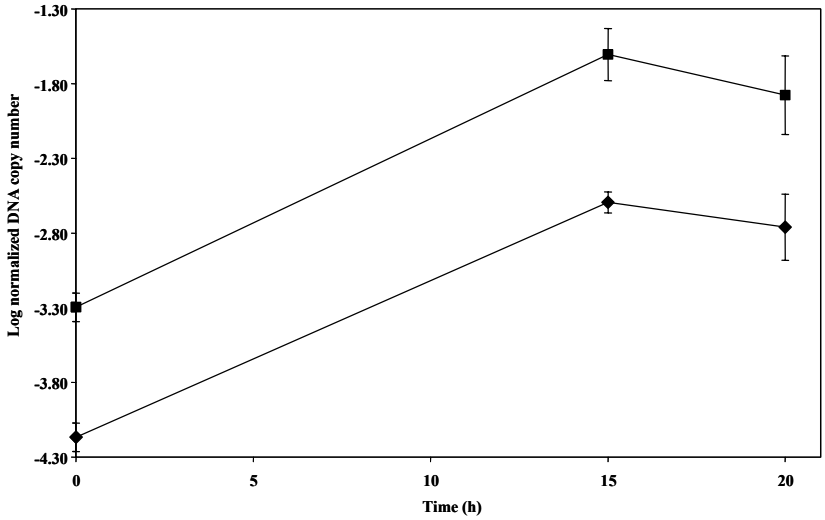

Fig. 15. Growth of *S. cerevisiae* at inoculation level 10^4 (♦) and 10^5 (■) cfu/g moist barley determined by real-time PCR when co-cultivated with *R. oligosporus* during barley tempeh fermentation (Feng, unpublished, n=3, ±SD).

The DNA copy numbers in *R. oligosporus* determined by real-time PCR agreed less well with the ergosterol content when the growth of *R. oligosporus* was inhibited by yeasts (Fig. 16) (**III**). We found that when yeast was inoculated at 10^5 cells/ g, the relative DNA copy numbers of *R. oligosporus* were higher than those inoculated at 10^4 cells/g (Fig. 17). In fact, the growth of *R. oligosporus* was inhibited at co-inoculation with 10^5 cells/ g of yeast. This suggests that DNA copy numbers may increase even when mycelial growth is inhibited. This phenomenon has also been found in *Aspergillus* spp. (Lin & Momany, 2004). Therefore, real-time PCR may need to be combined with other quantification methods to accurately determinate mould growth in mixed culture fermentation systems.

Fig. 16. Growth of *R. oligosporus* (inoculated at 10^4 spores/g moist barley) and *S. cerevisiae* (inoculated at 4 x 10^4 cfu/g moist barley) alone or co-inoculated with *R. oligosporus* (Feng, unpublished).

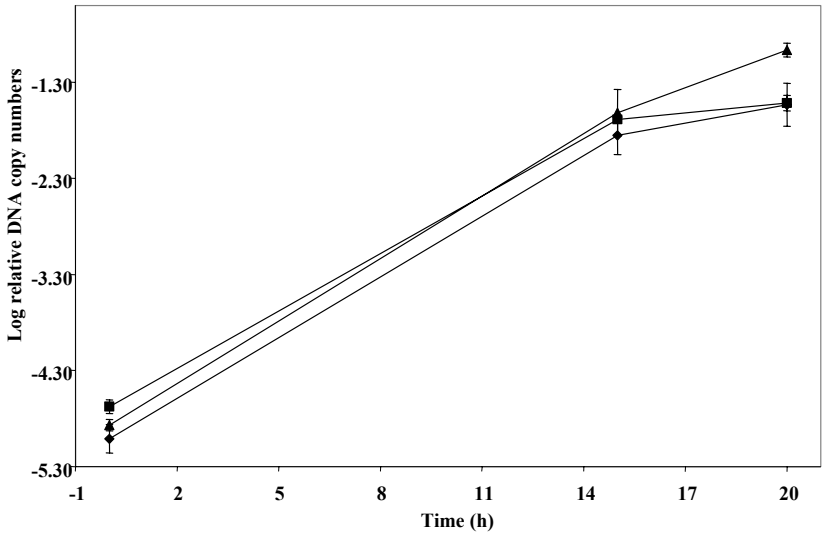

Fig. 17. Growth of *R. oligosporus* determined by real-time PCR in co-inoculation with *Saccharomyces cerevisiae* at different inoculation levels. *R. oligosporus* grown alone (♦), together with yeast at 10^4 cfu/g wet barley (■), together with yeast at 10^5 cfu/g wet barley (▲) (Feng, unpublished, n=3, ±SD).

The real-time PCR quantification developed in this thesis was also used to determine the growth of high-folate production yeasts strains (*Saccharomyces cerevisiae* CBS 7764 and an industrial *S. cerevisiae* strain IS1) during tempeh fermentation and their effects on the growth of *R. oligosporus*.

The growth of *R. oligosporus* in barley tempeh when inoculated alone was quantified by real-time PCR and cfu. The cfu determination basically followed the growth trend determined by real-time PCR (Fig. 18). In co-cultivation with yeasts, the growth of *R. oligosporus* quantified by real-time PCR was not affected by the two yeast strains (Fig. 18).

For comparison, the growth of yeasts was also estimated by cfu determination. The CBS strain, although the initial inoculation level was lower, reached higher levels than the IS1 strain after 20 h (Fig. 19). The real-time PCR data (log relative DNA copies) were highly correlated with cfu data for both strains (CBS: $r=0.96$, $n=16$, $P<0.001$; IS1: $r=0.96$, $n=16$, $P<0.001$).

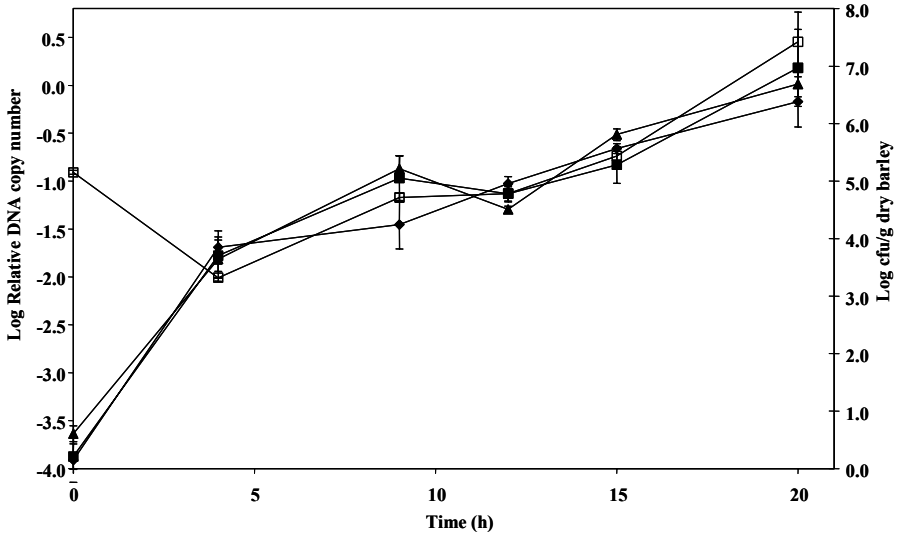

Fig. 18. Growth of *R. oligosporus* alone (♦ real-time PCR quantification, □ cfu) or together with CBS 7764 (■ real-time PCR quantification), or together with ISF (▲real-time PCR quantification) (Feng, unpublished, n=3, ±SD).

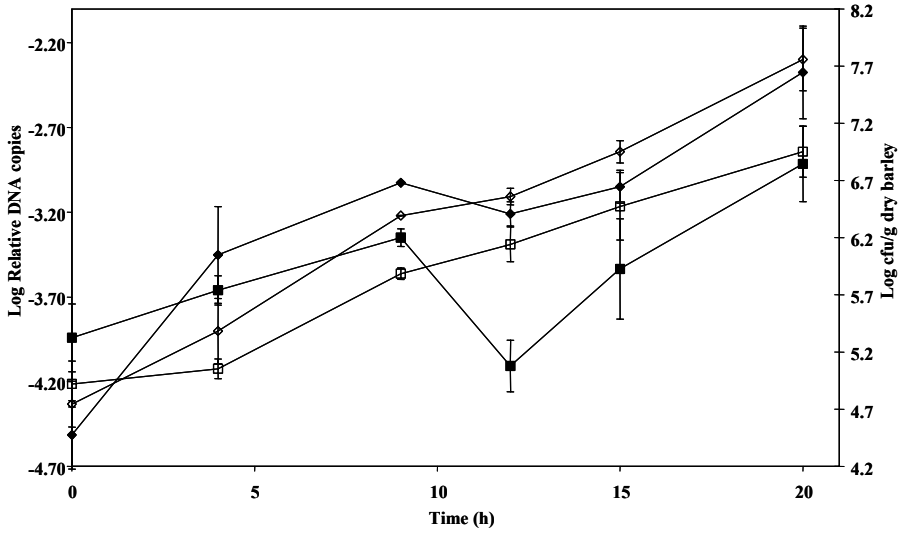

Fig. 19. Growth of *Saccharomyces cerevisiae* CBS 7764 (♦ real-time PCR quantification, ◊ cfu) and IS1 (■ real-time PCR quantification, □ cfu) when co-inoculated with *R. oligosporus* during tempeh fermentation (Feng, unpublished, n=3, ±SD).

Potential online fungal biomass measurement - capacitance and image analysis

The above-mentioned methods for determination of fungal growth during tempeh fermentation are not rapid enough to follow tempeh production on an industrial scale. Instead, online measurement is needed. A technique for measuring fungal growth based on the biomass-dependent changes in electrical capacitance at 0.30 MHz has been developed (Davey *et al.*, 1991; Penaloza *et al.*, 1992). However, with this technique, an electrode is inserted into the substrate, which increases the risk of contamination.

We have instead intended to develop a non-destructive image analysis method (**IV**). The images were taken with a digital colour camera under standardised conditions. Statistical measures sensitive to the colour and structure of the barley tempeh were analysed. The statistical measures were highly correlated with the ergosterol contents (**IV**), which indicates that image analysis could be used to survey the tempeh fermentation process. An image-processing algorithm was also developed to measure the changes in the number of visible grains during barley tempeh fermentation. This algorithm was generally in agreement with the statistical measures. The determination of the number of visible grains was optimised to the end product. Therefore, it was mostly suitable for determining when the final stage of the tempeh fermentation was reached. For barley tempeh fermentation, 5 visible barley grains per petridish was the threshold for onset of the final stage of the tempeh fermentation. This image analysis process could be further developed into online analysis by coupling a digital camera to a computer.

Mathematical modeling of fungal growth

Many mathematic modeling methods have been developed for predicting fungal growth in solid-state substrate fermentation. Mitchell *et al* (1991a) developed a method for describing the growth of *R. oligosporus* in a model solid-state fermentation system. Equations were presented for the release of glucoamylase, the diffusion of glucoamylase, the hydrolysis of starch, the generation and diffusion of glucose, and the uptake of glucose and conversion into new biomass. A cellular automata method was also described to model fungal growth in SSF systems (Laszlo & Silman, 1993).

Production of volatile compounds

Flavour changes during tempeh fermentation could be due to the formation of volatile compounds. A headspace diffusion method was used to collect volatile compounds from several strains of *R. oligosporus* grown on malt extract agar (MEA) plates, soybean tempeh, and barley tempeh (**II**). The ten *R. oligosporus* strains tested on MEA had very different colony morphologies, but still produced similar volatile profiles, except for slight variations among the minor volatile compounds (e.g. sesquiterpenes). Likewise, no differences in volatile profiles were

observed between three strains grown on soybeans. In contrast, the *R. oligosporus* volatile profile on soybean was different from that on barley from the same strain. The main volatile compounds produced by *R. oligosporus* on these three substrates were ethanol, acetone, ethyl acetate, 2-butanone 2-butanone, 2-methyl-1-propanol, 3-methyl-1-butanol and 2-methyl-1-butanol. Acetaldehyde, 2-methyl-propanal and ethyl acetate were also produced in noticeable amounts on MEA and barley, while 2-pentanone, methyl acetate, 2-butanol and 3-methyl-3-buten-1-ol were observed on soybeans. Ethanol, 2-methyl-1-butanol and 3-methyl-1-butanol were the most abundant volatile compounds produced on MEA and barley, while 2-butanone was the dominant volatile metabolite on soybeans (**II**).

Soybean has a beany flavour, probably due to the production of the volatiles hexanal and hexanol (Shogren *et al.*, 2003), while soybean tempeh often has a mushroom flavour (Nout & Rombouts, 1990). This mushroom flavour is generally attributed to the presence of 1-octen-3-ol and 3-octanone (NoelSuberville *et al.*, 1996). Whitaker (1978) suggested that the disappearance of the beany flavour is probably due to the proteolysis of those proteins to which the beany flavour compounds are bound. Nout & Rombouts (1990) hypothesized that the lack of beany flavour in tempeh was due to the result of inactivation of the lipo-oxygenases associated with the formation of such flavours during the boiling stage. Surprisingly, mushroom flavour compounds were not produced by microbial fermentation (**II**), but are present in soybean (Boue *et al.*, 2003) together with beany smell compounds (**II**). The increased mushroom flavour after tempeh fermentation was probably due to the reduction or elimination of beany compounds by the tempeh fungal fermentation (**II**).

Moroe (1985) observed that the production of volatile compounds from tempeh is temperature dependent. He found that the flavour compounds of soybean tempeh fermentated at 31°C included the original soybean components and newly formed 3-methylbutanal, 3-methylbutanol, 2,3-butanediol, acetoin, acetic acid, methylarbitol and iso-valeric acid. In contrast, at 38°C the original soybean components strongly decreased, and instead, 3-methylbutanol, acetoin, tetramethylpyrazine, 2,3-butanediol and iso-valeric acid dominated (Moroe, 1985). The aroma at 38°C is stronger than at 31°C. This suggests that a characteristic aroma of tempeh should be produced under stable temperature conditions.

Different volatile profiles were developed during barley and soybean tempeh fermentation (**II**). These different profiles might contribute to the different odours, e.g. sweet aroma for barley tempeh and mushroom-like aroma for soybean tempeh. Based on this knowledge, it may be possible to produce differently flavoured tempehs by mixing different substrates in different proportions.

The use of different starter cultures may also produce fermented products with different flavours (Supriyanto *et al.*, 1991). However, co-cultivation with *L. plantarum* did not change volatile production during barley tempeh fermentation (**II**). It might not change tempeh flavour.

Effects of fermentation on the nutritional parameters of tempeh

Removal of anti-nutritional compounds

Legume seeds contain a variety of anti-nutritional factors: 1) flatulence-producing factors, such as tri- and tetrasaccharides, raffinose and stachyose, verbascose; 2) protease inhibitors, such as trypsin and chymotrypsin inhibitors; 3) tannins; 4) phytic acid; 5) haemagglutinins or lectins; 6) favism-inducing factors (Nout & Rombouts, 1990). Cereal grains contain several anti-nutritional factors such as 1) phytic acid, 2) tannins, and 3) polyphenols (Sandberg & Svanberg, 1991; Svanberg *et al.*, 1993; Matuschek *et al.*, 2001). During the tempeh fermentation process, *R. oligosporus* can reduce or eliminate these anti-nutritional factors (Hachmeister & Fung, 1993; Rodríguez-Bürger *et al.*, 1998; **III**). Co-inoculation with *R. oryzae* may assist in the degradation of flatulence-producing compounds (Rehms & Barz, 1995; Wiesel *et al.*, 1997). LAB can degrade phytates in sourdough (de Angelis *et al.*, 2003) and reduce many other anti-nutritive factors in other fermented foods (Leroy & de Vuyst, 2004). Co-inoculation with selected LAB strains during barley tempeh fermentation might subsequently improve the nutritional quality of barley tempeh. In our study, 28% of the phytate was reduced during barley tempeh fermentation (**III**). Yeast can produce phytases, but co-fermentation of yeasts with *R. oligosporus* did not reduce phytate further than *R. oligosporus* fermentation alone (**III**). In contrast, 97% of the grain phytate could be removed by further modifying the pre-treatments of barley in the preparation of tempeh (Eklund-Jonsson *et al.*, 2006).

Improved nutrient bioavailability

Tempeh fermentation may increase the bioavailability of proteins, lipids, carbohydrates and minerals such as iron and zinc (Steinkraus *et al.*, 1983; Nout & Rombouts, 1990; Hachmeister & Fung, 1993; Rodríguez-Bürger *et al.*, 1998; Astuti *et al.*, 2000). The growth of the fungus reduced the concentration of low molecular carbohydrates and increased the dietary fibre content (Nout & Rombouts, 1990). Broiler chickens fed with barley tempeh gained more body weight than those fed with unfermented barley (Newman *et al.*, 1985). However, the total or essential amino acid contents and compositions were not changed during barley tempeh fermentation with *R. oligosporus* alone or together with yeasts (**III**).

Vitamin production by fungi during tempeh fermentation

Many vitamins, such as niacin (B_3), riboflavin (B_2), pyridoxine (B_6), pantothenic acid and thiamine, can be produced by the tempeh fungus *R. oligosporus* (Nout & Rombouts, 1990; Mugula, 1992; Shurtleff & Aoyagi, 2001; Nout & Kiers, 2005). *Rhizopus oryzae*, another important fungus frequently found in tempeh products (Samson *et al.*, 1987), has also been reported to produce niacin, vitamin K,

ergosterol, and tocopherol, pyridoxine, riboflavin and biotin (Mugula, 1992; Wiesel *et al.*, 1997). The mycelium of *R. oryzae* tends to be less dense, so it can not be used alone to produce tempeh (Sharma & Sarbhoy, 1984). However, the vitamin contents of tempeh increased when it was co-inoculated with *R. oligosporus* (Mugula, 1992; Wiesel *et al.*, 1997). *R. oryzae* is able to utilize α-galactosides as carbon sources, and therefore grows faster than *R. oligosporus* in mixed culture. Thus, in a mixed-culture fermentation, *R. oligosporus* should be inoculated to a higher level than *R. oryzae* to avoid overgrowth by *R. oryzae* (Wiesel *et al.*, 1997). *R. oryzae* is associated with undesirable sour off-flavours in tempeh due to amylase activity and lactic acid formation from glucose, especially if the tempeh is made from starch-containing raw materials (Hesseltine *et al.*, 1985). In conclusion, *R. oryzae* might not be suitable for barley tempeh production.

Yeasts can also produce various vitamins (Diplock *et al.*, 1961; Fleet, 1990). The addition of specifically selected yeasts can be expected to increase the vitamin contents in tempeh. Co-inoculating with *S. cerevisiae* slightly increased the vitamin B_6 and niacinamide contents during barley tempeh fermentation (**III**).

Vitamin production by bacteria during tempeh fermentation

Vitamin B_{12} is one of the most frequently studied vitamins produced by bacteria. It is normally present in insufficient amounts in vegetarian foods, while it is found in high amounts in animal food sources (Murphy & Allen, 2003). Bacteria such as *Klebsiella pneumoniae*, *Kl. pneumiae* spp. *ozenae*, *Kl. terrigena*, *Kl. planticola* and *Enterobacter cloacae* can produce vitamin B_{12} during tempeh fermentation (Okada *et al.*, 1985b). Especially *Kl. pneumoniae* (formerly *Aerobacter aerogenes*), is considered as being the main species producing vitamin B_{12} in soybean tempeh (Okada *et al.*, 1985b). It is a common organism on plant materials and can grow rapidly at 37°C and pH 5-7, with an optimum temperature for vitamin B_{12} production at 35°C (Suparmo, 1989). Growth of *Kl. pneumoniae* does not interfere with the growth of *R. oligosporus* (Steinkraus *et al.*, 1983). A mixed culture of *R. oligosporus* and *Kl. pneumoniae* has been used to produce a tempeh rich in vitamin B_{12} (Suparmo, 1989). *Citrobacter freundii* and *Brevibacterium epidermidis* can also produce vitamin B_{12} (Wiesel *et al.*, 1997). Other microbes that can produce vitamin B_{12} belong to the genera *Propionibacterium, Pseudomonas*, *Clostridium* and *Streptomyces* (Hachmeister & Fung, 1993). Recently, the probiotic *Lactobacillus reuteri* has also been shown to produce vitamin B_{12} (Taranto *et al.*, 2003). Unfortunately, the strain of *L. reuteri* evaluated by us did not grow well during barley tempeh fermentation (**I**). LAB can also produce other B-group vitamins (Leroy & de Vuyst, 2004; Sanna *et al.*, 2005). Therefore, introducing LAB to tempeh may enhance the vitamin content of tempeh.

Main findings of the thesis

1. Several lactic acid bacteria, including *Lactobacillus plantarum*, *Lactobacillus fermentum*, and yeasts, including *Saccharomyces cerevisiae*, *S. boulardii*, *Pichia anomala* and *Kluyveromyces lactis*, could grow together with *R. oligosporus* without influencing the growth of *R. oligosporus* when inoculated at 4 log cfu/g moist barley (**I** and **III**);
2. Yeast co-inoculation enhanced the ergosterol contents of barley tempeh by 7.1-17.5 µg/g dry tempeh (**III**);
3. *R. oligosporus* produced different volatile profiles in barley and soybean and different strains of *R. oligosporus* produced similar profiles in similar substrates. The mushroom flavour compounds of soybean tempeh, 3-octanone and 1-octen-3-ol, were present in soybean, and were not produced by *R. oligosporus* (**II**);
4. A DNA-extraction method suitable for yeast and filamentous fungus and a specific quantification of *S. cerevisiae* and *R. oligosporus* by a real-time PCR method were developed (**III**);
5. The *Rhizopus* genome copy relative to biomass might increase when fungal mycelial growth was inhibited by yeasts (**III**);
6. A non-destructive image analysis method based on digital images was developed to supervise the barley tempeh fermentation process. The image analysis data were found to be highly correlated with the ergosterol contents of barley tempeh (**IV**).

Future perspectives

Several new questions arise from my work. These mainly relate to the effects of co-inoculation of LAB and yeasts with *R. oligosporus*. My results only show a minimal effect of yeast growth on nutritional parameters of barley tempeh. However, this should be confirmed by feeding assays because the concentrations of nutrients are not always correlated with their bioavailability. It is also possible to use specific strains, such as high-folate producers, to enhance the concentration of a certain nutrient. The reduction of mineral binding phytate in cereals is desirable from a nutritional viewpoint. The phytate in barley tempeh might be reduced further by introducing LAB or yeasts with high phytase activities.

LAB and yeasts have the ability to inhibit the growth of a number of microorganisms. The specific effects of LAB and yeasts on unwanted microorganisms during barley tempeh fermentation should be investigated.

By quantifying the growth of *R. oligosporus* using real-time PCR, it was found that genome copy numbers were not always in good agreement with fungal growth. This was obviously due to a stress response resulting from inhibition by

the yeast. Reliable real-time PCR quantification requires an understanding of the circumstances under which this stress response occurs. Understanding the mechanism of this stress response also provides an interesting topic for basic research on fungal physiology.

Cereal grains, including barley, oat, wheat and rye, are the most important crops in Sweden and other European countries. It should be possible to adapt the techniques developed for barley tempeh to produce tempeh from other cereal grains, further improving the range of nutritionally beneficial plant-based products.

References

Amadi, E.N., Uneze, R., Barimalaa, I.S. & Achinewhu, S.C. 1999. Studies on the production of bambara groundnut (*Vigna subterranea*) tempe. *Plant Foods for Human Nutrition 53*, 199-208.

Anderson, J.W. 2003. Whole grains protect against atherosclerotic cardiovascular disease. *Proceedings of the Nutrition Society 62*, 135-142.

Andlid, T.A., Veide, J. & Sandberg, A.-S. 2004. Metabolism of extracellular inositol hexaphosphate (phytate) by *Saccharomyces cerevisiae*. *International Journal of Food Microbiology 97*, 157-169.

Anon, M.C. 1969. Tempeh: protein-rich food may increase disease resistance. *Agricultural Research (Washington) 17*, 5.

Ashenafi, M. 1991. Growth of *Listeria monocytogenes* in fermenting tempeh made of various beans and its inhibition by *Lactobacillus plantarum*. *Food Microbiology 8*, 303-310.

Ashenafi, M. & Busse, M. 1989. Inhibitory effect of *Lactobacillus plantarum* on *Salmonella infantis*, *Enterobacter aerogenes* and *Escherichia coli* during tempeh fermentation. *Journal of Food Protection 52*, 169-172.

Ashenafi, M. & Busse, M. 1991a. Development of microorganisms during cold storage of pea and chickpea tempeh and effect of *Lactobacillus plantarum* on storage microflora. *Journal of the Science of Food and Agriculture 56*, 71-78.

Ashenafi, M. & Busse, M. 1991b. Growth of *Bacillus cereus* in fermenting tempeh made from various beans and its inhibition by *Lactobacillus plantarum*. *Journal of Applied Bacteriology 70*, 329-333.

Ashenafi, M. & Busse, M. 1991c. Growth potential of *Salmonella infantis* and *Escherichia coli* in fermenting tempeh made from horsebean, pea and chickpea and their inhibition by *Lactobacillus plantarum*. *Journal of the Science of Food and Agriculture 55*, 607-615.

Ashenafi, M. & Busse, M. 1991d. Microbial development during tempeh fermentation from various beans and effect of *Lactobacillus plantarum* on the natural microflora. *International Journal of Food Science and Technology 26*, 501-506.

Ashenafi, M. & Busse, M. 1991e. The microflora of soak water during tempeh production from various beans. *Journal of Applied Bacteriology 70*, 334-338.

Ashenafi, M. & Busse, M. 1992. Growth of *Staphylococcus aureus* in fermenting tempeh made from various beans and its inhibition by *Lactobacillus plantarum*. *International Journal of Food Science and Technology 27*, 81-86.

Astuti, M., Meliala, A., Dalais, F.S. & Wahlqvist, M.L. 2000. Tempe, a nutritious and healthy food from Indonesia. *Asia Pacific Journal of Clinical Nutrition 9*, 322-325.

Badger, J.L., Stins, M.F. & Kim, K.S. 1999. *Citrobacter freundii* invades and replicates in human brain microvascular endothelial cells. *Infection and Immunity 67*, 4208-15.

Behall, K.M., Scholfield, D.J. & Hallfrisch, J. 2004a. Diets containing barley significantly reduce lipids in mildly hypercholesterolemic men and women. *American Journal of Clinical Nutrition 80*, 1185-1193.

Behall, K.M., Scholfield, D.J. & Hallfrisch, J. 2004b. Lipids significantly reduced by diets containing barley in moderately hypercholesterolemic men. *Journal of the American College of Nutrition 23*, 55-62.

Beolchini, F., Pagnanelli, F., Reverberi, A.P. & Veglio, F. 2003. Copper biosorption onto *Rhizopus oligosporus*: pH-edge tests and related kinetic and equilibrium modeling. *Industrial & Engineering Chemistry Research 42*, 4881-4887.

Berg, S., Olsson, J., Swanberg, M., Schnürer, J. & Eriksson, A. 2001. Method for the production of fermented cereal food products and products thereof. In *World Intellectual Property Organization*. Sweden: Olligon AB.

Berghofer, E., Grzeskowiak, B., Mundigler, N., Sentall, W.B. & Walcak, J. 1998. Antioxidative properties of faba bean-, soybean- and oat tempeh. *International Journal of Food Sciences and Nutrition 49*, 45-54.

Beuchat, L.R. 2001. Traditional fermented foods. In *Food Microbiology*. Edited by L.R.B. Michael P. Doyle, Thomas J. Montville. ASM press, American Society for Microbiology. Washington, DC. 701-719. pp.

Bishnoi, N.R. & Garima 2005. Fungus - An alternative for bioremediation of heavy metal containing wastewater: A review. *Journal of Scientific & Industrial Research 64*, 93-100.

Blakeman, J.P., McCrachen, A.R. & Seaby, D.A. 1988. Changes brought about in solid substrates after fermentation of mixtures of cereals and pulses with *Rhizopus oryzae*. *Journal of the Science of Food and Agriculture 45*, 109-118.

Bohn, T., Davidsson, L., Walczyk, T. & Hurrell, R.F. 2004. Phytic acid added to white-wheat bread inhibits fractional apparent magnesium absorption in humans. *American Journal of Clinical Nutrition 79*, 418-23.

Boue, S.M., Shih, B.Y., Carter-Wientjes, C.H. & Cleveland, T.E. 2003. Identification of volatile compounds in soybean at various developmental stages using solid phase microextraction. *Journal of Agricultural and Food Chemistry 51*, 4873-4876.

Buckenhüskes, H.J. 2001. Fermented vegetables. In *Food Microbiology*. Edited by L.R.B. Michael P. Doyle, Thomas J. Montville. ASM press, American Society for Microbiology. Washington, DC. 665-679. pp.

Bååth, E. & Söderström, B. 1980. Comparisons of the agar-film and the membrane-filter methods for the estimation of hyphal lengths in soil, with particular reference to the effect of magnification. *Soil Biology & Biochemistry 12*, 385-387.

Chango, A., Bau, H.M., Villaume, C., Mejean, L. & Nicolas, J.P. 1993. Effects of fermentation by *Rhizopus oligosporus* on chemical composition of sweet white lupin, sweet yellow lupin and bitter yellow lupin. *Sciences des Aliments 13*, 285-295.

Christen, P., Bramorski, A., Revah, S. & Soccol, C.R. 2000. Characterization of volatile compounds produced by *Rhizopus* strains grown on agro-industrial solid wastes. *Bioresource Technology 71*, 211-215.

Correia, R.T.P., McCue, P., Magalhaes, M.M.A., Macedo, G.R. & Shetty, K. 2004a. Production of phenolic antioxidants by the solid-state bioconversion of pineapple waste mixed with soy flour using *Rhizopus oligosporus*. *Process Biochemistry 39*, 2167-2172.

Correia, R.T.P., McCue, P., Vattem, D.A., Magalhaes, M.M.A., Macedo, G.R. & Shetty, K. 2004b. Amylase and *Helicobacter pylori* inhibition by phenolic extracts of pineapple wastes bioprocessed by *Rhizopus oligosporus*. *Journal of Food Biochemistry 28*, 419-434.

Cuevas-Rodriguez, E.O., MiIan-Carrillo, J., Mora-Escobedo, R., Cardenas-Valenzuel, O.G. & Reyes-Moreno, C. 2004. Quality protein maize (*Zea mays L.*) tempeh flour through solid state fermentation process. *Lebensmittel Wissenschaft Und Technologie Food Science and Technology 37*, 59-67.

Damiani, P., Gobbetti, M., Cossignani, L., Corsetti, A., Simonetti, M.S. & Rossi, J. 1996. The sourdough microflora. Characterization of hetero- and homofermentative lactic acid bacteria, yeasts and their interactions on the basis of the volatile compounds produced. *Lebensmittel-Wissenschaft Und -Technologie-Food Science and Technology 29*, 63-70.

Davey, C.L., Penaloza, W., Kell, D.B. & Hedger, J.N. 1991. Real-time monitoring of the accretion of *Rhizopus oligosporus* biomass during the solid-substrate tempe fermentation. *World Journal of Microbiology and Biotechnology 7*, 248-259.

David, I.M. & Jitendra, V. 1981. Modification of tempeh with the addition of bakla (*Vicia faba* Linn). *Journal of Food Technology 16*, 39-50.

de Angelis, M., Gallo, G., Corbo, M.R., McSweeney, P.L.H., Faccia, M., Giovine, M. & Gobbetti, M. 2003. Phytase activity in sourdough lactic acid bacteria: purification and characterization of a phytase from *Lactobacillus sanfranciscensis* CB1. *International Journal of Food Microbiology 87*, 259-270.

Diplock, A.T., Green, J., Edwin, E.E. & Bunyan, J. 1961. Tocopherol, ubiquinones and ubichromenols in yeasts and mushrooms. *Nature 189*, 749-750.

Druvefors, U. (2004). *Yeast biocontrol of grain spoilage moulds,* Swedish University of Agricultural Sciences.

Egounlety, M., Aworh, O.C., Akingbala, J.O., Houben, J.H. & Nago, M.C. 2002. Nutritional and sensory evaluation of tempe-fortified maize-based weaning foods. *International Journal of Food Sciences and Nutrition 53*, 15-27.

Eikenes, M., Hietala, A.M., Alfredsen, G., Fossdal, C.G. & Solheim, H. 2005. Comparison of quantitative real-time PCR, chitin and ergosterol assays for monitoring colonization of *Trametes versicolor* in birch wood. *Holzforschung 59*, 568-573.

Eklund-Jonsson, C., Sandberg, A.-S. & Alminger, M. 2006. Reduction of phytate content while preserving mineral content during whole grain cereal tempe fermentation. *Journal of Cereal Science 44*, 154-160.

Filion, M., St-Arnaud, M. & Jabaji-Hare, S.H. 2003. Direct quantification of fungal DNA from soil substrate using real-time PCR. *Journal of Microbiological Methods 53*, 67-76.

Filtenborg, O., Frisvad, J.C. & Thrane, U. 1996. Moulds in food spoilage. *International Journal of Food Microbiology 33*, 85-102.

Fleet, G.H. 1990. Yeasts in dairy-products. *Journal of Applied Bacteriology 68*, 199-211.

Fudiyansyah, N., Petterson, D.S., Bell, R.R. & Fairbrother, A.H. 1995. A nutritional, chemical and sensory evaluation of lupine (*L. angustifolius*) tempe. *International Journal of Food Science and Technology 30*, 297-305.

Gandjar, I. 1981. Soybean fermentation in Indonesia. In *The 6th international fermentation symposium*, Edited by M.a.R. Young, C. W. London, UK: Pergamon Press. 531-534. pp.

Gibson, R.S., Perlas, L. & Hotz, C. 2006. Improving the bioavailability of nutrients in plant foods at the household level. *Proceedings of the Nutrition Society 65*, 160-168.

Gobbetti, M. 1998. The sourdough microflora: Interactions of lactic acid bacteria and yeasts. *Trends in Food Science & Technology 9*, 267-274.

Gobbetti, M., Corsetti, A. & Rossi, J. 1994a. The Sourdough microflora: Interactions between lactic acid bacteria and yeasts - metabolism of amino acids. *World Journal of Microbiology & Biotechnology 10*, 275-279.

Gobbetti, M., Corsetti, A. & Rossi, J. 1994b. The Sourdough microflora: Interactions between lactic acid bacteria and yeasts - metabolism of carbohydrates. *Applied Microbiology and Biotechnology 41*, 456-460.

Goh, N., Sato, Z., Yaoita, T. & Aoyagi, K. 1978. Studies on the control of *Rhizopus* in the nursery cases of rice seedlings. In *Proc Assoc Plant Protect*. Shikoku90-94. pp.

Gonzalez, R.N., Wilson, D.J., Sickles, S.A., Zurakowski, M.J., Weybrecht, P.M. & Walsh, A.K. 2001. Outbreaks of clinical mastitis caused by *Trichosporon beigelii* in dairy herds. *Journal of the American Veterinary Medical Association 218*, 238.

Granfeldt, Y., Liljeberg, H., Drews, A., Newman, R. & Björck, I. 1994. Glucose and insulin responses to barley products - Influence of food structure and amylose-amylopectin ratio. *American Journal of Clinical Nutrition 59*, 1075-1082.

Grattepanche, F., Lacroix, C., Audet, P. & Lapointe, G. 2005. Quantification by real-time PCR of *Lactococcus lactis* subsp *cremoris* in milk fermented by a mixed culture. *Applied Microbiology and Biotechnology 66*, 414-421.

Hachmeister, K.A. & Fung, D.Y.C. 1993. Tempeh: a mold-modified indigenous fermented food made from soybeans and/or cereal-grains. *Critical Reviews in Microbiology 19*, 137-188.

Hallberg, L., Brune, M. & Rossander, L. 1989. Iron-absorption in man - ascorbic-acid and dose-dependent inhibition by phytate. *American Journal of Clinical Nutrition 49*, 140-144.

Han, B.Z., Ma, Y., Rombouts, F.M. & Nout, M.J.R. 2003. Effects of temperature and relative humidity on growth and enzyme production by *Actinomucor elegans* and *Rhizopus oligosporus* during sufu pehtze preparation. *Food Chemistry 81*, 27-34.

Harris, C.M. & Kell, D.B. 1985. The estimation of microbial biomass. *Biosensors 1*, 17-84.

Henkel, T.W. 2005. Parakari, an indigenous fermented beverage using amylolytic *Rhizopus* in Guyana. *Mycologia 97*, 1-11.

Hesseltine, C.-W. & Wang, H.-L. 1980. The importance of traditional fermented foods. *BioScience 30*, 402-404.

Hesseltine, C.W., Featherston, C.L., Lombard, G.L. & Dowell, V.R., Jr. 1985. Anaerobic growth of molds isolated from fermentation starters used for foods in Asian countries. *Mycologia 77*, 390-400.

Hesseltine, C.W., Smith, M., Bradle, B. & Djien, K.S. 1963. Investigations of tempeh, an Indonesian food. *Developments in Industrial Microbiology 4*, 275-287.

Hesseltine, C.W., Smith, M. & Wang, H.L. 1967. New fermented cereal products. *Developments in Industrial Microbiology 8*, 179-186.
Holzapfel, W.H. 2002. Appropriate starter culture technologies for small-scale fermentation in developing countries. *International Journal of Food Microbiology 75*, 197-212.
Homma, S., Aida, K. & Fujimaki, M. 1983. Food processing of winged bean (*Psophocarpus tetragonolobus*), Proceedings of the 6th International Congress of Food Science and Technology. In *The 6th International Congress of Food Science and Technology*94-95. pp.
Jennessen, J., Nielsen, K.F., Houbraken, J., Lyhne, E.K., Schnürer, J., Frisvad, J.C. & Samson, R.A. 2005. Secondary metabolite and mycotoxin production by the *Rhizopus microsporus* group. *Journal of Agricultural and Food Chemistry 53*, 1833-1840.
Jha, K. & Verma, J. 1980. Removal of flatulence principles from legumes by mold fermentation. *Indian Journal of Experimental Biology 18*, 658-9.
Jujin, W., Hanhu, J. & Zhenli, L. 2000. Study on tempeh sausage. *Meat Industry (In Chinese) 8*, 17-19.
Juntunen, K.S., Niskanen, L.K., Liukkonen, K.H., Poutanen, K.S., Holst, J.J. & Mykkanen, H.M. 2002. Postprandial glucose, insulin, and incretin responses to grain products in healthy subjects. *The American journal of clinical nutrition 75*, 254-62.
JuQin, W., Mingshen, D. & Hanhu, J. 2001. Study of tempeh icecream. *Science and Technology of Food Industry (In chinese) 22*, 47-48.
Järvi, A.E., Karlstrom, B.E., Granfeldt, Y.E., Bjorck, I.M.E., Vessby, B.O.H. & Asp, N.G.L. 1995. The influence of food structure on postprandial metabolism in patients with non-insulin-dependent diabetes-mellitus. *American Journal of Clinical Nutrition 61*, 837-842.
Kalavi, F.N.M., Muroki, N.M., Omwega, A.M. & Mwadime, R.K.N. 1996. Effect of tempe-yellow maize porridge and milk-yellow maize porridge on growth rate, diarrhoea and duration of rehabilitation of malnourished children. *East African Medical Journal 73*, 427-431.
Kariluoto, S., Aittamaa, M., Korhola, M., Salovaara, H., Vahteristo, L. & Piironen, V. 2006. Effects of yeasts and bacteria on the levels of folates in rye sourdoughs. *International Journal of Food Microbiology 106*, 137-143.
Keuth, S. & Bisping, B. 1993. Formation of vitamins by pure cultures of tempe moulds and bacteria during the tempe solid substrate fermentation. *Journal of Applied Bacteriology 75*, 427-434.
Keuth, S. & Bisping, B. 1994. Vitamin B12 production by *Citrobacter freundii* or *Klebsiella pneumoniae* during tempeh fermentation and proof of enterotoxin absence by PCR. *Applied and Environmental Microbiology 60*, 1495-1499.
Kiers, J.L., Nout, M.J.R., Rombouts, F.M., Nabuurs, M.J.A. & van der Meulen, J. 2002. Inhibition of adhesion of enterotoxigenic *Escherichia coli* K88 by soya bean tempe. *Letters in Applied Microbiology 35*, 311-315.
Kiers, J.L., Nout, R.M.J. & Rombouts, F.M. 2000. *In vitro* digestibility of processed and fermented soya bean, cowpea and maize. *Journal of the Science of Food and Agriculture 80*, 1325-1331.
Kimaryo, V.M., Massawe, G.A., Olasupob, N.A. & Holzapfel, W.H. 2000. The use of a starter culture in the fermentation of cassava for the production of "kivunde", a traditional Tanzanian food product. *International Journal of Food Microbiology 56*, 179-190.
Ko Swan, D. & Hesseltine, C.W. 1979. Tempe and related foods. *Economic Microbiology 4*, 115-140.
Ko Swan, D. & Kelholt, A.J. 1981. The inhibitive effect of *Rhizopus oligosporus* on toxin production by *Pseudomonas cocovenenans* in tempe bongkrek. *Annales Bogorienses 7*, 97-106.
Kobayasi, S.Y., Okazaki, N. & Koseki, T. 1992. Purification and characterization of an antibiotic substance produced from *Rhizopus oligosporus* IFO 8631. *Bioscience, Biotechnology, and Biochemistry 56*, 94-98.
Lambo, A.M., Öste, R. & Nyman, M.E.G.L. 2005. Dietary fibre in fermented oat and barley beta-glucan rich concentrates. *Food Chemistry 89*, 283-293.

Laszlo, J.A. & Silman, R.W. 1993. Cellular-automata simulations of fungal growth on solid substrates. *Biotechnology Advances 11*, 621-633.
Leal-Klevezas, D.S. & Martinez-Soriano, J.P. 2001. A modified and simple method for the extraction of genomic DNA from a wide variety of animal and plant tissues. *Australasian Biotechnology 11*, 32-34.
Leroy, F. & de Vuyst, L. 2004. Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends in Food Science & Technology 15*, 67-78.
Li-Te, L., Jian-hua, Z., Zai-gui, L. & Tat-sumi, E. 2003. The comparison of natto, tempeh and douchi. *China Condiment (In Chinese) 5*, 3-7.
Li-Te, L., Yin, L.J. & Saito, M. 2004. Function of traditional foods and food culture in China. *Jarq-Japan Agricultural Research Quarterly 38*, 213-220.
Liem, I.T.H., Steinkraus, K.H. & Cronk, T.C. 1977. Production of vitamin B-12 in tempeh, a fermented soybean food. *Applied and Environmental Microbiology 34*, 773-776.
Liljeberg, H., Granfeldt, Y. & Björck, I. 1992. Metabolic responses to starch in bread containing intact kernels versus milled flour. *European Journal of Clinical Nutrition 46*, 561-575.
Lin, X.R. & Momany, M. 2004. Identification and complementation of abnormal hyphal branch mutants ahbA1 and ahbB1 in *Aspergillus nidulans*. *Fungal Genetics and Biology 41*, 998-1006.
Mackay, I.M. 2004. Real-time PCR in the microbiology laboratory. *Clinical Microbiology and Infection 10*, 190-212.
Marquart, L., Wiemer, K.L., Jones, J.M. & Jacob, B. 2003. Whole grain health claims in the USA and other efforts to increase whole-grain consumption. *Proceedings of the Nutrition Society 62*, 151-159.
Matcham, S.E., Jordan, B.R. & Wood, D.A. 1985. Estimation of fungal biomass in a solid substrate by three independent methods. *Applied Microbiology and Biotechnology 21*, 108-112.
Matsuo, M. 1990. Suitability of 'okara tempe' as foodstuff. *Nippon Nogeikagaku Kaishi 64*, 1235-1236.
Matsuo, M. 2006. Suitability of quinoa fermented with *Rhizopus oligosporus* as an ingredient of biscuit. *Journal of the Japanese Society for Food Science and Technology-Nippon Shokuhin Kagaku Kogaku Kaishi 53*, 62-69.
Matuschek, E., Towo, E. & Svanberg, U. 2001. Oxidation of polyphenols in phytate-reduced high-tannin cereals: Effect on different phenolic groups and on *in vitro* accessible iron. *Journal of Agricultural and Food Chemistry 49*, 5630-5638.
Mayer, Z., Bagnara, A., Farber, P. & Geisen, R. 2003. Quantification of the copy number of nor-1, a gene of the aflatoxin biosynthetic pathway by real-time PCR, and its correlation to the cfu of *Aspergillus flavus* in foods. *International Journal of Food Microbiology 82*, 143-151.
McCue, P., Horii, A. & Shetty, K. 2003. Solid-state bioconversion of phenolic antioxidants from defatted soybean powders by *Rhizopus oligosporus*: Role of carbohydrate-cleaving enzymes. *Journal of Food Biochemistry 27*, 501-514.
McCue, P., Lin, Y.T., Labbe, R.G. & Shetty, K. 2004. Sprouting and solid-state bioprocessing by *Rhizopus oligosporus* increase the *in vitro* antibacterial activity of aqueous soybean extracts against *Helicobacter pylori*. *Food Biotechnology 18*, 229-249.
McKeown, N.M. 2004. Whole grain intake and insulin sensitivity: evidence from observational studies. *Nutrition Reviews 62*, 286-91.
Merk, K., Borelli, C. & Korting, H.C. 2005. Lactobacilli - bacteria-host interactions with special regard to the urogenital tract. *International Journal of Medical Microbiology 295*, 9-18.
Mitchell, D.A., Do, D.D., Greenfield, P.F. & Doelle, H.W. 1991. A semimechanistic mathematical-model for growth of *Rhizopus oligosporus* in a model solid-state fermentation system. *Biotechnology and Bioengineering 38*, 353-362.
Mitchell, D.A., Greenfield, P.F. & Doelle, H.W. 1991. An empirical-model of growth of *Rhizopus oligosporus* in solid- state fermentation. *Journal of Fermentation and Bioengineering 72*, 224-226.

Mokoena, M.P., Chelule, P.K. & Gqaleni, N. 2005. Reduction of fumonisin B-1 and zearalenone by lactic acid bacteria in fermented maize meal. *Journal of Food Protection 68*, 2095-2099.
Moroe, M. 1985. Asian symposium on non-salted soybean fermentation. Tsukuba, Japan: Tsukuba science. 210-218. pp.
Mugula, J.K. 1992. Evaluation of the nutritive value of maize-soybean tempe as a potential weaning food in Tanzania. *International Journal of Food Sciences and Nutrition 43*, 113-119.
Mugula, J.K. & Lyimo, M. 1999. Evaluation of the nutritional quality and acceptability of fingermillet-based tempe as potential weaning foods in Tanzania. *International Journal of Food Sciences and Nutrition 50*, 275-282.
Murphy, S.P. & Allen, L.H. 2003. Nutritional importance of animal source foods. *Journal of Nutrition 133*, 3932S-3935S.
Newman, R.K., Newman, C.W. & Eslick, R.F. 1985. Effect of fungal fermentation and other treatments on nutritional value of waxy barley fed to chicks. *Poultry Science 64*, 1514-1518.
Njoku, H.O., Ofuya, C.O. & Ogbulie, J.N. 1991. Production of tempeh from the African yam bean (*Sphenostylis stenocarpa Hams*). *Food Microbiology 8*, 209-214.
NoelSuberville, C., Cruz, C., Guinberteau, J. & Montury, M. 1996. Correlation between fatty acid content and aromatic compound release in fresh blewit (*Lepista nuda*). *Journal of Agricultural and Food Chemistry 44*, 1180-1183.
Nout, M.J.R. 1989. Effect of *Rhizopus* and *Neurospora* spp. on growth of *Aspergillus flavus* and *A. parasiticus* and accumulation of aflatoxin B1 in groundnut. *Mycological Research 93*, 518-523.
Nout, M.J.R. 1995. Fungal interactions in food fermentations. *Canadian Journal of Botany-Revue Canadienne De Botanique 73*, S1291-S1300.
Nout, M.J.R., Beernink, G. & Bonants van Laarhoven, T.M.G. 1987. Growth of *Bacillus cereus* in soyabean tempeh. *International Journal of Food Microbiology 4*, 293-301.
Nout, M.J.R., Bonants van Laarhoven, T.M.G., Jongh, P.D. & Koster, P.G.D. 1987a. Ergosterol content of *Rhizopus oligosporus* NRRL 5905 grown in liquid and solid substrates. *Applied Microbiology and Biotechnology 26*, 456-461.
Nout, M.J.R., Dreu, M.A.d., Zuurbier, A.M. & Bonants van Laarhoven, T.M.G. 1987b. Ecology of controlled soyabean acidification for tempe manufacture. *Food Microbiology 4*, 165-172.
Nout, M.J.R. & Kiers, J.L. 2005. Tempe fermentation, innovation and functionality: update into the third millenium. *Journal of Applied Microbiology 98*, 789-805.
Nout, M.J.R. & Rombouts, F.M. 1990. Recent developments in tempe research. *Journal of Applied Bacteriology 69*, 609-633.
Nowak, J. 1992. Oats tempeh. *Acta Biotechnologica 12*, 345-348.
Nowak, J. & Steinkraus, K.H. 1988. Effect of tempeh fermentation of peas on their potential flatulence productivity as measured by gas production and growth of *Clostridium perfringens*. *Nutrition Reports International 38*, 1163-1171.
O'Toole, D.K. 1999. Characteristics and use of okara, the soybean residue from soy milk production--a review. *Journal of Agricultural and Food Chemistry 47*, 363-71.
Okada, N., Hadioetomo, R.S., Nikkuni, S. & Itoh, H. 1985a. Isolation of bacteria producing vitamin B12 from fermented soybean tempeh from Indonesia. *Report of the National Food Research Institute [Shokuryo-Kenkyusho-Kenkyu-Hokoku] 46*, 15-20.
Okada, N., Hariantono, J., Hadioetomo, R.S., Nikkuni, S. & Itoh, H. 1985b. Survey of vitamin B12-producing bacteria isolated from Indonesian tempeh. *Report of the National Food Research Institute [Shokuryo-Kenkyusho-Kenkyu-Hokoku] 47*, 49-56.
Olson, F.C.W. 1950. Quantitative estimation of filamentous algae. *Transaction of the American microscopy society 59*, 272-279.
Oscarsson, M., Andersson, R., Salomonsson, A.C. & Åman, P. 1996. Chemical composition of barley samples focusing on dietary fibre components. *Journal of Cereal Science 24*, 161-170.
Othman, M.R. & Amin, A.M. 2003. Comparative analysis on equilibrium sorption of metal ions by biosorbent tempe. *Biochemical Engineering Journal 16*, 361-364.

Ouwehand, A.C. & Salminen, S.J. 1998. The health effects of cultured milk products with viable and non-viable bacteria. *International Dairy Journal 8*, 749-758.
Paredes Lopez, O., Harry, G.I. & Gonzalez Castaneda, J. 1990. Sensory evaluation of tempeh produced by fermentation of common beans. *Journal of Food Science 55*, 123-126.
Paredeslopez, O., Gonzalezcastaneda, J. & Carabeztrejo, A. 1991. Influence of solid substrate fermentation on the chemical-composition of chickpea. *Journal of Fermentation and Bioengineering 71*, 58-62.
Partida-Martinez, L.P. & Hertweck, C. 2005. Pathogenic fungus harbours endosymbiotic bacteria for toxin production. *Nature 437*, 884-888.
Pasanen, A.L., Yli-Pietila, K., Pasanen, P., Kalliokoski, P. & Tarhanen, J. 1999. Ergosterol content in various fungal species and biocontaminated building materials. *Applied and Environmental Microbiology 65*, 138-142.
Penaloza, W., Davey, C.L., Hedger, J.N. & Kell, D.B. 1992. Physiological studies on the solid-state quinoa tempe fermentation, using online measurements of fungal biomass production. *Journal of the Science of Food and Agriculture 59*, 227-235.
Petersson, S., Hansen, M.W., Axberg, K., Hult, K. & Schnürer, J. 1998. Ochratoxin A accumulation in cultures of *Penicillium verrucosum* with the antagonistic yeast *Pichia anomala* and *Saccharomyces cerevisiae*. *Mycological Research 102*, 1003-1008.
Pierides, M., El-Nezami, H., Peltonen, K., Salminen, S. & Ahokas, J. 2000. Ability of dairy strains of lactic acid bacteria to bind aflatoxin M-1 in a food model. *Journal of Food Protection 63*, 645-650.
Pimentel, D. & Pimentel, M. 2003. Sustainability of meat-based and plant-based diets and the environment. *American Journal of Clinical Nutrition 78*, 660S-663S.
Reddy, N.R., Sathe, S.K. & Salunkhe, D.K. 1982. Phytates in cereals and legumes. *Advances in Food Research 28*, 1-92.
Rehms, H. & Barz, W. 1995. Degradation of stachyose, raffinose, melibiose and sucrose by different tempe-producing *Rhizopus* fungi. *Applied Microbiology and Biotechnology 44*, 47-52.
Reyes-Moreno, C., Cuevas-Rodriguez, E.O., Milan-Carrillo, J., Cardenas-Valenzuela, O.G. & Barron-Hoyos, J. 2004. Solid state fermentation process for producing chickpea (*Cicer arietinum L*) tempeh flour. Physicochemical and nutritional characteristics of the product. *Journal of the Science of Food and Agriculture 84*, 271-278.
Rimsten, L., Haraldsson, A.K., Andersson, R., Alminger, M., Sandberg, A.S. & Åman, P. 2002. Effects of malting on beta-glucanase and phytase activity in barley grain. *Journal of the Science of Food and Agriculture 82*, 904-912.
Rodríguez-Bürger, A.P., Mason, A. & Nielsen, S.S. 1998. Use of fermented black beans combined with rice to develop a nutritious weaning food. *Journal of Agricultural and Food Chemistry*
Rozan, P., Villaume, C., Bau, H.M., Schwertz, A., Nicolas, J.P. & Mejean, L. 1996. Detoxication of rapeseed meal by *Rhizopus oligosporus* sp-T3: a first step towards rapeseed protein concentrate. *International Journal of Food Science &Technology 31*, 85-90.
Samson, R.A. 1985. *Taxonomic considerations of beneficial and harmful moulds in food.* Japan Scientific Societie Press. Arai, T., Tokyo, Japan pp.
Samson, R.A., Kooij, J.A.v. & Boer, E.d. 1987. Microbiological quality of commercial tempeh in the Netherlands. *Journal of Food Protection 50*, 92-94.
Sandberg, A.S. & Svanberg, U. 1991. Phytate hydrolysis by phytase in cereals - effects on invitro estimation of iron availability. *Journal of Food Science 56*, 1330-1333.
Sandström, B. & Sandberg, A.S. 1992. Inhibitory effects of isolated inositol phosphates on zinc absorption in humans. *Journal of Trace Elemments and Electrolytes in Health and Disease 6*, 99-103.
Sanna, M.G., Mangia, N.P., Garau, G., Murgia, M.A., Massa, T., Franco, A. & Deiana, P. 2005. Selection of folate-producing lactic acid bacteria for improving fermented goat milk. *Italian Journal of Food Science 17*, 143-154.

Scalabrini, P., Rossi, M., Spettoli, P. & Matteuzzi, D. 1998. Characterization of *Bifidobacterium* strains for use in soymilk fermentation. *International Journal of Food Microbiology 39*, 213-219.
Schipper, M.A.A. & Stalpers, J.A. 1984. A revision of the genus *Rhizopus*. *Studies in Mycology 25*, 1-34.
Schnürer, J. 1993. Comparison of methods for estimating the biomass of three food-borne fungi with different growth patterns. *Applied and Environmental Microbiology 59*, 552-555.
Schnürer, J., Clarholm, M. & Rosswall, T. 1985. Microbial biomass and activity in an agricultural soil with different organic matter contents. *Soil Biology & Biochemistry 17*, 611-618.
Schnürer, J. & Magnusson, J. 2005. Antifungal lactic acid bacteria as biopreservatives. *Trends in Food Science & Technology 16*, 70-78.
Seitz, L.M., Sauer, D.B., Burroughs, R., Mohr, H.E. & Hubbard, J.D. 1979. Ergosterol as a measure of fungal growth. *Phytopathology 69*, 1202-1203.
Sharma, A. & Kapoor, A.C. 1996. Levels of antinutritional factors in pearl millet as affected by processing treatments and various types of fermentation. *Plant Foods for Human Nutrition 49*, 241-252.
Sharma, R., John, S.J., Damgaard, M. & McAllister, T.A. 2003. Extraction of PCR-quality plant and microbial DNA from total rumen contents. *Biotechniques 34*, 92-4, 96-7.
Sharma, R. & Sarbhoy, A.K. 1984. Tempeh-a fermented food from soybean. *Current Science 53*, 325-326.
Shetty, P.H. & Jespersen, L. 2006. *Saccharomyces cerevisiae* and lactic acid bacteria as potential mycotoxin decontaminating agents. *Trends in Food Science & Technology 17*, 48-55.
Shimosato, T., Kimura, T., Tohno, M., Iliev, I.D., Katoh, S., Ito, Y., Kawai, Y., Sasaki, T., Saito, T. & Kitazawa, H. 2006. Strong immunostimulatory activity of AT-oligodeoxynucleotide requires a six-base loop with a self-stabilized 5 '-C. G-3 ' stem structure. *Cellular Microbiology 8*, 485-495.
Shogren, R.L., Mohamed, A.A. & Carriere, C.J. 2003. Sensory analysis of whole wheat/soy flour breads. *Journal of Food Science 68*, 2141-2145.
Shurtleff, W. & Aoyagi, A. 2001. *The book of tempeh*. 2 2. Ten Speed Press. Berkeley, California pp.
Skrede, G., Herstad, O., Sahlstrom, S., Holck, A., Slinde, E. & Skrede, A. 2003. Effects of lactic acid fermentation on wheat and barley carbohydrate composition and production performance in the chicken. *Animal Feed Science and Technology 105*, 135-148.
Slavin, J., Jacobs, D. & Marquart, L. 1997. Whole-grain consumption and chronic disease: Protective mechanisms. *Nutrition and Cancer: an International Journal 27*, 14-21.
Sparringa, R.A. & Owens, J.D. 1999a. Causes of alkalinization in tempe solid substrate fermentation. *Enzyme and Microbial Technology 25*, 677-681.
Sparringa, R.A. & Owens, J.D. 1999b. Glucosamine content of tempe mould, *Rhizopus oligosporus*. *International Journal of Food Microbiology 47*, 153-157.
Stahl, P.D., Parkin, T.B. & Eash, N.S. 1995. Sources of error in direct microscopic methods for estimation of fungal biomass in soil. *Soil Biology & Biochemistry 27*, 1091-1097.
Steinkraus, K.H., Cullen, R.E., Pederson, C.S., Nellis, L.F. & Gavitt, B.K. 1983. Indonesian tempeh and related fermentations. In *Handbook of indigenous fermented foods*. Edited by K.H. Steinkraus, R.E. Cullen, C.S. Pederson, L.F. Nellis & B.K. Gavitt. New York, Marcel Dekker. 1-94. pp.
Stien, G., Blanchard, F., Rondags, E. & Marc, I. 1999. An on-line method for the measurement of diacetyl and acetaldehyde in yoghurt, fermented milk, butter and margarine. *Lait 79*, 615-624.
Struve, C. & Krogfelt, K.A. 2004. Pathogenic potential of environmental *Klebsiella pneumoniae* isolates. *Environmental Microbiology 6*, 584-590.
Suparmo 1989. Vitamin B-12 formation in tempeh fermented by mixed-culture. *Dissertation Abstracts International,-B 49*, 4621.

Supriyanto, Wada, K., Hayakawa, I. & Fujio, Y. 1991. Principal component analysis of aroma profiles of tempeh produced by using various starters. *Hakkokogaku Kaishi-Journal of the Society of Fermentation Technology 69*, 331-336.
Svanberg, U., Lorri, W. & Sandberg, A.S. 1993. Lactic fermentation of non-tannin and high-tannin cereals - Effects on *in vitro* estimation of iron availability and phytate hydrolysis. *Journal of Food Science 58*, 408-412.
Takahashi, M., Iwasaki, S., Kobayashi, H., Okuda, S., Murai, T., Sato, Y., Haraguchi-Hiraoka, T. & Nagano, H. 1987. Studies on macrocyclic lactone antibiotics. XI. Anti-mitotic and anti-tubulin activity of new antitumor antibiotics, rhizoxin and its homologues. *The Journal of Antibiotics (Tokyo) 40*, 66-72.
Taranto, M.P., Vera, J.L., Hugenholtz, J., De Valdez, G.F. & Sesma, F. 2003. *Lactobacillus reuteri* CRL1098 produces cobalamin. *Journal of Bacteriology 185*, 5643-7.
Truswell, A.S. 2002. Cereal grains and coronary heart disease. *European Journal of Clinical Nutrition 56*, 1-14.
Tsuruo, T., Ohhara, T., Iida, H., Tsukagoshi, S., Sato, Z., Matsuda, I., Iwasaki, S., Okuda, S., Shimizu, F., Sasagawa, K., Fukami, M., Fukuda, K. & Arakawa, M. 1986. Rhizoxin, a macrocyclic lactone antibiotic, as a new antitumor agent against human and murine tumor-cells and their vincristine-resistant sublines. *Cancer Research 46*, 381-385.
Tuncel, G., Nout, M.J.R., Brimer, L. & Goektan, D. 1990. Toxicological, nutritional and microbiological evaluation of tempe fermentation with *Rhizopus oligosporus* of bitter and sweet apricot seeds. *International Journal of Food Microbiology 11*, 337-344.
Vaidehi, M.P. & Rathnamani, A. 1990. The shelf-life of soy-sunflower tempeh and its acceptability to Indian children. *Food and Nutrition Bulletin 12*, 53-56.
Vaidehi, M.P., Sumangala, S.G. & Vijayakumari, J. 1996. Tempe-based ready-to-prepare food mixes of high nutritional value. *Journal of Food Science and Technology 33*, 506-509.
Vaitilingom, M., Gendre, F. & Brignon, P. 1998. Direct detection of viable bacteria, molds, and yeasts by reverse transcriptase PCR in contaminated milk samples after heat treatment. *Applied and Environmental Microbiology 64*, 1157-1160.
Walker, G.M. 1999. *Yeast - Physiology and biotechnology*. John Wiley and Sons. Chichester pp.
van der Aa Kuhle, A., Skovgaard, K. & Jespersen, L. 2005. *In vitro* screening of probiotic properties of *Saccharomyces cerevisiae* var. *boulardii* and food-borne *Saccharomyces cerevisiae* strains. *International Journal of Food Microbiology 101*, 29-39.
Wang, H.L., Ruttle, D.I. & Hesseltine, C.W. 1968. Protein quality of wheat and soybeans after *Rhizopus oligosporus* fermentation. *Journal of Nutrition 96*, 109-114.
Wang, H.L., Ruttle, D.I. & Hesseltine, C.W. 1969. Antibacterial compound from a soybean product fermented by *Rhizopus oligosporus*. *Proceedings of the Society for Experimental Biology and medicine 131*, 579-583.
Varzakas, T. 1998. *Rhizopus oligosporus* mycelial penetration and enzyme diffusion in soya bean tempe. *Process Biochemistry 33*, 741-747.
Vattem, D.A., Lin, Y.T., Labbe, R.G. & Shetty, K. 2004. Antimicrobial activity against select food-borne pathogens by phenolic antioxidants enriched in cranberry pomace by solid-state bioprocessing using the food grade fungus *Rhizopus oligosporus*. *Process Biochemistry 39*, 1939-1946.
Vaughan, A., O'Sullivan, T. & van Sinderen, D. 2005. Enhancing the microbiological stability of malt and beer - A review. *Journal of the Institute of Brewing 111*, 355-371.
Whitaker, J.R. 1978. Biochemical changes occurring during the fermentation of high-protein foods. *Food Technology 32*, 175-180.
Wiesel, I., Rehm, H.J. & Bisping, B. 1997. Improvement of tempe fermentations by application of mixed cultures consisting of *Rhizopus* sp. and bacterial strains. *Applied Microbiology and Biotechnology 47*, 218-225.
Wilhelm, J. & Pingoud, A. 2003. Real-time polymerase chain reaction. *ChemBioChem 4*, 1120-1128.
Wilson, T., Rabie, C.J., Fincham, J.E., Steyn, P.S. & Schipper, M.A.A. 1984. Toxicity of rhizonin A, isolated from *Rhizopus microsporus*, in laboratory animals. *Food and Chemical Toxicology 22*, 275-281.

Wisselink, H.W., Weusthuis, R.A., Eggink, G., Hugenholtz, J. & Grobben, G.J. 2002. Mannitol production by lactic acid bacteria: a review. *International Dairy Journal 12*, 151-161.

Wood, P.J. 2004. Relationships between solution properties of cereal beta-glucans and physiological effects - a review. *Trends in Food Science & Technology 15*, 313-320.

Yiannikouris, A., Francois, J., Poughon, L., Dussap, C.G., Bertin, G., Jeminet, G. & Jouany, J.P. 2004. Adsorption of zearalenone by beta-D-glucans in the *Saccharomyces cerevisiae* cell wall. *Journal of Food Protection 67*, 1195-1200.

Yiannikouris, A., Poughon, L., Cameleyre, X., Dussap, C.G., Francois, J., Bertin, G. & Jouany, J.P. 2003. A novel technique to evaluate interactions between *Saccharomyces cerevisiae* cell wall and mycotoxins: application to zearalenone. *Biotechnology Letters 25*, 783-789.

Yu, X., Chai, L.Y. & Min, X.B. 2003. Removal of lead in wastewater by immobilized inactivated cells of *Rhizopus oligosporus*. *Journal of Central South University of Technology 10*, 313-317.

Yuksekdag, Z.N., Beyatli, Y. & Aslim, B. 2004. Antimicrobial activity of *Lactobacillus* species isolated from kefir. *Indian Veterinary Journal 81*, 687-690.