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review

The Use of the Mould *Rhizopus oligosporus* in Food Production

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Summary

Filamentous fungi are used in food production for protecting the surface of article against spoilage, as well as for the improvement of taste and the biological or nutritional value. A variety of moulds are used in the preparation of fermented foods. However, ***Rhizopus oligosporus*** *has been one of the most widely used and accepted moulds in fermentation of vegetal substrates. The fermented product made from soaked and cooked soybeans or cereal grains inoculated with a mould is called the tempeh. In the bioprocess, the substrate is bound together into a cake by dense white mycelium. An important function of mould in the fermentation process is the synthesis of enzymes that hydrolyse the components and contribute to the development of a desirable texture, flavour, and aroma of the product. Fermentation also eliminates antinutritional constituents, and the nutritional quality of the fermented product is thus improved. Tempeh is an attractive product from sensory, health and economical points of view.*

Keywords: fermented food, *Rhizopus oligosporus*, tempeh

Introduction

Current technology and new scientific advances have enabled researchers to test specific strains and numerous substrates used for the production of fermented products. The major part of total food production is of plant origin, the remainder being derived from animal, marine, and single-cell sources. There is an increasing interest in high quality plant foods as replacements or supplements to animal sources. Not only are plant sources nutritious, but they are more readily available and cheaper than animal sources. Solid state fermentation of plant materials offers a promising option in achieving such new, high quality products. Soybeans are reported to be the most frequent substrate, but many other legumes could be fermented to delicious products (*1*). In some regions of the world, cereals such as barley, wheat, sorghum, oats, rye, corn, and triticale are used as fermentation substrates (*2*). Fermentation of germinated soybeans was noted by Suparmo and Markakis (*3*). Mixtures of cereals, beans and groundnuts (*4*) are attractive from fermentation and nutritional points of view. Secondary materials like soybean, coconut and groundnut residues from the food industry (*1*), defatted rapeseed meal (*5*), sago starch (*6*), cassava fibres (*7*), corn gluten (*8*) and other could be also fermented into edible products by *Rhizopus oligosporus*. Mould food fermentation of plant origin substrates could be classified into the following categories:

1. Fermentation involving proteolysis of vegetable polymers by fungal enzymes followed by submerged fermentations (examples include soy sauce and miso). Moulds from the genus *Aspergillus* are predominantly used in such processes.

2. Fermentation producing a meat-like texture from cereal grain or legume substrate by means of a mycelium that knits the particles together (examples include tempeh, oncom, and tempeh bongkrek). Moulds from genus *Rhizopus* are predominantly used in such processes.

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Fungal growth and enzymatic activity are essential for an appropriate quality of product formation. The optimal conditions for *R. oligosporus* growth are 42 °C and pH = 4 (*9*). In the phase of germ protrusion, the presence of exogenous carbon and nitrogen is required. *R. oligosporus* grows faster than most moulds and quickly colonizes the substrate (*10*). Steinkraus *et al.* (*11*) suggested that a *Rhizopus* strain used for tempeh production should have the following characteristics:

1. Rapid growth at 37 °C
2. High lipolytic activity
3. Strong antioxidant activity
4. Inability to ferment sucrose
5. Ability to produce the typical tempeh flavour, aroma, and texture
6. High proteolytic activity, resulting in the release of free ammonia after 48 to 72 h of fermentation.

Some other desirable characteristics, such as biosynthesis of vitamins and inhibition of bacteria and aflatoxin-producing moulds, and low sporulation, are additionally important. There has never been a report of food poisoning or aflatoxin contamination in soybean tempeh, although some species of *Rhizopus* were found to be toxic for ducklings when grown for 21 days on maize meal (*12*). *R. microsporus* and *R. chinensi* were acutely toxic to rats, and *R. oryzae* strain 1 caused growth retardation. The formation of the toxin Rhizonin A was detected in *R. microsporus* biomass. The toxicity of the tested strains was much lower when grown on soybeans. *R. oligosporus* is also considered to be a potential human pathogen, because of its ability to grow at 37 °C. Inhalation of sporangiospores could cause potential pathogenicity (*12*). There is no information about the *in vivo* effect of inhaling *R. oligosporus* spores. There have been no reports of poisonings other than from a tempeh-like product made of coconut but clearly, to be safe, toxicological screening of *Rhizopus* strains grown on various substrates is recommended (*2*). Many authors have stated that bacterial and yeast microflora associated with *Rhizopus* fermentation in traditional conditions, have resulted in a better final quality of product and longer shelf life (*13,14*). Keuth and Bisping (*15*) and Berghofer and Werzer (*16*) investigated the formation of water soluble vitamins (vitamin B_{12}, riboflavin, thiamine, niacin, nicotinic acid and nicotinamide) during the fermentation of beans by various *Rhizopus* strains. The role of several strains of *Rhizopus oligosporus*, *R. arrhius* and *R. stolonifer*, and the role of several bacteria in the vitamin formation process were checked. Strains were isolated from Indonesian tempeh and soaking water. Although the mould increases the amount of physiologically active vitamins, the only source of vitamin B_{12} in tempeh products fermented by *Rhizopus oligosporus* are spoilage bacteria which appear during the soaking or fermentation of beans (*17,18*).

Bioconversion of Substrates to *Rhizopus oligosporus* Fermented Products

Soy sauce production in China and Japan and miso fermentation in Japan have become giant commercial industries. Making tempeh in Indonesia is a household art. Indigenous fermented foods constitute a group of foods that are produced in homes, small industries, and also commercial processing plants. Generally, *Rhizopus oligosporus* fermentation can be conducted by two types of process: traditional fermentation in rural conditions, and industrial fermentation.

Traditional Soybean Fermentation

In most developing countries, soybeans are available and are a major source of protein in their nutrition. Soybeans are soaked in tap water overnight (*c.* 12 hours) until the hulls can be removed easily by rubbing. During soaking, spontaneous fermentation by bacteria from the water occurs (*19*). Bacterial microflora, in which *Lactobacillus* species are dominant, cause acidification of the beans and inhibition of some pathogenic bacteria (*20,21*). After dehulling, the soybeans are boiled in the excess water for *c.* 30 minutes, drained, and surface dried. The cotyledons are then inoculated with small pieces of tempeh from a previous batch, or specially prepared sporulated tempeh starter (22). The usual method of fermenting is to wrap a handful of soybeans into a package with banana leaves (*23*). Although wilted banana leaves are most commonly used to wrap the soybeans, other large leaves of tropical plants can be substituted. These packages are then incubated at room temperature for 24 to 48 h, until the soybeans are covered with white mycelium and the cotyledons are bound together.

Industrial Production of Tempeh

Although in the past, and also nowadays in rural conditions, tempeh is prepared for sale using the previously described traditional process, major improvements in processing tempeh on a larger scale have occurred. Soybeans are dehulled dry by passing through a Burr mill. Hulls can be removed from the cotyledons by passing through an aspirator, or over a gravity separator. However, the soybeans can be soaked and dehulled wet by passing through an abrasive vegetable peeler (*24*). Acidification of soybeans on a large scale is accomplished by adding pure cultures of *Lactobacillus* bacteria or organic acids directly to the soak and cook water. De Reu *et al.* (*25*) found that the lag phase of *Rhizopus oligosporus* during the tempeh process depends on spore viability, temperature, concentration of undissociated organic acids and pH. By increasing the initial temperature and the optimal concentration of acetic acid, a shorter lag phase can be obtained. The best results were obtained when the mass fraction of lactic acid in the soak water was 2.1% and acetic acid 0.3%. The pasteurised soybeans are then drained, cooled, and inoculated with a pure culture of mould.

Pure culture fermentation is necessary for industrial tempeh manufacture. The inoculum can be prepared by various procedures. Spore suspension of *R. oligosporus* grown on potato dextrose agar slants for 5 to 7 days could be applied. Steinkraus *et al.* (*24*) suggested a powdered, freeze dried inoculum made by growing pure cultures on sterilized, hydrated soybeans. Wang *et al.* (*26*) developed a tempeh inoculum in powdered form.

The inoculated soybeans are then spread on dryer trays which should be covered and incubated at 37 °C and 90% relative humidity. Fermentation trays are wooden, stainless steel or metal (*2*). Various times and temperature combinations may be applied in order for the fermentation to be completed in 24–36 h. Physiological and organoleptic changes occurring during the incubation period, and histological data on frequency and depth of hyphal penetration, may be additional parameters for fermentation time and temperature optima determination. Excellent tempeh could be made in perforated plastic bags and plastic tubes or containers (*27*). The imperative incubation condition could be described as that of oxygen starvation, allowing mycelia growth but not sporulation. In practice that is achieved by perforating the container with evenly spaced tiny punctures of approximately 1 mm diameter. In the absence of perforation, anaerobic growth of bacterial and yeast species occurs but only a little mycelium will grow until all the oxygen is consumed. On the other hand, if perforations are too large and close, fungal growth will be extremely fast and the resulting heat will increase the temperature of the substrate beyond the optimum (*1*). This favours thermophilic spoilage bacteria which cause proteolysis, resulting in a soggy, smelly incoherent mass. Other causes of excessive heat production are insufficient packing density with consequent pockets of air, and heavy inoculation. Bioreactors for *Rhizopus* fermentation have renewed major interest and attention from the engineering point of view (*28*). De Reu (*29*) has developed a pilot rotating drum bioreactor. The reactor is placed in a temperature-controlled incubator. During fermentation, a discontinuous rotation scheme is used to avoid the substrate exceeding optimal temperatures. The process resulted in a product which does not have the same consistency as that made by static fermentation. Kovač (*30*) tested a static solid state reactor for cultivation of *Rhizopus* under controlled conditions. Key process parameters such as temperature, relative humidity, oxygen and carbon dioxide concentration were controlled and regulated by computer. Fermentation by *R. oligosporus* resulted in a short cultivation period and a unique fermented product.

Nutritional Quality

A great deal of attention has been paid to biochemical changes during fermentation having a crucial effect on the nutritional quality of the final product. The mycelium of *R. oligosporus* penetrates several layers into the soybean cotyledon. Mycelia colonize the intercellular lamellar material and solubilize it by the activity of extracellular enzymes. *Rhizopus* spp. produce lipases, proteases, phytases and a variety of carbohydratases.

According to Yokotsuka (*33*), the average protein efficiency ratio of soybeans fermented by *Rhizopus oligosporus* is 2.4, as compared to casein, which is 2.5. The biological value is 58.7 as compared to that of meat at 80. The net protein utilisation of soybeans tempeh is 56 as compared to that of chicken meat at 65. The digestibility quotient of tempeh is 86.1% (*33*). As mentioned, tempeh is very interesting for consumers and nutritionists because of the physiologically active vitamin B_{12} (*38*). Many antinutritional factors can be found in unfermented legume seeds. During soaking and heating, and because of fungal enzymatic activity, almost all the antinutritious factors, such as protease inhibitors, tannins, phytates, lectins, are removed (*39,40*). The allergic activ-

Table 1. Enzyme activities of *R. oligosporus* after 24 h fermentation at 37 °C

Enzyme	Activity / (U/g of lyophilised product)	Substrate	Reference
Cellulase	3	soybean	De Reu *et al.* (*25*)
Polygalacturonase	7	soybean	De Reu *et al.* (*25*)
Xylanase	5	soybean	De Reu *et al.* (*25*)
Lipase	25.4*	soybean	Wang *et al.* (*31*)
Protease	27.8**	soybean	Nowak, Szebiotko (*32*)
α-amylase	100	rice	Yokotsuka (*33*)
Phytase	0.116 U/mL***	rice	Wang *et al.* (*34*)

* the product was not dried or lyophilised
** 26 hours fermentation, estimated at pH = 7.6
*** the mould was grown on rice liquid media

Table 2. Chemical changes during soybean tempeh fermentation

Component	Mass fraction/ % of dry matter		Fermentation conditions	Reference
	Soybeans	Tempeh		
Soluble nitrogen	0.00	4.60	24 h 37 °C	Nowak, Szebiotko (*32*)
Total nitrogen	44.10	53.80	65 h 37 °C	De Reu *et al.* (*35*)
Free amino acids		8.30 fold increase	70 h 32 °C	Baumann, Bisping (*36*)
Lipids	28.00	20.00	69 h 37 °C	De Reu *et al.* (*37*)
Free fatty acid	11.70	4.30	69 h 37 °C	De Reu *et al.* (*37*)
Glyceride fatty acid	22.30	11.50	69 h 37 °C	De Reu *et al.* (*37*)
Hemicellulose	2.01	1.13	no data	Nout, Rombouts (*1*)
Fibre	3.70	4.80	no data	Nout, Rombouts (*1*)
Water-soluble solids	13.00	21.00	no data	Nout, Rombouts (*1*)

Table 3. Increase in vitamin content in a fermented product (2)

Vitamin	Soybeans[a]	Fermented[b] product	Increase (mass ratio)
Thiamine (B_1)	0.48 mg	0.28 mg	0.6
Riboflavin (B_2)	0.15 mg	0.65 mg	4.3
Niacin (B_3)	0.67 mg	2.52 mg	3.8
Pantothenic acid	0.43 mg	0.52 mg	1.2
Pyridoxine (B_6)	0.18 mg	0.83 mg	4.6
Folacin (folic acid)	25.0 μg	100 μg	4.0
Cobalamin (B_{12})	0.15 ng	3.9 μg	26000.0
Biotin	35.0 μg	53.0 μg	1.5

[a] vitamin mass in 100 g of soybean
[b] vitamin mass in 100 g of fermented product

ity of raw substrate to leguminoses-allergic individuals is decreased or removed (*41*). The bioavailability of minerals was evaluated by *in vivo* rat feeding tests. The availability of zinc was better than in boiled beans (*42*). *In vitro* iron adsorption from tempeh increased 2–5 fold (*43*). Due to the high protein content and amino acid composition, many *Rhizopus* fermented products, especially those from soybeans, surpass the FAO/WHO amino acid reference pattern. Some desirable effects on human health are obtained in *Rhizopus oligosporus* products. Reduction of the serum cholesterol level is obtained by a tempeh diet in rats (*44*). The effect of decreasing cholesterol is presumably attributable to the lecithin, niacin, sitosterol and unsaturated fatty acids in tempeh. *Rhizopus oligosporus* produces components with high antioxidative activity (*45,46*). Nutritional rehabilitation of chronic diarrhetic children with tempeh-based (58% tempeh) and milk-based (12.5% skim milk) formulas, was evaluated. Recovery from diarrheal disease was reported to be faster with the tempeh-based formula and resulted also in better weight gain, immunological values and increase of haemoglobin (*1*). Tempeh is distributed deep frozen, pasteurised or canned and used as a meat substitute or a snack. Tempeh could be consumed deep fat fried, in sandwiches, burgers, with eggs, cooked for salads, in sauces, or added to soups. Tempeh could be added to many meat products to prevent autooxidation (*47*).

Conclusions

Rhizopus oligosporus is suitable for the modification of a variety of substrates into tasty products. We expect an increasing international interest in tempeh technology and increasing consumption. The increase in world-wide need for healthy and nutritious food can be met with proteins from fungal origin. Plant protein is not readily consumable, and does not have high PER value and digestibility. Fermentation produces an attractive product from sensory, health and economical points of view. Priority areas of research relate to its safety and health aspects.

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Uporaba glive *Rhizopus oligosporus* v proizvodnji hrane

Povzetek

Filamentozne glive uporabljamo v proizvodnji hrane z namenom zaščite površine izdelka pred zunanjimi vplivi ali izboljšanja osnovnega okusa živila ob hkratnem povečanju biološke in prehranske vrednosti. V pripravi fermentiranih oziroma bioprocesiranih jedi so uporabne številne glive. Med njimi je za biološko obogatitev rastlinskih substratov **Rhizopus oligosporus** *najbolj sprejemljiva in široko uporabna. Iz namočenih in toplotno obdelanih zrn soje ali žit dobimo izdelek, ki se imenuje »tempeh«. V bioprocesu gliva poveže sojina ali žitna zrna z gostim belim micelijem. Gliva s svojo metabolno aktivnostjo z izločanjem ekstracelularnih encimov hidrolizira substrat in odločilno vpliva na teksturo ter organoleptične lastnosti izdelka. Pri tem se med bioprocesom iz substrata odstranijo neželjeni antinutricijski faktorji in poveča prehranska vrednost. Z zdravstvenega, nutricionističnega in ekonomskega aspekta je tempeh obetaven in atraktiven izdelek.*