Dormancy, activation and viability of *Rhizopus oligosporus* sporangiospores

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Contents

Abstract

The production of fungal fermented foods requires inoculum or starter to initiate the fermentation. One specific Asian fermented soybean food is tempe; as a starter, sporangiospores of the mould *Rhizopus oligosporus* are mostly used. With the objective of understanding factors limiting productivity and shelf-life of tempe starters, a study was made of the nature of dormancy of *R. oligosporus*, and transitions due to activation, into vital and viable and germinating sporangiospores. Freshly produced rice-grown starter contained 6.3×10^9 spores/g, of which only 5-6% were viable, most (85-90%) of the remaining spores being dormant. An optimum harvesting age of 4-5 days was determined.

For activating dormant spores, heat treatments were unsuccessful, but malt extract broth (MEB) was effective, with 80% of dormant spores being activated. Also peptone and yeast extract, but not glucose could activate dormant spores. During storage up to 3 months, some dormant spores were activated. Activation studies of long-stored spores showed that L-alanine is a highly effective activator that can serve as a sole source of carbon and nitrogen. L-leucine and L-isoleucine slightly favour spore germination while L-arginine and L-lysine do not have any stimulating effect. L-proline, on the other hand, inhibits alanine uptake, resulting in apparent low viability. The stimulatory role of glucose was only evident in the presence of phosphate (in minimal medium). Phosphate plays a facilitating role in spore germination. Soybeans subjected to traditional preparation for tempe making are heavily leached; germination of starter spores on such beans is sub-optimal, so there is scope for optimization of soybean preparation for tempe fermentation.

We established that the shelf-life of tempe starter was not limited by the death of spores, but rather by sublethal damage and dormancy of spores, and that both sublethally damaged and dormant spores can be resuscitated. During storage, dormancy and sublethal damage increased with corresponding decline of metabolic activity. After very long (30 months) storage of tempe starter, sublethally damaged spores could still be activated but could not germinate anymore, whereas its dormant spores would not be activated anymore.

SDS-PAGE 2-D protein profiles of dormant and activated sporangiospores of *Rhizopus oligosporus* showed that 18 dormancy related proteins disappear during activation, whereas 4 germination-specific proteins become detectable as early as 2 h after onset of activation with a strong increase to 17 proteins after 4 h activation.

Foreword

It was the award obtained from NUFFIC (The Netherlands organization for international cooperation in higher education) MHO-7 project between Can Tho and Wageningen University that enabled me to start the MSc. study (1998) and continue this Ph.D. study in a sandwich program at the Laboratory of Food Microbiology, Department of Agrotechnology and Food Sciences, Wageningen University from 2001. Upon completion of this study, I wish to express my sincere gratitude to those without whose help I would not have finished this study.

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Nguyen Van Thanh

Wageningen, The Netherlands
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This thesis is dedicated to

my parents

my wife

and my children

Chapter 1

Introduction

Fungal food fermentation

Fungi have long been exploited, either as edible fungi (mushrooms), in fungal fermented foods as well as in brewing. More recently, developments in the fermentation industry have yielded an increasing range of valuable products in medicine (antibiotics, drugs), agriculture (fungicides, plant growth regulators), vitamins and enzymes.

Koji moulds are used as starters for oriental food fermentations (koji or tane koji, meaning mouldy rice). Soybean koji (in which soybeans were used as substrate) is used in the fermentations of soy sauce (Yokotsuka, 1985), soybean paste (Flegel et al., 1981), sufu (Yuan, 1994), and fish sauce (Togano et al., 1978). Rice koji or starter cakes consisting of mixed cultures of moulds and yeasts are used in the fermentation of alcoholic foods and beverages. Ang-kak (red rice) or anka koji is widely used for imparting flavour and colouring to a variety of fermented products (red soybean cheese, fermented fish, red rice wine and red Chinese liquor), and miso rice koji is used for about 80% of the total miso production in Japan (Abiose et al., 1982).

Tempe

Tempe is a traditional Indonesian fermented food in which fungi, particular *Rhizopus* spp., play an essential role. Fresh tempe is a compact and sliceable mass of cooked particles of raw material covered, penetrated and held together by dense non-sporulated mycelium of *Rhizopus* spp. (Nout and Rombouts, 1990). The major desirable aspects of tempe are its actractive flavour (mushroomy or nutty ordour), texture, and easy digestibility (Shurtleff and Aoyagi, 1979; Steinkraus, 1996).

With its high protein content (40-50% of dry matter) tempe serves as a tasty protein complement to starchy staples. In Indonesia, tempe is consumed as a protein-rich meat substitute by all economic groups. The Netherlands have a sizeable population of former Indonesians who continue to produce and consume tempe as is done in Indonesia. In the United States, vegetarians produce and consume tempe as a major protein source to replace meat (Steinkraus, 1996).

Several tempe-making processes have been described for different localities and countries (Shurtleff and Aoyagi, 1986; Steinkraus, 1996). The essential stages in the preparation of tempe include: cleaning the beans, hydration/acid fermentation, dehulling, cooking, draining, cooling, surface drying, inoculation with the starter, incubation in fermentation containers (fermentation), harvesting and cooking or frying prior to consumption.

It is generally accepted that fungal growth (*Rhizopus*) is essential for tempe formation, but also that bacteria levels of 10^8-10^9 colony-forming-units (cfu)/g are common in the final product (Mulyowidarso et al., 1990). The role of this 'accompanying' flora of bacteria as well as some yeasts in the quality of tempe is only partly understood. Whereas high numbers of e.g., bacilli result in spoilage, others such as lactic acid bacteria and *Klebsiella* spp. play a role in prolonging shelf-life and flavour development, and influence the chemical composition through substrate modifications and synthesis of vitamins (Nout and Rombouts, 1990; Keuth and Bisping, 1993; Wiesel et al., 1997). During the fungal fermentation stage, the mycelium of *Rhizopus* spp. penetrates several layers of cells into the soybean cotyledon. Penetration occurs to a depth of 2 mm in 40 hours for soybean tempe (Varzakas, 1998). An important function of the mould in the fermentation process is the synthesis of enzymes (Hachmeister and Fung, 1993). Lipases, proteases, phytases and a variety of carbohydrases are produced (Sarrette et al., 1992) and because of the enzymatic degradation of macromolecules into substances of lower molecular weight, the cell walls and intracellular material are partly solubilised (Nout and Rombouts, 1990; Kovac and Raspor, 1997). Furthermore, enzymatic hydrolysis also may decrease or eliminate antinutritional constituents; consequently, the nutritional value and digestibility might be improved (Hachmeister and Fung, 1993).

Starter cultures for tempe fermentation

Several types of tempe fermentation starters can be distinguished. Natural starters made with plant leaves (e.g. *Hisbiscus* spp.) and soybeans, known as "usar" are still widely used in Indonesia. In addition, powdered starter cultures are now commercially available as single or mixed pure cultures. Mixed pure cultures of e.g., *R. oligosporus* and *Klebsiella pneumoniae* were used (Suparmo, 1988) experimentally to produce tempe with increased vitamin B_{12} content. Most fungal species produce numerous spores and the production of a pure culture starter would therefore be easy to achieve (Samson, 1993).

The use of pure culture starters (containing approx. 10^8 cfu/g) was also advocated for large-scale industrial tempe making (Tanuwidjaja and Roestamsjah, 1985). Pure culture starters are prepared by growing e.g., a pure culture *Rhizopus* strain on sterile substrate (e.g. soya beans, wheat or rice), followed by dehydration and pulverizing (Ko and Hesseltine, 1979).

Besides pure culture starter, semi-pure culture starters are frequently used; they are prepared by growing a pure culture *Rhizopus* strain on traditionally cooked or steamed substrate, mostly rice (Ko, 1985; Tanuwidjaja, 1985) or soya beans (Usmani and Noorani, 1986; Tunçel et al., 1989). After incubation, the moist starter is dehydrated to a final moisture content of about 5% (Ko, 1985). During incubation of semi-pure culture starters, fungi but also accompanying bacteria develop. To reduce the development of undesirable types of bacteria in the starters, the use of biologically acidified substrate significantly decreases total aerobic bacteria (Tunçel et al., 1989).

Pure culture starter development

Solid state fermentations are widely used for spore production of filamentous fungi (Cuero et al., 1985). Mass production of *R. oligosporus* spores on several solid substrates was studied earlier (Wang et al., 1975). Rusmin and Ko (1974) developed a simple method for preparation of a semi-pure culture inoculum for tempe fermentation. Cooked rice was inoculated with a spore suspension and spread to a loose layer of approximately 1 cm thickness in a covered and perforated aluminium tray. It was then incubated at 37 °C. Concomitant with fungal growth and formation of sporangia, the initial substrate moisture content of 67% gradually decreased to 5% or less during the incubation period. The moulded, dried rice was then pulverized to the final product that contained 10^8 to 10^9 spores per gram. One gram of this inoculum was used to inoculate 2 kg of cooked soybeans for tempe fermentation. It was found that the best storage conditions for preservation of the inoculum were at a low temperature (4 °C) and low relative humidity (near 0%). However, the inoculum could also be stored in sealed dry containers at room temperature. The inoculum remained active for more than one year and its method of preparation would be adaptable to small-scale factory production (Ko and Hesseltine, 1979).

The characteristics of a good tempe starter were summarized (Hesseltine et al., 1976) as follows: (1) produces large quantities of spores; (2) uniform viability and genetic stability over a period of at least several months; (3) a high percentage of spore germination in a short time after inoculation; (4) pure culture or correct proportion of strains where mixed pure cultures are used; (5) ready dispensability of spores in the fermentation substrate; (6) freedom from contaminating organisms and if possible, ability to protect itself against contamination; and (7) ability to yield the same amount of desired product repeatedly under a given set of fermentation conditions. In the case of tempe, it is also important that the starter does not contain a mould that sporulates prematurely; the mycelium should be strong, dense, fragrant, and pure white.

Production of tempe inoculum

Because of the need to produce stable, preferably powdered tempe inocula, a number of research laboratories experimented on starter production methods. Some (Steinkraus et al., 1983) basically used the tempe process to produce inocula, except that the soybean cotyledon substrate was sterilized and aseptic conditions were maintained during the processing of the inoculum. Others (Rusmin and Ko, 1974) used hydrated polished rice as a substrate while making no attempt to maintain sterility of subtrate or aseptic conditions. Their inoculum contained large numbers of accompanying bacteria. It was recommended (Wang et al., 1975) to grow *R. oligosporus* on polished rice, on rice:wheat bran (4:1), or wheat:wheat bran (4:1) at a substrate-to-water ratio of 10:6 for 4 days at 32 °C. The substrates were sterilized (20 min at 121 °C) in Erlenmeyer conical flasks. Following sporulation, the cultures were freeze-dried and pulverized.

Rice or rice:wheat bran would appear to be the preferred substrates for production of tempe inoculum (Hesseltine et al., 1976), giving a higher yield of viable spores than on

cooked soybeans. As a result, the number of spores surviving freeze-drying is also higher. The dried spore powder retained good viability for at least 6 months when stored at 22 °C.

Factors affecting the activity of starters

The quantity of starter required for adequate inoculation of a fermentation substrate is determined by the concentration of spores that are able to germinate and produce mycelial biomass. A confusing terminology including terms such as "dead, moribund, starved, dormant, resting, quiescent, viable but non-culturable, injured, sublethally damaged, inhibited, resuscitable, living, active, and vital" has evolved to describe the physiological state of microorganisms (Kell et al., 1998). The terms of relevance to this thesis are described below.

Viability

Viability can be defined as the capability of performing all cell functions necessary for survival under given conditions. Survival can be defined as the continuing existence of the species. To be viable, microorganisms must have: (1) an intact cytoplasmic (plasma) membrane which functions as a barrier between the cytoplasm and the extracellular environment, (2) DNA transcription, and RNA translation, (3) generation of energy for maintenance of cell metabolism, biosynthesis of proteins, nucleic acids, polysaccharides, and other cell components, and, eventually, (4) growth and multiplication (after activation if needed). Methods for assessment of cell viability are based on these requirements (Breeuwer and Abee, 2000). Viability is most commonly determined by the plate count culturing method. However, this method may underestimate the numbers of truly viable microorganisms (because some are sublethally damaged, viable but non-culturable, dormant, or inactive) (Breeuwer and Abee, 2000) and the culturing method can also be frustrated by clumping, inhibition by neigh- bouring cells and composition of the growth media used (Mason et al., 1986).

Fluorescence techniques are used for the rapid assessment of viability of microorganisms. Their advantages are high sensitivity, a high time resolution and potential to analyze individual cells in combination with fluorescence microscopy or with flow cytometry (FCM) (Ritz *et al.*, 2001). Viability fluorescence probes such as cFDA (carboxy-fluorescein diacetate), PI (propidium iodide), and TOTO-1 {1'-(4,4,7,7-tetramethyl-4,7-diazaundecamethylene)-bis-4-[3-methyl-2,3dihydro (benzo-1,3-oxazole)-2-methylidene]-1-(3'-trimethylammoniumpropyl)-pyridinium tetraiodide} have been used recently; especially the combination of cFDA and TOTO-1 enabled to distinguish between live and dead cells of lactic acid bacteria (Bunthof et al., 2001).

The detection of viability of microorganisms is useful for applications such as detection and enumeration of food spoilage microorganisms, evaluation of inactivation treatments, quality assessment of starter cultures, biodegradation, production of antibiotics, and others (Breeuwer and Abee, 2000).

Essential for long-term survival are low rates of metabolism, absence of further cytological activities, and sometimes the presence of thick protective walls. With its vital

processes thus suspended, the spore remains relatively inactive and in many species resistant to unfavourable environmental and nutritive conditions as well as to toxic substances. Metabolic inactivity is, however, only relative, and respiration proceeds at a very slow rate so that energy sources and then other vital compounds are eventually depleted and the spores lose their viability and their ability to germinate (Gottlieb, 1978).

Dormancy

The development of fungal spores can be arbitrarily distinguished into several stages: formation, maturation, dormancy, after-ripening, activation and germination (Griffin, 1994). Dormancy is a common strategy to survive unfavourable external conditions, and is a rest period or reversible interruption of the phenotypic development of the organism in the life cycle of a fungus (Sussman, 1965). To become germinable after dormancy, many (fungal) spores require an after-ripening period (e.g., a cold period) and/or activation treatment.

The latter may include thermal, chemical, or light activation (Griffin, 1994). Some spores are exogenously dormant; their failure to germinate is due to unfavourable environmental conditions. In such case, germination will proceed as soon as environmental conditions are satisfactory. In contrast, endogenously (or constitutively) dormant spores do not germinate even under ideal environmental conditions because the dormancy depends on structural or metabolic features of the spore. Spores of this type may require particular or unusual conditions - either a period of aging or specific treatments to activate the germination process - to terminate dormancy (Griffin, 1994; Carlile et al., 2001). The distinction between exogenous and endogenous dormancy is not always clear and it is likely that in many spores both have a role (Carlile et al., 2001).

The period of dormancy may, depending on circumstances, last only a few hours, or many years. During dormancy morphological changes do not occur and the metabolic rate is much lower than in vegetative cells of the same species (Carlile et al., 2001).

Dormant spores can, however, retain viability at low temperatures, such as those occurring with liquid nitrogen refrigeration, where metabolism- and indeed virtually all chemical reactions- cannot occur. Some fungal spores have remarkable capacities for prolonged viability in the absence of metabolism (Carlile et al., 2001)

Tempe potential in Vietnam

The food consumption surveys of the Vietnamese population in 1985 showed that inadequate energy intake occurred in 15% on average, and protein intake was low. Most protein came from rice; the consumption of meats, beans and fish was negligible (Hop, 2003). Malnutrition among infants is a major problem in Vietnam (28.4%, 2003) in general, and in the Mekong delta in specific. The lack of quality complementary foods in addition to breast milk at affordable prices is one of the main causes as reported by the National Institute of Nutrition, Ministry of Health, Hanoi, Vietnam.

Tempe offers one way of producing protein-rich meat substitutes that are easily digestible, nutritionally adequate and inexpensive (Steinkraus, 1996). The use of local

soybeans (about 31,000 tons yearly in the Mekong delta) (General Statistics Office, 2003) for tempe fermentation probably has a great potential for reducing this problem, because incorporation of tempe in infant formulas would help to decrease the overall incidence of diarrhoea, improve nutrition and thus improve infant/child growth rates and health.

Aim of the thesis

The general objective of the present work described in this thesis is to contribute to the development of efficient starters for tempe making. The scientific objective is to study factors that limit their productivity and shelf-life stability. In specific, the thesis will address the nature and mechanism of dormancy, activation and viability of *Rhizopus oligosporus* and the way they are affected by culturing, processing and storage conditions.

Outline of the thesis

Chapter 2 describes the effect of harvesting age and processing conditions on biomass, spore yield and viability of *Rhizopus oligosporus* in order to prepare tempe starter with a maximum of viable sporangiospores that can be stored without significant loss of viability. Fluorescence probes and conventional culturing methods were applied simultaneously to assess live, dead and dormant spores at harvest, and after each treatment in the process of making tempe starter culture, followed by a period of storage.

Chapter 3 assesses the extent of dormancy, and factors that could result in activation such as heat treatments and nutrient supplementation. Transitions of spore categories (viable, dead, dormant) resulting from nutrient supplementation are discussed.

Chapter 4 describes the role of glucose, phosphate and individual amino acids in activation and germination of sporangiospores of *R. oligosporus* in tempe starter that had been stored for 12 months. The relation between germination of spores and their ability to take up individual amino acids and/or glucose is presented.

Chapter 5 describes the viability and physiological state transitions of *R. oligosporus* sporangiospores in tempe starter culture having been stored for long periods (8, 10, 16, and 30 months) using fluorescence probes in combination with flow cytometry. The relation between shelf-life of tempe starter, and the presence of sublethally damaged and dormant spores, the transitions of spores to different physiological states, and some characteristics of sublethally damaged and dormant spores exposed to activation are examined. A model of physiological state transitions of *R. oligosporus* sporangiospores is proposed.

Chapter 6 presents a preliminary proteomics approach to compare dormant, activated and germinated *R. oligosporus* sporangiospores.

In Chapter 7, the data presented in this work, the relations observed, remaining unsolved problems, and recommendations for further research are discussed.

References

Abiose, S. H., Allan, M. C., and Wood, B. J. B. (1982) Microbiology and biochemistry of miso (soy paste) fermentation. *Advances in Applied Microbiology* **28**, 239-255.

Breeuwer, P., and Abee, T. (2000) Assessment of viability of microorganisms employing fluorescence techniques. *International Journal of Food Microbiology* **55**, 193-200.

Bunthof, C. J., Bloemen, K., Breeuwer, P., Rombouts, F. M., and Abee, T. (2001) Flow cytometric assessment of viability of lactic acid bacteria. *Applied Environmental Microbiology* **67**, 2326-2335.

Carlile, M. J., Watkinson, S. C., and Gooday, G. W. (2001) Spores, Dormancy and Dispersal. In *The Fungi* ed. G. W. Gooday, pp. 185-243. San Diego: Academic Press.

Cuero, R. G., Smith, J. E., and Laley, J. (1985) A novel containment system for laboratory scale solid particulate fermentations. *Biotechnology Letters* **6**, 55-60.

Flegel, T. W., Bhumiratana, A., and Srisutipruti, A. (1981) Problematic occurrence of tyrosine crystal in the Thai soybean paste tao chieo. *Applied and Environmental Microbiology* **41**, 746-751.

General Statistics Office (2003) *Statistical Yearbook 2002*, Ha Noi, Vietnam: Statistical Publishing House.

Gottlieb, D. (1978) Summary. In *The germination of fungus spores* ed. D. Gottlieb, pp. 143-151. Durham, England: Meadowfield Press Ltd, Patterns of Progress Microbiology.

Griffin, D. H. (1994) Spore dormancy and germination. In *Fungal physiology* ed. D. H. Griffin, pp. 375-398. New York: John Wiley & Sons, Inc.

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

Hesseltine, C. W., Swain, E. W., and Wang, H. L. (1976) Production of fungal spores as inocula for oriental fermented foods. *Developments in Industrial Microbiology* **17**, 101-115.

Hop, L. T. (2003) Programs to improve production and consumption of animal source foods and malnutrition in Vietnam. *The Journal of Nutrition* **133 (11 suppl 2)**, 4006S-4009S.

Kell, D. B., Kaprelyants, A. S., Weichart, D. H., Harwood, C. R., and Barer, M. R. (1998) Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. *Antonie van Leeuwenhoek* **73**, 169-187.

Keuth, S., and Bisping, B. (1993) Formation of vitamines by pure cultures of tempe moulds and bacteria during the tempe solid substrate fermentation. *Journal of Applied Bacteriology* **75**, 427-434.

Ko, S. D. (1985) Some microbiological aspects of tempe starters. *Proceedings, Asian Symposium on Non-salted Soybean Fermentation. Tsukuba, Japan, July 1985. pp. 101-109. Tsukuba Science City: National Food Research Institute.*

Ko, S. D., and Hesseltine, C. W. (1979) Tempe and related foods. In *Economic Microbiology, Vol. 4, Microbial Biomass* ed. A. H. Rose, pp. 115-140. London: Academic Press.

Kovac, B., and Raspor, P. (1997) The use of the mould *Rhizopus oligosporus* in food production. *Food Technology and Biotechnology* **35**, 69-73.

Mason, C. A., G., H., and Bryers, J. D. (1986) The death and lysis of microorganisms in environmental processes. *FEMS Microbiol. Rev.* **39**, 373-401.

Mulyowidarso, R. K., Fleet, G. H., and Buckle, K. A. (1990) Association of bacteria with the fungal fermentation of soybean tempe. *Journal of Applied Bacteriology* **68**, 43-47.

Nout, M. J. R., and Rombouts, F. M. (1990) Recent developments in tempe research. *Journal of Applied Bacteriology* **69**, 609-633.

Ritz, M., Tholozan, J. L., Federighi, M., and Pilet, M. F. (2001) Morphological and physiological characterization of *Listeria monocytogenes* subjected to high hydrostatic pressure. *Applied and Environmental Microbiology* **67**, 2240-2247.

Rusmin, S., and Ko, S. D. (1974) Rice-grown *Rhizopus oligosporus* inoculum for tempeh fermentation. *Applied and Environmental Microbiology* **28**, 347-350.

Samson, R. A. (1993) The exploitation of moulds in fermented foods. In *Exploitation of Microorganisms* ed. D. G. Jones, pp. 321-341. London: Chapman & Hall.

Sarrette, M., Nout, M. J. R., Gervais, P., and Rombouts, F. M. (1992) Effect of water activity on production and activity of *Rhizopus oligosporus* polysaccharidases. *Applied Microbiology and Biotechnology* **37**, 420-425.

Shurtleff, W., and Aoyagi, A. (1979) *The book of tempe, a Super soyfood from Indonesia*, New York: Harper & Row.

Shurtleff, W., and Aoyagi, A. (1986) *Tempeh production, a craft and technical manual*, Lafayette: Harper & Row.

Steinkraus, K. H. (1996) *Handbook of indigenous fermented foods*, Second, revised and expanded ed./Ed., New York: Marcel Dekker, Inc.

Steinkraus, K. H., Cullen, R. E., Pederson, C. S., Nellis, L. F., and Gavitt, B. K. (1983) Indonesian tempe and related fermentations. In *Handbook of indigenous fermented foods* ed. K. H. Steinkraus, pp. 1-94. New York: Marcel Dekker.

Suparmo (1988) Vitamin B12 formation in tempeh fermented by mix-culture. *Ph.D, Thesis, Michigan State University. Dissertation Abstracts International B49, 4621 (1989)*.

Sussman, A. S. (1965) Physiology of dormancy and germination in the propagules of cryptogamic plants. In *Encyclopedia of Plant Physiology* ed. A. Lang, Vol. 15, pp. 933-1025. Berlin: Springer.

Tanuwidjaja, L. (1985) Large scale tempe inoculum production. *Proceedings, Asian Symposium on Non-salted Soybean Fermentation. Tsukuba, Japan, July 1985. pp. 305-309. Tsukuba Science City: National Food Research Institute*.

Tanuwidjaja, L., and Roestamsjah (1985) Preparation and utilization of powder form inoculum for tempe fermentation. *ASEAN Food Journal* **1**, 22-24.

Togano, T., Nakamura, M., and Sanchez, P. C. (1978) Fish sauce in S.E. Asia. *5th International Congress in Food Science and Technology, Kyoto, Japan*.

Tunçel, G., Nout, M. J. R., and Rombouts, F. M. (1989) Effect of acidification on the microbiological composition and performance of tempe starter. *Food Microbiology* **6**, 37-43.

Usmani, N. F., and Noorani, R. (1986) Studies on soybean tempeh. II. Propagation and preservation of *Rhizopus oligosporus* spores for commercial production of tempeh from soybean. *Pakistan Journal of Scientific and Industrial Research* **29**, 148-150.

Varzakas, T. (1998) *Rhizopus oligosporus* mycelial penetration and enzyme diffusion in soya bean tempe. *Process Biochemistry* **33**, 741-747.

Wang, H. L., Swain, E. W., and Hesseltine, C. W. (1975) Mass production of *Rhizopus oligosporus* spores and their application in tempeh fermentation. *Journal of Food Science* **40**, 168-170.

Wiesel, I., Rehm, H. J., and Bisping, B. (1997) Improvement of tempe fermentations by application of mixed cultures consisting of *Rhizopus* sp. and bacteria. *Applied Microbiology and Biotechnology* **47**, 218-225.

Yokotsuka, T. (1985) Fermented protein foods in the Orient, with emphasis on shoyu and miso in Japan. In *Microbiology of Fermented Foods* ed. B. J. B. Wood, Vol. 1, pp. 197-247. London: Elsevier Applied Science Publishers.

Yuan, G. F. (1994) Isolation and identification of microorganisms used for fermented soy cheese (sufu) in Taiwan. *Proceedings of the International Worshop on Application and Control of Microorganisms in Asia, 14-18 March, Tokyo, Japan.*

Chapter 2

Rhizopus oligosporus biomass, sporangiospore yield and viability as influenced by harvesting age and processing conditions

Abstract

The objective of tempe starter preparation is to obtain a maximum of viable sporangiospores that can be stored in dry conditions without significant loss of viability. The aim of this research was to establish relationships between spore-harvesting age, their numbers, yield, viability and survival of processing and storage conditions. We observed that within 6 days of incubation on cooked rice substrate, about 8% w/w of fungal biomass was formed, containing 6.3×10^9 spores per gram. Of these spores, only 5-6% were viable. The remaining spores did not show damage to cytoplasmic membranes, but they were probably dormant. Of the processing conditions, mild oven-drying had little negative effect on viability unlike pulverisation which caused mechanical damage and loss of viability. The age of spores at harvest influences their storage stability. This is evidenced by spores harvested after 3 days suffering bigger losses during 2 and 3 months storage compared to spores harvested after 4 or 5 days.

INTRODUCTION

Tempe (also spelled as tempeh) is a mould-fermented soybean product originating from Indonesia (Ko and Hesseltine, 1979; Usmani and Noorani, 1986; Nout and Rombouts, 1990). Due to its excellent nutritional properties and its agreeable taste, it is gaining increasing popularity in other regions. Whereas much tempe is still made using the traditional mixed-strain fungal cultures on plant leaves (Nout et al., 1992), the use of defined tempe starter cultures has the advantage of selecting high quality strains. Several studies on the preparation of tempe starter cultures have been reported previously (Hesseltine et al., 1976; Lotong and Suwanarit, 1983; Shambuyi et al., 1992). It was (Steinkraus et al., 1983) reported the preservation of freeze-dried fermented soybeans for use as inoculum. Rusmin and Ko (1974) developed the inoculum by growing *Rhizopus oligosporus* on cooked rice. The mass production of spores using rice, pearled wheat bran and cracked soybean and preserving the spores after freeze drying and grinding the fermented mass was also reported (Wang et al., 1975). The rice inoculum produced more spores and had a longer shelf-life (4 months at 25-30 °C) than spores grown on tapioca waste, or on mixtures of tapioca waste and soybean flour as reported by (Tanuwidjaja and Roestamsjah, 1985). Rice-based starters were reported to yield tempe of higher acceptability but had a shorter shelf-life (2.5 months) compared to soya bean based starters (7 months) (Tunçel et al., 1989).

This study was designed to evaluate the effect of processing conditions such as incubation period, dehydration and pulverisation on biomass formation and its spore-forming productivity, and the viability of the resulting rice-based tempe starter culture.

MATERIALS AND METHODS

Culture

Rhizopus oligosporus LU 575 (NRRL 5905) was grown and maintained on malt extract agar (MEA, Oxoid CM 59) slants. Incubation was at 30 °C for 1 week, and storage at 5 °C.

Preparation of inoculum for making starter culture

Spores from the slants mentioned above were inoculated onto the surface of MEA in 90-mm Petri dishes and incubated at 30 °C for 1 week. Spores of *R. oligosporus* were harvested by adding 10 ml of sterile distilled water containing 0.1% (v/v) Tween 80, on the surface of the culture and gently rubbing with a sterile bent glass rod. The spore suspension was removed from the culture and the flooding procedure was repeated twice. Pooled suspension was diluted with sterile water to give approximately 10^5 spores per ml. This suspension ("A") was used as inoculum for substrate used to prepare tempe starter culture.

Procedure for making tempe starter

Solid state fermentation of polished broken rice was used to prepare tempe starter culture. In each 1000-ml Roux bottle, 50 g of rice and distilled water (0.6 g per g of dry substrate) were mixed and allowed to stand at room temperature for 1 hr with frequent but slow manual shaking with the aim to facilitate water absorption.

The cotton-plugged Roux bottles were steam-sterilized for 20 min at 121 °C. The bottles were taken from the autoclave and shaken until the rice was broken loose, or the rice was broken up with a spatula, aseptically. The sterilized rice was allowed to cool to room temperature.

To add nitrogen source and adjust the pH to 4, a volume of 1.5 ml of sterilized ammonium sulfate 1.5 M solution (0.0067 g $(NH_4)_2SO_4$ per g dry substrate) and 1 ml of sterilized H_2SO_4 0.5 M solution were added to the sterilized rice.

Each batch of sterilized rice (50 g) was inoculated and thoroughly mixed with 0.4 ml of *R. oligosporus* spore suspension "A" (about 1250 spores per g dry substrate). The number of spores per g dry substrate was chosen on the basis of previous trials. The inoculated substrate was spread in 0.5-cm layers in the Roux bottles, which had sterile cotton plugs covered with aluminum foil, perforated by puncturing.

The inoculated rice was incubated at 40 °C for 2 hours, followed at 30 °C, during 2 - 6 days, during which time the rice was covered with mycelium and black spores. Cultures were harvested after 2, 3, 4, 5 and 6 days of incubation to determine the effect of harvesting age on biomass, spore yield and viability.

Drying and grinding

The mouldy mass was broken up and transferred into sterile Petri dishes. The entire mass of substrate, mycelium and spores was dried at a mild temperature of 42 °C for 48 hrs using a forced-air oven.

The dried material was then ground in a disinfected sample grinder (Fritsch, type Pulverisette 14, Germany with a 1.0 mm screen, treated with ethanol 70%), at high speed for a short time to obtain a fine powder, and this was stored in air of 40% relative humidity for one day. The samples were analyzed in triplicate.

Spore yield

Microscopic counts were made at harvest (2, 3, 4, 5 and 6 days after inoculation) and after drying and grinding. One gram of the sample was placed in 99 ml of sterile distilled water containing 0.1% (v/v) Tween 80. The extraction of spores was carried out by vigorous agitation. The spore suspensions were diluted as appropriate and counted using a Bürker-Türk counting chamber.

Viability

Viable spores were determined as colony forming units by surface-plating triplicate 0.1-ml aliquots on RBCC: Rose Bengal Chloramphenicol Agar Base (Oxoid CM 549) with

addition of 0.2 g/l Rose Bengal (Fluka AG, Switzerland) and on MEA: Malt Extract Agar (Oxoid, CM 59). After incubation at 37 °C and 30 °C respectively, for 1-2 days, colonies were counted.

Fluorescent markers

The *R. oligosporus* spore suspensions were washed twice by centrifugation in an Eppendorf centrifuge in phosphate buffer (K_2HPO_4 50 mM, adjusted to pH 4.0 with citric acid 50 mM). Subsequently, the suspensions were incubated for 20 min. in the presence of 5-(and-6) carboxyfluorescein diacetate (Molecular Probes Europe, Leiden, The Netherlands) 10 mg/ml acetone (cFDA 0.22 mM) or propidium iodide (Sigma Chemical Co. St. Louis, USA) (PI 1.5 mM) at 40 °C. They were then put on ice and counted in a Bürker-Türk counting chamber with fluorescence microscopy, using an Axioskop epifluorescence microscope equipped with a 50-W mercury arc lamp, a fluorescein isothiocyanate filter set (excitation wavelength 450 to 490 nm; emission wavelength >520 nm), an x100 1.3 numerical-aperture Plan-Neofluar objective lens, and a camera (Carl Zeiss, Oberkochen, Germany).

Fungal biomass

Biomass of the mould was determined by enzymatic digestion of the mouldy rice substrate and weighing of the undissolved residue, using sterile cooked rice and purified mycelium as controls. The digestion protocol was described earlier (Kiers et al., 2000), specifically step 1 of their in vitro digestion method was used, involving subsequent incubation with artificial saliva for 30 min, with artificial gastric juice at pH 4.0 for 60 min, and with pancreatic solution at pH 6.0 for 30 min, all at 37 °C.

The estimated weight of mould biomass in moulded rice was calculated as:

$$M = (R_0 - R_1)/ R_m$$

in which

M = biomass of the mould in the moulded rice.
R_0 = residue of moulded rice after enzymatic digestion.
R_1 = residue of control cooked rice after enzymatic digestion.
R_m = residue of pure mycelium grown on Malt Extract Agar (dried and ground),
after enzymatic digestion.
The accuracy of the biomass estimation is 90 – 95%.

Statistical analysis

Experiments were in duplicate, analyses in triplicate. The results were reported as means of triplicates with standard deviation using unrelated *t*- test to test significance of differences.

RESULTS AND DISCUSSION

Effect of harvesting age on biomass, number and viability of sporangiospores of
R. oligosporus

Table 1 summarizes biomass content of the moulded rice, and the number and viability of spores harvested after 2-6 days of incubation. A comparison is made of freshly harvested and processed spores. During the first day of incubation, the inoculated spores germinated and the subsequent growth covered the rice lumps with a white, woolly mycelial layer. On the second day the colour turned to light gray due to the formation of sporangia. During the following days more sporangia were formed and the colour of the mass changed to dark gray. From day 5 the colour of mass began to change into brownish.

A gradual increase of biomass was observed until 5 days when a maximum was reached. This was followed by a decrease that coincided with development of brownish colour both of which may be due to nutrient depletion. Already after 2 days of cultivation the majority of microscopically visible sporangiospores had been formed, but it took up to 4 days cultivation to achieve the highest level of viability of the spores.

It should be noted that rather low levels of viability (2.8 - 6% of total number of spores) were obtained. This appears significantly lower than the viability of 69% reported elsewhere (Rusmin and Ko, 1974). However, their absolute number of germinating spores was of a similar order of magnitude, and they quantified germinating spores microscopically. Other discrepancies may arise from sensitivity to processing (drying and pulverising) as well as aggregation causing single colonies from multiple spores. It appeared that the older the spores that were harvested, the less resistant they were to processing. After 5 days or longer, the yield of viable processed spores decreased considerably.

Effect of drying and pulverization on viability of sporangiospores:

Table 2 summarizes the effect of drying and pulverization on the viability of sporangiospores of *R. oligosporus* harvested after 4 days. As this experiment was carried out independently, corresponding values differ slightly from those in Table 1.

It was observed that of the processing steps, drying caused a slight reduction to 69% of the freshly harvested spores. Rusmin and Ko (1974) reported that at the time the spores were prepared, the germination percentage was 69% in inoculum pieces. Our result is in good agreement with these studies of semi-pure starter culture using rice substrate. On the other hand, pulverizing caused a higher reduction of the number of visible sporangiospores. The same trend was seen when looking at the viable spores (RBCC).

Fluorescence with cFDA indicates esterase activity within the spores. This fluorescent marker has been used in several yeasts and other microorganisms as an indicator for metabolic activity. It was reported (Breeuwer et al., 1997) that the viability of *R. oligosporus* sporangiospores could be assessed by fluorescence techniques (cFDA), and

Table 1. Effect of harvesting age on biomass, number and viability of sporangiospores of *R. oligosporus*

Incubation (days)	Biomass[1],* (% w/w) Avg.	S.D.	Harvested[2] Visible spores* (microscopic) Log N.g⁻¹ dry wt Avg.	S.D.	Viable spores* (RBCC) Log cfu.g⁻¹ dry wt Avg.	S.D.	Viability of visible spores %	Dried, Pulverized[3] Visible spores* (microscopic) Log N.g⁻¹ dry wt Avg.	S.D.	Viable spores* (RBCC) Log cfu.g⁻¹ dry wt Avg.	S.D.	Viability of visible spores %
2	5.76ᵃ	± 0.06	9.13ᵃ	± 0.10	7.54ᵃ	± 0.02	2.8	8.77ᵃ	± 0.06	6.40ᵃ	± 0.09	0.43
3	6.79ᵃ	± 0.32	9.63ᵇ	± 0.08	8.12ᵇ	± 0.06	3.0	9.09ᵇ	± 0.15	7.74ᵇᶜ	± 0.11	4.4
4	7.71ᵇ	± 0.30	9.65ᵇ	± 0.03	8.43ᶜ	± 0.07	6.0	9.24ᵇᶜ	± 0.02	7.86ᵇ	± 0.09	4.2
5	8.17ᵇ	± 0.08	9.72ᵇᶜ	± 0.12	8.39ᶜ	± 0.04	4.6	9.35ᶜ	± 0.10	7.81ᵇ	± 0.04	2.8
6	8.11ᵇ	± 0.24	9.80ᶜ	± 0.07	8.39ᶜ	± 0.02	4.0	9.49ᶜ	± 0.24	7.71ᶜ	± 0.03	1.7

[1] Biomass dry weight % in moulded rice.

[2] Analyzed immediately after harvest.

[3] Analyzed after 24 h equilibration period at 21 °C.

* Results reported as means of triplicates with standard deviation.

In each column, the data that have the same alphabets are not significantly different (p ≤ 5%, 1 tail unrelated *t*- test)

Table 2. Effect of drying and pulverization on viability of sporangiospores of *R. oligosporus* harvested after 4 days

	Visible spores* Log N.g^{-1} dry wt		Viable spores* (RBCC) Log cfu.g^{-1} dry wt		fluorescent spores* (cFDA) Log N.g^{-1} dry wt		fluorescent spores* (PI) Log N.g^{-1} dry wt	
	Avg.	S.D.	Avg.	S.D.	Avg.	S.D.	Avg.	S.D.
Harvested	9.47[a]	± 0.03	8.29[a]	± 0.04	8.35[a]	± 0.03	7.54[a]	± 0.09
Dried	9.45[a]	± 0.02	8.13[b]	± 0.08	8.18[b]	± 0.02	7.24[b]	± 0.07
Pulverized	9.04[b]	± 0.04	7.43[c]	± 0.02	7.33[c]	± 0.09	7.33[b]	± 0.06

* Results reported as means of triplicates with standard deviation using unrelated *t*- test.

In each column, the data that have the same alphabets are not significantly different (p $\leq$ 5%, 1 tail *t*- test).

that the fluorescence intensity of the spores could be used as an indication of metabolic (enzymatic) activity. Spores which are not fluorescent did not germinate.

The results reported here were in general agreement with those of Breeuwer et al. (1997). In the present study, it appears that the loss of viability measured on RBCC is very well correlated with the same trend in fluorescence with cFDA. However, not all fluorescent spores formed colonies. We assume that colonies were formed by those spores emitting stronger fluorescence intensity.

The fluorescence marker PI can enter cells where the cytoplasmic membranes have been damaged. This can be useful as a marker for dead cells. The strong decrease of cFDA value was accompanied by an increase of dead spores as observed with PI. This confirms that drying and particularly pulverising are important causes of decreasing viability of spores. In the present study, the number of spores that give a fluorescent reaction with PI, is quite low, which would indicate that loss of membrane integrity is not the primary reason for the low level of viability of the sporangiospores. Alternatively, it could be that PI is not an appropriate marker for *R. oligosporus* and that other fluorescent markers for membrane integrity give higher values. This should be tested in future.

In conclusion, it is likely that the majority of sporangiospores tested are in a state of dormancy, having intact membranes but lacking enzyme activity.

Effect of harvesting age and storage conditions on viability

Table 3 shows the effects of harvesting age and storage conditions on viability of processed (dried and pulverized) sporangiospores of *R. oligosporus.*

On the whole, the storage experiment showed that spores retained their viability slightly better at 30 °C than at 5 °C. Spores harvested after 3 days lost more of their viability during storage, whereas there was no difference between days 4 and 5. After 2 and 3 months of storage, verifications using MEA and cFDA showed that on a less specific medium such as MEA, the same order of magnitude of viability was observed. Likewise spore fluorescence was maintained after 3 months of storage. Storage was also carried out under nitrogen. This gave very similar results (data not shown).

In conclusion, the following general remarks can be made: first, there is an age effect, which is reflected in a maximum of viability of spores and a different resistance to processing conditions; second, drying at mild temperatures such as done in this study, gives very little loss of viability; third, on the other hand, pulverization can give rise to considerable losses, so this is a step that can and must be optimized for practical applications; fourth, the majority of spores were in a "dormant" stage and it will be of interest to study the nature of this dormancy; fifth, cFDA fluorescence gives a very good correlation with culturing viability.

Table 3. Effect of harvesting age and storage conditions on viability of sporangiospores of *R. oligosporus* (dried and pulverized)

harvest age	storage period	storage temperature (°C)											
		5						30					
		Viable spores* (RBCC) Log cfu.g⁻¹ dry wt		Viable spores (MEA) Log cfu.g⁻¹ dry wt	fluorescent spores (cFDA) Log N.g⁻¹ dry wt			Viable spores* (RBCC) Log cfu.g⁻¹ dry wt		Viable spores (MEA) Log cfu.g⁻¹ dry wt	fluorescent spores (cFDA) Log N.g⁻¹ dry wt		
days	months	Avg.	S.D.					Avg.	S.D.				
3	0[1]	7.74[a]	± 0.11	nd	nd			7.74[a]	± 0.11	nd	nd		
	1	6.82[b]	± 0.02	nd	nd			6.87[b]	± 0.03	nd	nd		
	2	6.77[c]	± 0.03	7.4	7.5			6.80[b]	± 0.06	7.2	7.4		
	3	6.59[d]	± 0.04	6.8	7.2			6.81[b]	± 0.08	6.7	7.2		
4	0[1]	7.86[e]	± 0.09	nd	nd			7.86[c]	± 0.09	nd	nd		
	1	7.40[f]	± 0.07	nd	nd			7.31[d]	± 0.07	nd	nd		
	2	7.39[f]	± 0.07	7.7	7.6			7.26[d]	± 0.04	7.5	7.7		
	3	7.10[g]	± 0.02	7.0	7.3			7.23[d]	± 0.03	7.3	7.4		
5	0[1]	7.81[h]	± 0.04	nd	nd			7.81[e]	± 0.04	nd	nd		
	1	7.28[i]	± 0.02	nd	nd			7.29[f]	± 0.06	nd	nd		
	2	7.23[j]	± 0.02	7.5	7.4			7.26[f]	± 0.05	7.6	7.4		
	3	7.03[k]	± 0.02	7.2	7.2			7.10[g]	± 0.08	7.2	7.4		

nd = not determined

[1] Moisture content of the stored samples were ≈ 7.5%

* Results reported as means of triplicates with standard deviation.

Of each harvesting age, data that have the same alphabets are not significantly different (p ≤ 5%, 1 tail unrelated *t*- test).

REFERENCES

Breeuwer, P., De Reu, J. C., Drocourt, J.-L., Rombouts, F. M., and Abee, T. (1997) Nonanoic acid, a fungal self-inhibitor, prevents germination of *Rhizopus oligosporus* sporangiospores by dissipation of the pH gradient. *Applied and Environmental Microbiology* **63**, 178-185.

Hesseltine, C. W., Swain, E. W., and Wang, H. L. (1976) Production of fungal spores as inocula for oriental fermented foods. *Developments in Industrial Microbiology* **17**, 101-115.

Kiers, J. L., Nout, M. J. R., and 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.

Ko, S. D., and Hesseltine, C. W. (1979) Tempe and related foods. In *Economic Microbiology, Vol. 4: Microbial Biomass* ed. A. H. Rose, pp. 115-140. London: Academic Press.

Lotong, N., and Suwanarit, P. (1983) Production of soy sauce koji mold spore inculum in plastic bags. *Applied and Environmental Microbiology* **46**, 1224-1226.

Nout, M. J. R., Martoyuwono, T. D., Bonné, P. C. J., and Odamtten, G. T. (1992) Hibiscus leaves for the manufacture of Usar, a traditional inoculum for tempe. *Journal of the Science of Food and Agriculture* **58**, 339-346.

Nout, M. J. R., and Rombouts, F. M. (1990) Recent developments in tempe research. *Journal of Applied Bacteriology* **69**, 609-633.

Rusmin, S., and Ko, S. D. (1974) Rice-grown *Rhizopus oligosporus* inoculum for tempeh fermentation. *Applied and Environmental Microbiology* **28**, 347-350.

Shambuyi, M., Beuchat, L. R., Hung, Y. C., and Nakayama, T. (1992) Evaluation of subtrates and storage conditions for preparing and maintaining starter cultures for tempeh fermentation. *International Journal of Food Microbiology* **15**, 77-85.

Steinkraus, K. H., Cullen, R. E., Pederson, C. S., Nellis, L. F., and Gavitt, B. K. (1983) Indonesian tempe and related fermentations. In *Handbook of indigenous fermented foods* ed. K. H. Steinkraus, pp. 1-94. New York: Marcel Dekker.

Tanuwidjaja, L., and Roestamsjah (1985) Preparation and utilization of powder form inoculum for tempe fermentation. *ASEAN Food Journal* **1**, 22-24.

Tunçel, G., Nout, M. J. R., and Rombouts, F. M. (1989) Effect of acidification on the microbiological composition and performance of tempe starter. *Food Microbiology* **6**, 37-43.

Usmani, N. F., and Noorani, R. (1986) Studies on soybean tempeh. II. Propagation and preservation of *Rhizopus oligosporus* spores for commercial production of tempeh from soybean. *Pakistan Journal of Scientific and Industrial Research* **29**, 148-150.

Wang, H. L., Swain, E. W., and Hesseltine, C. W. (1975) Mass production of *Rhizopus oligosporus* spores and their application in tempeh fermentation. *Journal of Food Science* **40**, 168-170.

Chapter 3

Dormancy, activation and viability of

Rhizopus oligosporus sporangiospores

Abstract

Interruption of dormancy to improve viability of *Rhizopus oligosporus* sporangiospores is crucial for the application of stored starter cultures for fungal (tempe) production. We aimed to assess the extent of dormancy and factors that could result in activation. Whereas heat treatments were unsuccessful, Malt Extract Broth (MEB) showed to be a good activation medium, with 80% of dormant spores being activated as measured by fluorescence microscopy using a fluorescent marker, compared with 11% of the control. Peptone and yeast extract but not glucose played an important role in activating dormant spores. Metabolically active (fluorescent) and swollen spores, followed by germ tubes were obtained after activation in MEB for 25 min., 2 h and 4 h, respectively, at 37 °C. Simultaneously, some interesting transitions took place. Dormant spores represent 85-90% of the total spores at harvest and after drying. Their number decreased to 21-32% after activation with MEB with a concomitant increase of metabolically active spores. As a result of storage, some dormancy was lost, yielding an increase of active spores from 11.2% at harvest to 28.8% after 3 months storage. Levels of active spores were well correlated with their viability. By activation of dormant spores, their viability increased; levels of viable and active spores were maximum in 1 month old starter (61.7 and 75.9% of total spores, respectively) but gradually decreased with concomitant increase of the number of dead spores.

N.V.Thanh and M.J.R. Nout

INTRODUCTION

The development of fungal spores can be arbitrarily distinguished into several stages: formation, maturation, dormancy, after-ripening, activation and germination (Griffin, 1994). Dormancy is a common strategy to survive unfavourable external conditions. To become germinable after dormancy, many (fungal) spores require an after-ripening period (e.g., a cold period) and/or activation treatment. The latter may include thermal, chemical (detergents, organic acids, and amino acids, etc.), or light activation (Sussman and Halvorson, 1966; Griffin, 1994).

For example, *Phycomyces blakesleeanus* spores do not germinate in a suitable culture medium unless they are activated by one of a range of treatments, such as heating for 3 min at 44 °C (Halbsguth and Rudolph, 1959; Van Laere et al., 1980), γ radiation (Van Assche et al., 1977), dithionite treatment (Van Assche et al., 1978), or treatments with acetate, azide and ammonia (Van Laere et al., 1980). Likewise, n-alcohols and high pressure supported the heat activation of *P. blakesleeanus* (Thevelein et al., 1979) and of *Neurospora tetrasperma* ascospores (Belmans et al., 1983). During activation, glycerol formation was observed in *P. blakesleeanus* spores (Van Schaftingen and Van Laere, 1985). Heat treatment was related to trehalase activity in dormant and activated spores of *P. blakesleeanus* (Van Assche et al., 1972; Van Assche and Carlier, 1975).

Glucose was involved in the initiation of germination of *Mucor racemosus* sporangiospores (Tripp and Paznokas, 1982a). Furthermore, glucose induced trehalase activity and trehalose mobilization during early germination of *P. blakesleeanus* spores (Thevelein et al., 1983). The availability of glucose also affected events during germination of *Syncephalastrum racemosum* sporangiospores (Hobot and Gull, 1977).

Whereas amino acids and endogenous protein stimulated germination of *Mucor racemosus* sporangiospores (Tripp and Paznokas, 1981, 1982b), no single amino acid was as effective as glucose or peptone at triggering germination. So it was suggested that glucose may trigger germination by signalling the breakdown of endogenous protein reserves, while the subsequent increase of free amino acids may be the dormancy-breaking factor.

Earlier studies on the germination of *R. oligosporus* sporangiospores (Medwid and Grant, 1984) revealed that two phases: I (swelling) and II (germ tube protrusion), could be distinguished. Initial swelling during phase I occurred only in the presence of a suitable carbohydrate, while subsequent production of germ tubes during phase II required exogenous sources of both carbon and nitrogen. It was also shown (Breeuwer et al., 1997) that germination of *R. oligosporus* sporangiospores is prevented by nonanoic acid, a fungal self-inhibitor. Despite this knowledge, there is a lack of understanding relating to the dormancy and activation of the tempe-mould *R. oligosporus*. In this paper, we present physical and nutritional conditions affecting the activation and germination and the accompanying morphological changes of *R. oligosporus*. We demonstrate shifts in physiological categories (i.e., dormant, metabolically active, viable and dead) of *R. oligosporus* sporangiospores during storage.
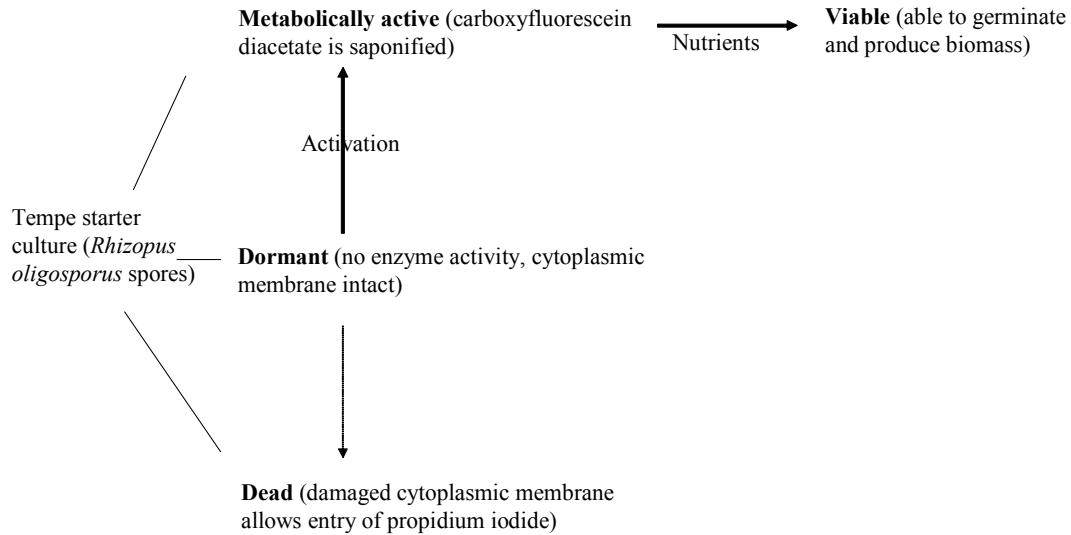
Metabolically active (carboxyfluorescein diacetate is saponified) — Nutrients → **Viable** (able to germinate and produce biomass)

Activation ↑

Tempe starter culture (*Rhizopus oligosporus* spores)

Dormant (no enzyme activity, cytoplasmic membrane intact)

Dead (damaged cytoplasmic membrane allows entry of propidium iodide)

Figure 1. Physiological categories of *R. oligosporus* sporangiospores

Like in other fungal spores, dormancy occurs in sporangiospores of *R. oligosporus*, especially during storage time; dormancy may well be one of reasons for the limited shelf-life of tempe starter cultures as were reported by several authors, e.g. 4 months at 25-30ºC (Tanuwidjaja and Roestamsjah, 1985), or 2.5 months (Tunçel et al., 1989). In powdered tempe starters we can distinguish 3 categories by fluorescence microscopy, namely: metabolically active (green fluorescence with carboxyfluorescein diacetate), dead (red fluorescence with propidium iodide) and dormant (no fluorescence). In rice-based spore powders, typically more than 90% of spores were dormant, and 5-6% are metabolically active (Thanh and Nout, 2002). Many, but not all, metabolically active spores are viable, i.e. able to germinate and produce colonies of biomass. To facilitate the discussion, the relation between these categories is illustrated in figure 1.

MATERIALS AND METHODS

Fluorescent probes and media

cFDA: 5-(and-6)-carboxyfluorescein diacetate (Molecular Probes Europe, Leiden, The Netherlands), 10 mg/ml acetone, (cFDA 0.22 mM) was used as a fluorescent marker for

metabolically active spores (see also "Activation of dormant spores" and "Fluorescent counts etc." below). **PI:** Propidium iodide 95-98% (TLC) (Sigma Chemical Co. St. Louis, USA) 1- mg/ml distilled water (30 µM) was used as a fluorescent marker for dead spores (see also "Activation of dormant spores" and "Fluorescent counts etc." below). **RBCC**: Rose-Bengal chloramphenicol Agar Base, Oxoid, CM 549 with addition of 0.2 g/l Rose Bengal (Fluka AG, Switzerland) was used for viability tests (see "Viability of spores" below). **MEA:** Malt extract agar (malt extract 30 g/l, mycological peptone 5 g/l, agar 15 g/l), Oxoid, CM 59 was used in the preparation of tempe starter (see "Preparation of tempe starter" below) and for viability tests (see "Viability of spores" below). **MEB:** malt extract broth (malt extract 17 g/l, mycological peptone 3 g/l), Oxoid, CM 57 was used for activation of spores (see also "Activation of dormant spores" below). **CDM:** Czapek-Dox liquid medium Oxoid, CM 95 containing sodium nitrate 2 g/l, potassium chloride 0.5g/l, magnesium glycerophosphate 0.5g/l, ferrous sulphate 0.01g/l, potassium sulphate 0.35g/l and sucrose 30g/l was used for activation of spores (see also "Activation of dormant spores" below). **PPS:** Peptone physiological salt solution containing neutralised bacteriological peptone 1g/l (Oxoid, L34) and NaCl 8.5 g/l was used as a diluant for viability tests (see "Viability of spores" below).

Preparation of tempe starter

Tempe starter was prepared as described earlier (Thanh and Nout, 2002). Briefly, the procedure was as follows: *Rhizopus oligosporus* LU 575 (NRRL 5905) was grown on MEA plates during 1 week at 30 °C, and spores were harvested by washing with sterile water. The spore suspension was diluted to approximately 10^5 spores per ml. This suspension "A" was used as inoculum for substrate used to prepare tempe starter. Polished broken rice was steam-sterilized, allowed to cool and sterilized ammonium sulfate and sterilized H_2SO_4 were added to adjust the pH to 4, this is the optimal pH for spore germination of *Rhizopus oligosporus* (Medwid and Grant, 1984; Breeuwer et al., 1997). The sterile broken rice (50 g) was inoculated and thoroughly mixed with 0.4 ml of spore suspension "A". The inoculated rice was incubated at 40 °C for 2 hours, followed by incubation for 4 days at 30 °C. The mouldy mass was broken up and the entire mass of substrate, mycelium and spores was dried at 42 °C for 48 h in a forced-air drying cabinet. Only crushing by sterile pestle and mortar, but no fine grinding was used in order to avoid misjudging between spores and rice particles with similar size and shape. The starter powder was stored in screw-cap glass tubes, protected from light, in a silicagel desiccator at 25 °C, as these conditions were considered to be more representative of practical usage than storage at low temperature. Moreover, it was shown that spores survive better at ambient (25-30 °C) temperature than at 5 °C (Thanh and Nout, 2002). The samples were analyzed in triplicate after a defined period of storage.

Activation of dormant spores

Sporangiospores in crushed rice powder were diluted with sterile water to suspensions (approximately 10^6 spores/ml), and were washed twice by centrifugation at 13000 x g for 3

min in an Eppendorf centrifuge. After decanting, the spores in pellets were re-suspended in phosphate buffer (K_2HPO_4 50 mM, adjusted to pH 4.0 with citric acid 50 mM), CDM, and MEB, respectively. Subsequently, the suspensions were either plated on RBCC and MEA to quantify viable spores (see "Viability of spores" below), metabolically active, or dead spores (see "Fluorescent counts etc." below). At each sampling time point during the activation process, spore suspensions were stained (see "Fluorescent counts etc." below).

Total number of spores

One gram of crushed rice-based spore starter was placed in 99 ml of sterile distilled water containing 0.1% (v/v) Tween 80. The suspension of spores was carried out by vigorous agitation and filtration with a Millipore membrane filter [(fluorassure); Chem filter 15, REF: 100-C2003-01; Chemunex]. Spore suspensions were diluted as appropriate and counted using a Bürker-Türk counting chamber. Microscopic counts were also made similarly to determine fluorescent spores, as described in the next section.

Fluorescent counts of metabolically active and dead spores

Spore suspensions were washed twice by centrifugation at 13000 x g for 3 min in an Eppendorf centrifuge in phosphate buffer (K_2HPO_4 50 mM, adjusted to pH 4.0 with citric acid 50 mM). Subsequently, the suspensions were incubated for 20 min. in the presence of cFDA and PI at 40 °C. They were then put on ice and counted in a Bürker-Türk counting chamber by fluorescence microscopy, with an Axioskop epifluorescence microscope equipped with a 50-W mercury arc lamp, a fluorescein isothiocyanate filter set (excitation wavelength 450 to 490 nm; emission wavelength >520 nm), an x100 1.3 numerical-aperture Plan-Neofluar objective lens, and a camera (Carl Zeiss, Oberkochen, Germany). Fluorescent, non-fluorescent and spores showing germ tubes were counted. Their values were presented in decade log units and percent of total spores. Fluorescent spores (cFDA) were considered as metabolically active, or dead in case of PI fluorescence; non-fluorescent spores were considered as dormant; spores with germ tubes were considered as germinated spores when the extension of the germ tube was to a length equal to one-half the diameter of the spores (Medwid and Grant, 1984).

Viability of spores

Viable spores were determined as colony forming units by surface-plating triplicate 0.1-ml aliquots of decimal dilution series in PPS, on RBCC and MEA. After incubation at 37 °C for 12 h (MEA) or 24 h (RBCC), colonies were counted.

Statistical analysis

Experiments were in duplicate, analyses in triplicate. The results were reported as means of triplicates with standard deviation using unrelated *t*-test to test significance of differences.

RESULTS AND DISCUSSION

Effect of heat and nutrients on activation

In Table 1, the effects of incubating 2-month old rice-grown as well as fresh MEA-grown spores of *R. oligosporus*, in MEB and buffer are shown. MEB has a significant activation effect as shown by higher numbers of metabolically active (cFDA fluorescent) spores. Attempts to activate spores by heat treatments at at 50 and 60 °C were unsuccessful. This is in contrast with the very successful activation of *Phycomyces blakesleeanus* spores after such heat treatment (Halbsguth and Rudolph, 1959). We conclude that heat activation is not suitable for activation of dormant sporangiospores of *Rhizopus oligosporus*.

Table 1. Effect of heat treatments on metabolic activation of fresh and stored spores of *Rhizopus oligosporus*

Activation treatment	Metabolically active[1] spores of *R. oligosporus* grown on rice and stored for 2 months at 25 °C		Metabolically active spores of *R. oligosporus* grown on MEA for 4 days, at 37 °C	
	Log N/g dry wt rice powder Avg. ± S.D	% of total number of spores[2]	Log N/ml Suspension Avg. ± S.D	% of total number of spores[2]
MEB[3] (37 °C, 4h)	8.57 [a] ± 0.01	79.4	8.68[a] ± 0.01	93.3
Buffer[4] (37 °C, 4h) (Control)	7.7 [b] ± 0.04	10.7	8.5[b] ± 0.04	61.7
Buffer[4] (50 °C,10 min)	7.48 [c] ± 0.08	6.5	n.d.[5]	n.d.
Buffer[4] (60 °C, 3 min)	7.46 [c] ± 0.04	6.2	8.46[b] ± 0.23	56.2
Buffer[4] (60 °C, 10min)	7.37 [c] ± 0.06	5.0	n.d.	n.d.

[1] Fluorescent with cFDA; [2] Log total spores = 8.71; [3] Malt Extract Broth, Oxoid CM 57; [4] Buffer was prepared by K_2HPO_4 50 mM adjusted to pH 4.0 with citric acid 50 mM; [5] n.d. = not determined. Data reported as means of triplicates with standard deviation. In each column, data with the same indicators are not significantly different (p ≤ 5%, 1 tail unrelated *t*- test).

Table 2 presents data on the effects of control buffer, CDM (Czapek-Dox liquid medium), and MEB (Malt Extract Broth). Incubation in CDM did not result in appreciable activation (low number of fluorescent metabolically active spores). This suggests that its main components, sodium nitrate and sucrose, play no role as activating compounds. The number of fluorescent, i.e. active spores increased strongly by incubation in MEB at 37 °C. In an earlier study (Breeuwer et al., 1997) it was observed that the majority of freshly harvested spores had become fluorescent after 3 h incubation at 37 °C in MEB. Our data in Table 2 show that activation with MEB proceeds very quickly. We found that MEB is suitable for activation of dormant spores, even after prolonged storage of 2 months. This suggests that problems relating to limited shelf-life reported earlier (Tanuwidjaja and Roestamsjah, 1985; Tunçel et al., 1989), might have been caused by dormancy and could have been overcome by activation under appropriate conditions. The results of the control in Table 1 show that the number of active spores in fresh MEA-grown spores (61.7%) was much higher than that of rice-grown spores stored for 2 months (10.7%) and when activated in MEB, fresh spores can be activated up to 93%. This means that not only stored spores, but also fresh spores benefit from MEB activation.

Table 2. Effect of incubation time in activation solutions on the metabolic activation of rice-grown spores of *R. oligosporus* stored for 2 months at 25 °C

Incubation time	Buffer[1] (control)		CDM[2]		MEB[3]	
	Log N/g dry wt[4]	% of total[5]	Log N/g dry wt[4]	% of total[5]	Log N/g dry wt[4]	% of total[5]
5 min[6]	7.75	12.0	7.48	6.5	8.52	70.8
2 h[6]	7.72	11.2	7.65	9.6	8.54	74.1
4 h[6]	7.51	6.9	7.74	11.8	8.58	81.3
6 h[6]	7.53	7.2	7.84	14.8	8.57	79.4
8 h[6]	7.47	6.3	7.80	13.5	8.55	75.9

[1] Buffer: K_2HPO_4 50 mM adjusted to pH 4.0 with citric acid 50 mM
[2] Czapek-Dox medium, Oxoid CM 95
[3] Malt Extract Broth, Oxoid CM 57
[4] N= cFDA fluorescent spores; dry wt: dry weight of sample
[5] Log total spores = 8.67
[6] Measured after 20 min for fluorescent staining at 40 °C in buffer[1]

The mechanistic base for dormancy of *R. oligosporus* is not known. In *Aspergillus oryzae*, it has been shown (Horikoshi, 1964) that spore coats contain higher levels of glucosamine and protein; spore walls of *Rhizopus stolonifer* are multi-layered (Hawker and Abbott, 1963) and superficial lipid on the asexual spores of *Rhizopus stolonifer* prevents wetting (Fisher et al., 1972). Such physico-chemical conformations of the spore surface could prevent nutrient uptake and activation. Considering the rapid activation of dormant spores of *R. oligosporus* as shown in Table 2, we conclude that its dormancy is not caused by such factors, but rather by deficiency of certain nutrients.

We also tested whether MEB may still be limited in its concentrations of activating component(s), by carrying out the activation experiments of Table 2 with MEB (normal strength) to which D-Glucose (0.5%), NH_4Cl (0.1%), and KH_2PO4 (0.15%) were added, and with 1.5-fold concentrated MEB. Similar numbers of fluorescent and germinated spores were found (data not shown) which makes us conclude that the levels in MEB are adequate to activate dormant spores.

Activation, germination and viability as affected by exposure to MEB

The activation, swelling and germination sequence followed by mycelial growth of *R. oligosporus* sporangiospores is shown in Table 3. The number of activated spores quickly exceeded 9 decade log units (42% of total spores) already after 5 min of exposure to MEB, whereas the maximum was reached after 6 h (70% of total spores). Spore swelling, the first visible phase of germination, had started between 20 min and 2 h. Next, the emergence of germ tubes followed after 4 h, with 8.99 decade log unit increasing to 9.27 decade log unit (55% of total spores) after 6 h. However, after 8 h the outgrowth of germ tubes caused such entwinement that counting was not reliable anymore. Our results agree with earlier reports (Ekundayo and Carlile, 1964; Medwid and Grant, 1984; Breeuwer et al., 1997) that germination proceeded through two distinguishable phases, viz. phase 1 the enlargement of the sporangiospores and phase 2 the emergence of a germ tube. Table 3 shows that the number of viable spores increased from 8.63 log units (12.6% of total) after 20 min activation, to its highest value of 9.28 log units (56.2% of total) after 4 h and then decreased slightly. We expect this decrease to be an artefact caused by mycelial entwinement and physical damage to germ tubes during surface plating; both will decrease the number of colonies to some extent. After 4 h we obtained higher levels of viability than visible germ tubes probably because the spores that had swollen started to form germ tubes during plating on MEA medium. This assumption is supported by the increase of germinated spores between 4 h and 6 h.

Table 3. Activation, germination and viability of rice-grown spores of *R. oligosporus* stored for 3 months at 25 °C after exposure to Malt Extract Broth at 37 °C

Time	Active spores[1]		Germinated spores[2]		Viable spores[3]	
	Log N/g dry wt[4]	% of total[5]	Log N/g dry wt[6]	% of total[5]	Log CFU/g dry wt[6]	% of total[5]
0 min [7]	0	0	0	0		
5 min [7]	9.15	41.7	0	0		
10 min [7]	9.16	42.7	0	0		
15 min [7]	9.19	45.7	0	0		
20 min [7]	9.21	47.9	0	0	8.63	12.6
2 h [7]	9.25	52.5	0 (swollen)	0	8.96	26.9
4 h [7]	9.32	61.7	8.99	28.8	9.28	56.2
6 h [7]	9.37	69.2	9.27	55.0	9.16	42.7
8 h [7]	Not countable[8]		Not countable[8]			

[1] Fluorescent with cFDA; [2] Showing germ-tubes; [3] Producing colonies on MEA and RBCC; [4] N= fluorescent spores; [5] Log total spores = 9.53; [6] Dry wt = dry weight of spore powder; [7] Measured after 20 min for fluorescent staining at 40 °C in buffer (K_2HPO_4 50 mM adjusted to pH 4.0 with citric acid 50 mM); [8] Caused by mycelial entwinement.

Effect of some carbon and nitrogen sources on viability of stored (11 months) spores

As shown above, MEB containing peptone and maltose, is a good activation medium. The effect of complex carbon and nitrogen sources on germination and colony formation (viability) of dormant spores is shown in Table 4 for (1) MEB as a control, (2) glucose 2%, (3) peptone 1%, (4) yeast extract 1%, (5) casamino acids 1%, and (6) combined glucose and peptone. Without incubation or after an incubation during 4 h at 37 °C of the dormant spores, the activated spores were spread-plated on the same solutions to which 1% w/v Agar Bacteriological (Oxoid L11) had been added. Colonies were counted after 12 h. The

data show the effects of activation (incubation in activation solution) and of individual components.

Table 4. Effect of some carbon and nitrogen sources on viability of rice-grown spores of *R. oligosporus* stored for 11 months at 25 °C

Activation solution	Without activation		Activation[1] at 37[0]C, 4 h	
	Log CFU/g	% of	Log CFU/g	% of
	Avg. ± S.D	total[2]	Avg. ± S.D	total[2]
1. MEB[3]	8.53[a] ± 0.03	36.3	8.63[a] ± 0.05	45.7
2. Glucose[4] (2 %)	<7.0[c]	<1.1	<7.0[c]	<1.1
3. Peptone[5] (1 %)	8.51[a] ± 0.04	34.7	8.63[a] ± 0.04	45.7
4. Yeast extraxt[6] (1 %)	8.52[a] ± 0.06	35.5	8.59[a] ± 0.05	41.7
5. Casamino acids[7] (1 %)	7.96[b] ± 0.05	12.0	8.25[b] ± 0.06	19.1
6. Glucose[4] (2%) + peptone[5] (1%)	8.49[a] ± 0.04	33.1	8.59[a] ± 0.04	41.7

[1]Spores were incubated in activation solution; [2]Log total spores = 8.97; [3]MEB: Malt Extract Broth, Oxoid CM 57; [4]D(+)-Glucose-Monohydrate, Merck, Art. 1.08342; [5]Peptone aus Casein, Merck, Art. 7213; [6]Yeast Extract, Oxoid L21; [7]Bacto vitamin-free casamino acids, Difco, Art. 0288-01. Data reported as means of triplicates with standard deviation. In each column, data having the same indicators are not significantly different (p ≤ 5%, 1 tail unrelated *t*- test).

On glucose alone, viability was very low. Activation in casamino acids increased viability from 12.0 to 19.1% but the viability was significantly lower than in MEB, Peptone, Yeast extract and Glucose + peptone which were not significantly different. It was reported (Ekundayo and Carlile, 1964) that the initiation of germination of *R. arrhizus* sporangiospores required the presence of glucose or fructose and that germinating spores were transferred to a medium lacking glucose, swelling soon ceased. Glucose was also essential for initiation of spore swelling and germ tube emergence in *Syncephalastrum racemosum* (Hobot and Gull, 1977); both quickly ceased when glucose-grown cultures shifted down to non-permissive glucose-free medium. Our data also show that only glucose is insufficient to allow colony formation. Obviously, additional nitrogen and mineral sources will be required for germination and colony formation. In early studies on the slime mold *Dictyostelium discoideum* (Cotter and Raper, 1966) it was noted that the spores were constitutively dormant and did not release myxamoeba if merely suspended in buffer; however, each spore released a simple myxamoeba if peptone were added to the buffer. When the component parts of peptone were tested (exclusive of peptides), the

amino acids were mainly responsible for germination whereas vitamins, salts and glucose had no effect. Our data suggest a similar response by *R. oligosporus*. It was interesting to note that even after 11 months of storage, the viability of spores activated by MEB was still rather high (45.7% of total). This is partly due to the fact that the moulded rice was not finely milled since we observed that fine grinding gives considerable losses of viability (Thanh and Nout, 2002).

Dormancy, activation and viability of spores during a production and storage trial

We studied metabolic activity and viability from production up to 16 months storage of rice-based spore concentrate by measuring total number of spores, metabolically active spores, dormant spores, viable spores, and dead spores. Figure 2 shows that at harvest most spores (85.1%) are in the dormant stage, and only 11.2% are active. Of the dormant spores, more than 60% could be activated.
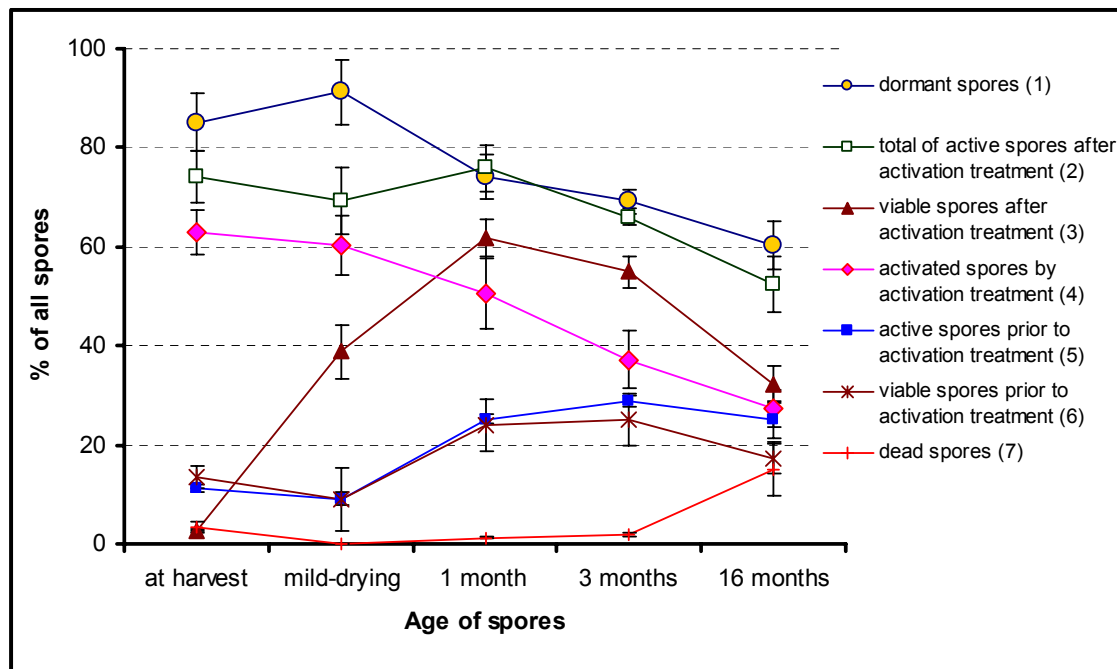

Figure 2. Activity, dormancy, and viability of *R. oligosporus* sporangiospores in a rice-based spore concentrate

(1) spores lacking enzyme activity, but with intact cytoplasmic membrane; (2) sum of active spores prior to activation treatment and activated spores; (3) sum of viable spores prior to activation treatment and increased viability by activation treatment; (4) spores activated from dormancy by activation treatment; (5) metabolically active spores without activation treatment; (6) viability without activation treatment; (7) spores with damaged cytoplasmic membrane allowing entry of propidium iodide.

Activation treatment did not increase the viability of freshly harvested spores; we assume that they were too sensitive to the staining temperature (40 °C) or they were too young. After mild drying, a general but slight decrease of active and viable spores was observed. As noticed earlier (Thanh and Nout, 2002) this must be caused by the drying temperature of 42 °C and the loss of moisture which renders rehydration more difficult. We found however, that the viability of dried spores could be enhanced considerably by activation.

After 1 month storage and thereafter, no dead germ tubes were observed anymore, indicating that the spores were maturing, stabilizing their structure and possibly recovering from the drying treatment. After 1-month storage, viability after activation was highest at 8.74 log units (61.7% of total), and decreased gradually during further storage to 32.4% after 16 months. This is conformity with earlier data (Rusmin and Ko, 1974; Wang et al., 1975) showing that the germination percentage of processed spores decreased rapidly during the early storage period. After 3 months of storage, the number of active spores had increased to 8.41 log units (28.8%), with concomitant decrease of dormant spores. This would suggest that during storage, some dormant spores woke up, i.e. changed to active spores. After 16 months of storage, a considerable number of stored spores had died (8.13 log units, or 15.1% of total), and the number of viable spores had strongly decreased to 8.2 log units (17.4%), whereas activation was not very effective any longer. The data after 16 months indicate that dormant spores could still be activated but that active spores could hardly germinate anymore.

In conclusion, we found that the majority of sporangiospores of *R. oligosporus* in rice-based starter are in a dormant stage. During storage, some of the dormant spores become active. The numbers of active and viable spores correlate well. Most dormant spores can be activated by MEB, peptone or yeast extract, with concomitant increase of viable spores.

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REFERENCES

Belmans, D. L., Van Laere, A. J., and Van Assche, J. A. (1983) Effect of n-alcohols and high pressure on the heat activation of *Neurospora tetrasperma* ascospores. *Archives of Microbiology* **134**, 49-51.

Breeuwer, P., De Reu, J. C., Drocourt, J.-L., Rombouts, F. M., and Abee, T. (1997) Nonanoic acid, a fungal self-inhibitor, prevents germination of *Rhizopus oligosporus* sporangiospores by dissipation of the pH gradient. *Applied and Environmental Microbiology* **63**, 178-185.

Cotter, D. A., and Raper, K. B. (1966) Spore germination in *Dictyostelium discoideum. Proceedings of the National Academy of Science U. S.* **56**, 880-887.

Ekundayo, J. A., and Carlile, M. J. (1964) The germination of sporangiospores of *Rhizopus arrhizus*: spore swelling and germ-tube emergence. *Journal of General Microbiology* **35**, 261-269.

Fisher, D. J., Holloway, P. J., and Richmond, D. V. (1972) Fatty acid and hydrocarbon constituents of the surface and wall lipids of some fungal spores. *Journal of General Microbiology* **72**, 71-78.

Griffin, D. H. (1994) Spore dormancy and germination. In *Fungal physiology* ed. D. H. Griffin, pp. 375-398. New York: John Wiley & Sons, Inc.

Halbsguth, W., and Rudolph, H. (1959) Untersuchungen über die Wärmeaktivierung der Sporangiosporen von *Phycomyces blakesleeanus. Archives of Mikrobiology* **32**, 296-308.

Hawker, L. E., and Abbott, P. M. (1963) Fine structure of vegetative hyphae of *Rhizopus. Journal of General Microbiology* **30**, 401-408.

Hobot, J. A., and Gull, K. (1977) The influence of glucose availability on events during germination of *Syncephalastrum racemosum* sporangiospores. *Journal of General Microbiology* **98**, 431-437.

Horikoshi, K., Iida, S. (1964) Isolation and composition of the spore coats of *Aspergillus oryzae. Biochimica et Biophysica Acta* **83**, 197-203.

Medwid, R. D., and Grant, D. W. (1984) Germination of *Rhizopus oligosporus* sporangiospores. *Applied and Environmental Microbiology* **48**, 1067-1071.

Rusmin, S., and Ko, S. D. (1974) Rice-grown *Rhizopus oligosporus* inoculum for tempeh fermentation. *Applied Microbiology* **28**, 347-350.

Sussman, A. S., and Halvorson, H. O. (1966) *Spores, their dormancy and germination.*, New York: Harper & Row Publishers.

Tanuwidjaja, L., and Roestamsjah (1985) Preparation and utilization of powder form inoculum for tempe fermentation. *ASEAN Food Journal* **1**, 22-24.

Thanh, N. V., and Nout, M. J. R. (2002) Biomass and yield, viability and dormancy of sporangiospores of *Rhizopus oligosporus* as a function of tempe starter preparation conditions. *Food Microbiology* **19**, 91-96.

Thevelein, J. M., Van Assche, J. A., Carlier, A. R., and Heremans, K. (1979) Heat activation of *Phycomyces blakesleeanus* spores: thermodynamics and effect of alcohols, furfural, and high pressure. *Journal of Bacteriology* **139**, 478-485.

Thevelein, J. M., Van Laere, A. J., Beullens, M., Van Assche, J. A., and Carlier, A. R. (1983) Glucose-induced trehalase activation and trehalose mobilization during early germination of *Phycomyces blakesleeanus* spores. *Journal of General Microbiology* **129**, 719-726.

Tripp, M. L., and Paznokas, J. L. (1981) Relationship between sporulation medium and germination ability of *Mucor racemosus* sporangiospores. *Journal of General Microbiology* **127**, 35-43.

Tripp, M. L., and Paznokas, J. L. (1982a) Glucose-initiated germination of *Mucor racemosus* sporangiospores. *Journal of General Microbiology* **128**, 477-483.

Tripp, M. L., and Paznokas, J. L. (1982b) Role of amino acids and endogenous protein in the germination of *Mucor racemosus* sporangiospores. *Journal of General Microbiology* **128**, 485-495.

Tunçel, G., Nout, M. J. R., and Rombouts, F. M. (1989) Effect of acidification on the microbiological composition and performance of tempe starter. *Food Microbiology* **6**, 37-43.

Van Assche, J. A., and Carlier, A. R. (1975) Some properties of trehalase from *Phycomyces blakesleeanus. Biochimica et Biophisica Acta* **391**, 154-161.

Van Assche, J. A., Carlier, A. R., and Dekeersmaeker, H. I. (1972) Trehalase activity in dormant and activated spores of Phycomyces blakesleeanus. *Planta (Berl.)* **103**, 327-333.

Van Assche, J. A., Carlier, A. R., and Van Tieghem, L. C. (1977) The effect of gramma radiation on breaking of dormancy in *Phycomyces* spores. *Archives of Microbiology* **113**, 95-97.

Van Assche, J. A., Van Laere, A. J., and Carlier, A. R. (1978) Trehalose metabolism in dormant and activated spores of *Phycomyces blakesleeanus. Burgeff. Planta* **139**, 171-176.

Van Laere, A. J., Van Assche, J. A., and Carlier, A. R. (1980) Metabolism and chemical activation of Phycomyces blakesleeanus sporangiospores. *Experimental Mycology* **4**, 260-268.

Van Schaftingen, E., and Van Laere, A. J. (1985) Glycerol formation after the breaking of dormancy of Phycomyces blakesleeanus spores. *European Journal of Biochemistry* **148**, 399-404.

Wang, H. L., Swain, E. W., and Hesseltine, C. W. (1975) Mass production of *Rhizopus oligosporus* spores and their application in tempeh fermentation. *Journal of Food Science* **40**, 168-170.

Chapter 4

Effect of individual amino acids and glucose on activation and germination of *Rhizopus oligosporus* sporangiospores in tempe starter

Abstract

The aim of this study was to determine the role of individual amino acids and glucose in the activation of dormant sporangiospores in tempe starter. Using fluorescence microscopy of labeled spores and culturing to detect metabolic activity and viability, respectively, we observed that (1) L-alanine plays an important role (of the same order as that of peptone) in stimulation of germination of dormant spores, and can satisfy the requirements of carbon as well as nitrogen for spore germination; (2) L-proline, on the other hand, inhibits alanine uptake by blocking/congesting transporters of spore cells, resulting in apparent low viability on agar media; (3) L-leucine and L-isoleucine slightly favour spore germination while L-arginine and L-lysine do not have any stimulating effect; (4) The stimulatory role of glucose was only evident in the presence of phosphate (in minimal medium); (5) Phosphate plays a facilitating role in spore germination. In conclusion, the ability and rate of germination of dormant/old sporangiospores of *R. oligosporus* depend on their uptake of individual amino acids and/or glucose. In tempe starter which has been stored, a majority of sporangiospores is dormant, but can be activated. This will enhance the effectiveness of these starters in tempe manufacture and will allow their longer storage.

INTRODUCTION

A spore must first germinate to develop into the vegetative stages of the fungus. The most important and consistent feature in the germination process is the shift from a state of very low metabolic activity into one of high activity. This shift depends on at least four factors: the presence of endogenous regulatory mechanisms; essential nutrients, either stored or available in the immediate environment; the presence of endogenous enzymes for respiration and synthesis; and the appropriate environmental physical conditions. Germination can be prevented by any one or a combination of a number of limiting factors. These may be developmental, such as spore maturation, senescence, and dormancy; nutritional, such as the absence of the proper carbon and nitrogen sources; environmental physical limitations, such as unfavourable temperature or osmotic pressure, or the absence of water; and the presence of inhibitory compounds in the spores or its environment. The process of germination varies with the species of fungus, but the essential feature is still the change from an inactive to an active phase (Gottlieb, 1978).

The components necessary for the initiation of germination have been studied in several fungal and bacterial spores. The requirement for exogenous carbon and nitrogen for germination of spores may depend on their density, e.g., at low density (10^3/ml) macroconidia of *Fusarium solani* did not require exogenous carbon or nitrogen for complete germination in phosphate-buffered inorganic salts medium, but at 10^6/ml they required exogenous nutrients (Griffin, 1970a). High extent of germination took place in chlamydospores that had been formed at low conidial density and required only an exogenous source of carbon, whereas chlamydospores formed at high conidial density required both exogenous carbon and nitrogen (Griffin, 1970b). The triggering of germination of *Mucor racemosus* sporangiospores was also affected by previous cultivation conditions (Tripp and Paznokas, 1981).

Single amino acids were less effective than glucose or peptone to trigger germination, and glucose may trigger germination by initiating the breakdown of endogenous protein reserves. The subsequent increase of free amino acids may be the dormancy-breaking step (Tripp and Paznokas, 1982). Glucose was the main factor in the germination medium responsible for the activation of trehalase (Thevelein et al., 1983). Protein is a major endogenous reserve in *M. racemosus* sporangiospores and its turnover is a necessary event for glucose-triggered germination (Tripp and Paznokas, 1982).

It was found (Ekundayo and Carlile, 1964) that initiation of germination of sporangiospores of *R. arrhizus* Fischer required the presence of glucose or fructose; in addition, maximum germination required a nitrogen source, phosphate ions and potassium or sodium ions.

Fungal spores germinated most rapidly in mixtures of amino acids; L-proline and L-alanine were the most effective for *R. oligosporus* (Medwid, 1984) and *A. flavus* (Orlowski and Sypherd, 1978). Similarly as with mixtures of amino acids, single amino acids also play a role as stimulators, such as L-leucine that induced germination of *Trichophyton mentagrophytes* microconidia (Hashimoto et al., 1972); proline combined

with phosphate effectively stimulated germination of spores of *R. stolonifer* Lind (Weber, 1962) and *R. arrhizus* Fisher (Weber and Ogawa, 1965); L-alanine initiated the germination of spores of *Bacillus subtilis* (Wax and Freese, 1968). Ornithine and arginine were good stimulators of spore germination of *R. arrhizus* in contrast to glutamic acid (Weber and Ogawa, 1965).

Moreover, mixtures of carbon and nitrogen sources are good activators e.g., glucose plus NH_4Cl supported a high extent of germination of *A. flavus* conidia, with exogenous nitrogen having the biggest influence (Pass and Griffin, 1972). Maximum germination of *R. arrhizus* spores occurred in the presence of utilizable carbon and nitrogen sources together with phosphate, sulphate, potassium and magnesium ions (Ekundayo, 1966).

The understanding of physical and nutritional conditions promoting activation and germination will contribute to a better control of production and optimization of storage procedures for tempe starters.

Most biochemical studies on *R. oligosporus* sporangiospores described previously have been carried out with freshly harvested spores in nutritionally complex media. We carried out studies in a defined minimal medium (Sorenson and Hesseltine, 1966) to determine carbon and nitrogen requirements for activation and germination of sporangiospores in stored starter.

MATERIALS AND METHODS

Preparation of tempe starter

Tempe starter was prepared as described earlier (Thanh and Nout, 2002). Briefly, the procedure was as follows: *Rhizopus oligosporus* LU 575 (NRRL 5905) was grown on Malt Extract Agar [MEA (malt extract 30 g/l, mycological peptone 5 g/l, agar 15 g/l), Oxoid, CM 59] plates during 1 week at 30 °C, and spores were harvested by washing with sterile water. The spore suspension was diluted to approximately 10^5 spores per ml. This suspension "A" was used as inoculum for substrate used to prepare tempe starter. Polished broken rice was steam-sterilized, allowed to cool and sterilized ammonium sulfate and sterilized 0.5 M H_2SO_4 were added to adjust the pH to 4, which is the optimum pH for spore germination of *Rhizopus oligosporus* (Medwid and Grant, 1984; Breeuwer et al., 1997). The sterile broken rice (50 g) was inoculated and thoroughly mixed with 0.4 ml of spore suspension "A". The inoculated rice was incubated at 40 °C for 2 hours, followed by incubation for 4 days at 30 °C. The mouldy mass was broken up and the entire mass of substrate, mycelium and spores was dried at 42 °C for 48 h in a forced-air drying cabinet. Only crushing by sterile pestle and mortar, but no fine grinding was used in order to avoid misjudging during microscopic counting between spores and rice particles of similar size and shape. The starter powder was stored in closed screw-cap glass tubes, protected from light, in a silicagel desiccator at 25 °C, as these conditions were considered to be more representative of practical usage than storage at low temperature. Moreover, it was shown

that spores survive better at ambient (25-30 °C) temperature than at 5 °C (Thanh and Nout, 2002).

Preparation of spore suspensions

Sporangiospores in crushed rice powder were diluted with sterile water to suspensions (approximately 10^7 spores/ml) by vigorous agitation and coarse filtration with a Millipore membrane filter (Fluorassure, Chem filter 15, Ref: 100-C2003-01, Chemunex, France) to remove mycelium and other debris. The filtrate containing the spores was washed twice by centrifugation at 13000 x g for 3 min in an Eppendorf centrifuge. After decanting, the spores in pellets were re-suspended in experimental treatments for (a) effect of individual amino acids on viability, and (b) amino acid and glucose uptake of dormant spores.

Preparation of samples for analysis of activation and colony formation

Pellets of spore suspension were re-suspended in minimal medium with added L-amino acid and glucose at 10 mM (for activation and colony formation experiments) or specific concentrations (for L-amino acids and glucose uptake) in 1ml aliquots in Eppendorf tubes. They were incubated in a water bath at 37 °C. Sampling was carried out at time intervals, and supernatants were obtained after centrifugation at 13000 x g for 3 min in an Eppendorf centrifuge immediately after sampling. The supernatants were put on ice prior to analysis.

Minimal Mineral Medium

All treatments contained the following minimal medium (mineral base) (Sorenson and Hesseltine, 1966) in which KH_2PO_4 was replaced by H_3PO_4 0.01 M to adjust the pH at 4 as basal solvent. The mineral base contained: H_3PO_4 / K_2HPO_4 buffer at pH 4 (0.01 M final concentration); $MgSO_4$ (1.25 mM); $ZnSO_4$. $7H_2O$ (0.3 mM); $MnSO_4$. H_2O (0.2 mM); $FeCl_3$. $6H_2O$ (0.09 mM); $CuSO_4$. $5H_2O$ (0.03 mM); Ethylene diamine tetraacetic acid (EDTA) (1.5 mM). The mineral base, except the phosphates, was sterilized by autoclaving. The phosphates were autoclaved separately and added aseptically after cooling. Sugar and amino acids were sterilized by filtration with membrane filters (Schleicher & Schuell FP 30/0,2 CA-S; 0.2μm, 7 bar max.; Ref. No. 10 462 200) and added aseptically.

Analysis of activated spores and viability (colony formation)

Spores were incubated at 37 °C (in a water bath) suspended in amino acid and/or glucose-containing solution (10 mM) or in complex nutrient medium such as malt extract broth [MEB (malt extract 17 g/l, mycological peptone 3 g/l), Oxoid, CM 57], peptone [peptone from soybean meal, enzymatic digest, Fluka 70178], casamino acids [Bacto vitamin-free casamino acids, Difco, Art. 0288-01]. Activated (fluorescent) spores were measured after 0, 2 and 4 h. Colony formation (viable spores) was determined by plating the samples after 4 h activation as described below.

Counts of fluorescent (activated) spores

Spore suspensions were washed twice by centrifugation at 13000 x g for 3 min in an Eppendorf centrifuge in KPi buffer pH 4 (K_2HPO_4 50 mM, adjusted to pH 4.0 by KH_2PO_4 50 mM). Subsequently, the suspensions were incubated at 40 °C for 20 min in the presence of 0.22 mM cFDA [cFDA: 5-(and-6)-carboxyfluorescein diacetate (Molecular Probes Europe, Leiden, The Netherlands), 10 mg/ml acetone]. They were then put on ice and counted in a Bürker-Türk counting chamber by fluorescence microscopy, with an Axioskop epifluorescence microscope equipped with a 50-W mercury arc lamp, a fluorescein isothiocyanate filter set (excitation wavelength 450 to 490 nm; emission wavelength >520 nm), an x100 1.3 numerical-aperture Plan-Neofluar objective lens, and a camera (Carl Zeiss, Oberkochen, Germany). Total, fluorescent, and non-fluorescent spores, as well as spores showing germ tubes were counted. Their values were presented as percentages of total spores.

Viable spores

Colony formation was measured on samples that had been incubated during 4 h at 37 °C; the activated spores were spread-plated on the same solutions (or defined solutions with/without adding amino acids and glucose to which 1% (3% when minimal medium pH 4 was used) w/v Bacteriological Agar (Oxoid L11) had been added or on MEA [Malt extract agar (malt extract 30 g/l, mycological peptone 5 g/l, agar 15 g/l), Oxoid, CM 59]. Colonies were counted after incubation at 37 °C for 18 h (on activation medium + agar) or 12 h (on MEA) respectively.

Amino acid and glucose uptake by spores

Experimental

Pellets of spore suspension as described in "Preparation of spore suspensions" above, were used for amino acid and glucose uptake experiments. To each pellet (10^7 spores) in an Eppendorf tube, 1 ml of L-amino acid and/or glucose-containing solution (based on minimal medium) was added. Concentrations such as 0.025, 0.05, 0.25, 1.25, or 2.5 mM of alanine or alanine+glucose or alanine+proline for alanine uptake; 0.05, 0.25, 1.25, 2.5 mM of glucose or glucose+alanine for glucose uptake; and 0.25, 0.5, 1.0 mM of proline or proline+alanine for proline uptake were used. Triplicate pellets were used for each concentration; pelleted spores were re-suspended completely by high speed vortex shaking. All these Eppendorf tubes were incubated at 37 °C (in a water bath) for 0 min and 120 min. Then the sampled Eppendorfs were immediately centrifuged at 13000 x g for 3 min at 4 °C. The supernatants were put on ice prior to analysis. Each sample was analyzed in triplicate, as follows.

Alanine

Alanine was measured by the ECB-ninhydrin method for the determination of free alpha amino nitrogen (Lie, 1973).

Proline

Proline was measured by ninhydrin determination after extraction with toluene (Magné and Larher, 1992).

Glucose

Glucose was determined by a reducing sugar method (Somogyi, 1952).

Materials

L-Alanine 98% (Sigma, A-7627); L-Arginine (Sigma, A-5006); L-Isoleucine 98% (Sigma, I-2752); L-Leucine 98% (Sigma, L-8000); L-Proline 99% (Sigma, P-0380); D(+)Glucose-Monohydrate (Merck, 1.08342). H_3PO_4 (Merck, 1.00563); K_2HPO_4 (Merck, 1.05104); $MgSO_4 \cdot 7H_2O$ (Merck, 5886.1000); $ZnSO_4 \cdot 7H_2O$ (Merck, 8883); $MnSO_4 \cdot H_2O$ (Merck, 5963.0100); $FeCl_3$ (Merck, 803945); $CuSO_4 \cdot 5H_2O$ (Merck, 2790.0250); Ethylene diamine tetraacetic acid (EDTA) (Merck, 1.08418.1000); Ninhydrin (Sigma, N-4876); $Na_2HPO_4 \cdot 2H_2O$ (Merck, 6580); KH_2PO_4 (Merck, 1.04873.1000); D(-)Fructose (Merck, 1.04007.100); KIO_3 (Merck, 5051); Ethanol 96% (Merck, 1.00971.2500); Glacial acetic acid (Merck, 1.00063.2500); Toluene p.a. (Janssen Chimica, 17.685.31); K-Na-tartrate 99% (Merck, 8087); Sodium-carbonate (Merck, 1.0639.1000); Sodium-hydrogen-carbonate (Merck, 1.06329.1000); Ammonium-molybdate (VI) tetrahydrate (Janssen Chimica, 20.595.21 C.A.S. 12054-85-2); $Na_2HAsO_4 \cdot 7H_2O$ (Sigma 50g, S-9663); H_2SO_4 95-97% R.G. (Riedel-deHaen, 30743, UN-No. 1830).

RESULTS

The effects of complex organic components, individual amino acids and glucose on activation of spores and colony formation on MEA are shown in Table 1. MEB (malt extract broth), peptone, casamino acids and buffer were used as controls. In MEB and peptone, spores were activated quickly, followed by emergence of germ-tubes with maxima after 2 h (40% of spores in peptone) and 4 h (53% in MEB). Colony formation in activation solution with 1% added agar showed that MEB and peptone gave very similar results and contained sufficient nutritive substances that activated dormant spores to germinate and grow into colonies (45%), which was much higher than with casamino acids (31%).

Table 1. Effect of complex organic compounds and L-amino acids[1] (10mM) with or without adding glucose (1%) on activation and colony formation of *R. oligosporus* spores (stored for 12 months)

Activation medium	Activation time				Colony formation (%)	
	2 h		4 h			
	fluorescent spores (%)	spores with germ tubes (%)	fluorescent spores (%)	spores with germ tubes (%)	In activation medium + 1% agar	In MEA
Buffer[2] pH 4	37.2		45.7	13.8	< 1	19.1
MEB	64.6	swollen	66.1	52.5	45.7	45.7
Peptone (1%)	60.3	39.8	61.7	45.7	44.7	45.7
Casamino acids (1%)	46.8	swollen	57.5	43.7	30.9	44.7
Glucose	56.2	0	58.9	16.2	< 1	25.7
Arginine	nd		nd			34.7
Arg + glucose	nd		nd			22.4
Isoleucine	55.0		64.6			52.5
Ileu + glucose	58.9		55.0			50.1
Leucine	63.1		41.7			30.2
Leu + glucose	56.2		61.7			40.7
Alanine	60.3		58.9			43.7
Ala + glucose	55.0		51.3			44.7
Lysine	77.1		77.6			28.8
Lys + glucose	57.7		63.1			12.9
Proline	66.1		63.1			20.4
Pro + glucose	64.6		58.9			9.12
Phenylalanine	58.9		46.8			26.3
Phe + glucose	53.7		44.7			30.9
Tryptophan	54.9		61.7			41.7
Trp + glucose	44.7		50.1			33.1
Tyrosine[3]	58.9		60.3			26.3
Tyr + glucose	53.7		41.7			15.9

[1] These amino acids are present at high levels in peptone (Fluka 70178). Data reported as means of triplicates. (*continued next page*)

[2] Buffer pH 4 was prepared from K_2HPO_4 50 mM and citric acid 50 mM. For non-activation: fluorescent spores = 13.8% and colony formation = 14.5%.

[3] L-tyrosine: supernatant from saturated solution was used in experiment.

nd: not determined (solution became fluorescent as soon as cFDA was added).

Thus, for colony formation, peptone is a superior source of amino acids. Free amino acids were subsequently investigated individually (with or without adding glucose) for their effect on activation and colony formation.

Glucose, in comparison to buffer, had some positive effect on dormant spores after incubating for 4 h at 37 °C, with slightly higher numbers of activated (fluorescent) spores, germ-tubes and colonies formed on MEA. However, unlike with peptone or casamino acids, glucose alone could not support colony formation with added 1% agar. This indicates that glucose may energize dormant spores but an additional nitrogen supply is needed to support colony formation. Individual L-amino acids were tested for their effect on activation. In general, the highest levels of activated (fluorescent) spores were obtained with lysine (78%) followed by isoleucine (65%), proline (63%), tryptophan (62%), tyrosine (60%), alanine (59%), phenylalanine (47%) and leucine (42%). Colony formation on MEA was highest after activation in isoleucine (53%), followed by alanine (44%), arginine (35%), leucine (30%), lysine (29%), phenylalanine (26%), tyrosine (26%), tryptophan (21%), and proline (20%). Mixtures of individual L-amino acids and glucose showed that adding glucose did not improve the activation of spores or colony formation. These results also show that there is no linear correlation between the numbers of activated spores and colonies formed on agar (MEA) medium.

The effects of individual amino acids and glucose on activation and colony formation in minimal medium are shown in Table 2. Minimal medium contained (only) mineral base (Sorenson and Hesseltine, 1966) in which KH_2PO_4 was replaced by H_3PO_4 10 mM to obtain pH 4. All media (activating solutions and agar media) were based on minimal medium with or without the addition of individual amino acids and/or glucose. The relative effectiveness of individual amino acids and glucose on activation and germination of 12-month-old sporangiospores was assessed by counting, based on the number of activated (fluorescent) spores obtained after incubation for 2 and 4 h at 37 °C in minimal medium to which individual amino acids were added, and by counting colonies formed on agar media.

L-alanine, L-arginine, L-isoleucine, L-leucine, L-lysine, L-proline (10 mM final concentration) were selected because they predominate in peptone which was shown to be one of the best activators of dormant spores of *R. oligosporus* (Thanh and Nout, 2004). The percentage of fluorescent (activated) spores was similar for all of treatments (about 58-68%) after 2 h or 4 h of incubation. However, differences were observed with the emergence of germ tubes between 2 or 4 h of activation; in lysine no germ tubes developed, in glucose approx 26% of spores showed germ tubes after 4 h, whereas with proline 44%, with alanine 26%, with leucine 22%, and with isoleucine 26%. Combinations of amino acids showed increased germ tube formation: 36% (Pro+Ala); 39% (Leu+ Ileu); 35% (Ala+Ileu+Leu) compared to glucose only. In comparison with Table 1, these results

show that the inclusion of minimal medium, especially phosphates, has an overall positive effect on activation. In addition, it is shown that individual amino acids have combinational effects on germ tube formation. As mentioned earlier in Table 1, numbers of activated spores and colonies obtained from plating these activated spores on agar media were not correlated. Activated spores when plated on agar media showed quite different abilities to form colonies in defined agar media: (1) minimal, (2) minimal plus glucose, (3) minimal plus amino acid(s) which was/were used in activation solution, and (4) glucose added to (3). On the agar media (2) and (4), all activated spores gave comparable numbers of colonies while medium (3), limited, and (1) allowed only a few treatments to give rise to colony formation. The ability to form colonies decreased from agar medium containing glucose (2) and (4), to those containing amino acid(s) (3) and was lowest in minimal agar medium (1). This showed the important role of glucose and certain amino acids in spore germination and colony formation.

L-alanine is the most effective amino acid in germination of spores (stored for 12 months). Minimal agar medium (1) was designed to study the role of individual amino acids in activation and viability (colony formation) of long-term stored spores. No colony formation occurred after the treatments with L-arginine, or L-lysine on agar medium (3) from spores that were activated in minimal medium, or in minimal medium plus each of these amino acids. It can be concluded that L-arginine and L-lysine play no role in colony formation. Of the other amino acids when tested on minimal agar medium (1), only L-alanine was effective in promoting spore germination, while L-isoleucine, L-leucine, and L-proline did not support germination. This indicated that L-alanine only could support colony formation, presumably because it can be used as C- as well as N-source. It was observed however, that in a mixture of L-alanine and L-proline no colony formation took place on minimal agar medium (1), while colony formation was obtained in a mixture of alanine-leucine-isoleucine solution, although no colonies were found on leucine or isoleucine or a mixture of leucine and isoleucine. This would suggest that proline counteracts alanine, and keeps dormant spores from germination.

Glucose is essential for germination of spores (stored for 12 months). Of spores activated in minimal medium without added glucose, only those incubated in minimal medium with added glucose (2) germinated and formed colonies. On minimal agar medium with added glucose (2) colonies were formed irrespective of the activation treatment given, including amino acids that failed in causing germination such as arginine or lysine, as well as minimal buffer control (Table 2). This reconfirmed the essential role of glucose in germination of (activated dormant) spores. Glucose was taken up either earlier (during activation) or later (on agar medium) and used for spore germination even without added amino acids, this can imply that glucose is an effective carbon source to activate the assimilation of endogenous nitrogen sources.

Table 2. Effect of L-amino acids[1] (10mM) and D-glucose (1%) on activation and colony formation of *R. oligosporus* spores (stored for 12 months) in minimal medium

Activation medium	Activation time					Colony formation (on the same activated solution + agar (3%) and incubated at 37 °C) (%)			
	0 h	2 h		4 h				Minimal Medium	
	fluorescent spores (%)	germ tubes (%)	fluorescent spores (%)	germ tubes (%)	fluorescent spores (%)	+ Agar	+ Agar +Glucose	+ Agar + aa[2]	+ Agar +Glucose + aa[2]
						(1)	(2)	(3)	(4)
Minimal medium[3]	41.7	14.1	51.3	35.5	58.9	<1	11.0[4]	17.0[5] <1 (Arg; Leu; & Lys)[5]	22.4[6]
+ Glucose	43.7	5.9	57.5	26.3	66.1	15.5	16.6	20.9	21.4
+ Lysine	52.5	0	57.5	0	60.3	<1	2.8	<1	6.0
+ Arginine	Nd[7]	nd	nd	nd	nd	<1	23.4	<1	12.9
+ Leucine	53.7	4.2	67.6	21.9	67.6	<1	15.5	13.8	20.4
+ Isoleucine	58.9	6.8	66.1	26.3	67.6	<1	17.8	22.4	19.1
+ Alanine	55.0	14.5	60.3	25.7	72.4	15.5	24.0	17.0	20.4
+ Proline	53.7	5.6	66.1	43.7	70.8	<1	7.8	9.8	7.8
+ Pro + Ala	60.3	13.8	69.2	36.3	70.8	<1	14.5	6.8	10.7
+ Leu + Ileu	67.6	14.8	74.1	38.9	75.9	<1	20.9	21.9	19.5
+ Ala + Ileu + Leu	60.3	7.8	72.4	34.7	74.1	17.4	30.9	35.5	31.6

[1] These amino acids were selected from results of earlier experiments (Table 1). Data represent means of triplicates. [2] one, two or three amino acids were used [same as the corresponding amino acid used in the activation medium (column 1)]; [3] Sorenson and Hesseltine (1966) in which the pH = 4 was adjusted with H_3PO_4 10mM; [4] Average of 10 plate counts of spores activated in minimal medium plated on Glucose-agar-minimal medium; [5] No colonies were found on plate counts of spores activated in minimal medium, plated on minimal glucose agar with Arg, Leu or Lys; with all other amino acids, colonies (17% average) were obtained; [6] Average of 10 plate counts of spores activated in minimal medium plated on Glucose-agar-minimal medium supplemented with any amino acid(s); [7] not determined (solution became fluorescent as soon as cFDA was added).

Table 3. Effect of soybean processing on activation and colony formation of *R. oligosporus* spores (stored 12 months) in minimal medium

Activation medium	Activation time					Colony formation (on the same activated solution + agar (3%) and incubated at 37 °C) (%) Minimal Medium			
	0 h	2 h		4 h		+ Agar	+ Agar +Glucose	+ Agar + Soy-ext[1]	+ Agar +Glucose + Soy-ext[1]
	fluorescent spores (%)	fluorescent spores (%)	germ tubes (%)	fluorescent spores (%)	germ tubes (%)	(1)	(2)	(3)	(4)
I. Soybean extract (rich[2])	57.5	77.6		81.3	7.1	12.9	16.6	34.7	27.5
II. Soybean extract (poor[3])	64.6	72.4		85.1	26.9	5.5	12.3	21.4	31.6
III. Soybean cooking water	60.3	69.2	4.7	81.3	6.5	26.3	20.9	37.2	33.9

Soybean extract: 150 g soaked overnight (with demi-water), cooked 3 h, blended, centrifuged, filtered, finally yielding 1000 ml extract. [1]Soy-ext: soybean extract/ soybean cooking water was added to agar medium [soy-extract is indicated by matching to that of in the 1st column (activation medium)]; [2]rich nutrition extract: during the process of making the extract, all by-products i.e. soak and cooking water were reused and added up to the extract solution; [3]poor nutrition extract: soak and cooking water were not reused. This is representative for cooked soybeans used in traditional tempe making processes.

The amino acid(s) and glucose uptake during spore activation is shown in Fig. 1. Spores were incubated in individual L-amino acids (10 mM) and/or D-glucose solutions, and uptake rates were analyzed after incubation during 120 min. Alanine, proline, isoleucine, leucine and lysine were selected on the basis of their effects on colony formation on agar medium (1) (Table 2) being representative for stimulation (alanine), slight stimulation (leucine, isoleucine), neutral (lysine) and inhibition (proline) of germination of dormant spores. The uptake rate (μmoles/min) of alanine depended on its concentration in the solution; at higher concentrations the uptake rate was higher, with a maximum of 0.23 μmoles/min at a concentration of 1.25 mM. Fig. 1 shows that alanine uptake was not affected by the presence of glucose, but was reduced in the presence of proline. In the mixture of alanine and proline (both at 1.25 mM), alanine uptake rate (0.09 μmoles/min) was only 38% of its individual uptake rate (0.23 μmoles/min).

Fig. 2 shows that the proline uptake rate was very low from a solution containing only proline and from a mixture of proline and alanine. Unlike for alanine, proline uptake was not influenced measurably by its concentration. We suppose that L-proline might not inhibit the alanine function, but inhibits alanine uptake by congesting the alanine transport mechanism; the higher the proline concentration, the more alanine uptake is blocked. Similarly to proline, we observed very low uptake rates for leucine, isoleucine and lysine (data not shown).

Fig. 1 shows that the uptake rate of glucose increased with higher glucose concentrations with a maximum of 0.65 μmoles/min at 0.25 mM. At higher concentration of glucose its uptake appears to be decreased. Uptake of glucose was practically independent of the presence of alanine, and was nearly 3 times higher than alanine uptake rate and much higher than uptake rates of the other amino acids. However, at the higher concentrations its uptake rate slightly decreased in mixtures of glucose and alanine. These uptake data explain the inability to form colonies in medium (1) (Table 2) after activation in leucine, isoleucine and proline because of their poor uptake. Proline blocks the uptake of alanine, explaining the inability to form colonies in medium (1) after activation with a mixture of alanine and proline.

The starter spores ultimately need to perform during the tempe fermentation. The first steps in the tempe making process (Nout and Rombouts, 1990) include soaking of soybeans in water overnight, followed by discarding the soak water, and cooking the beans in clean water. It has been shown that this results in a strong reduction of soluble dry matter in the beans from 64.3% fat-free dry matter in raw beans to 7.0% in cooked beans (Kiers et al., 2000). To what extent do these losses (of e.g. simple sugars, amino acids, minerals) affect the activation and colony (biomass) formation by *R. oligosporus* spores ? Table 3 shows that in beans that were heavily leached (II), the activation was still good but colony formation (medium 3) was considerably reduced compared with that on less leached beans (I). This shows that there is scope for optimization of the bean pretreatment process in adjustment to the physiological requirements of the starter.
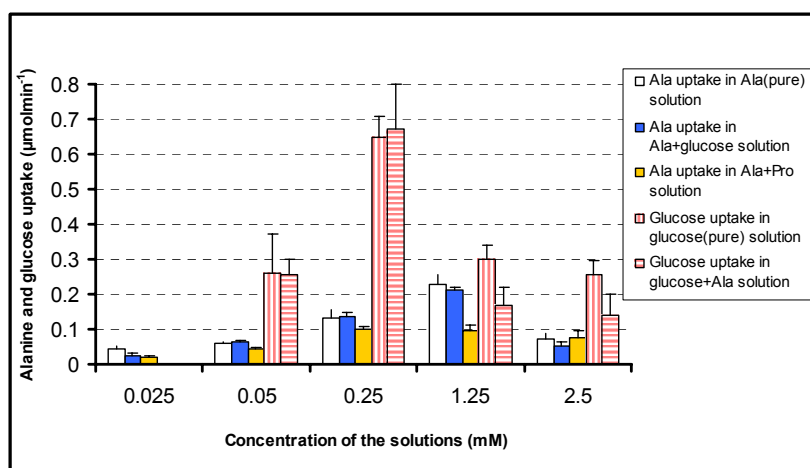
Fig. 1. Uptake of alanine and glucose by dormant spores of *Rhizopus oligosporus*.
Uptake rates were calculated after exposure during 120 min at 37 °C and are expressed as
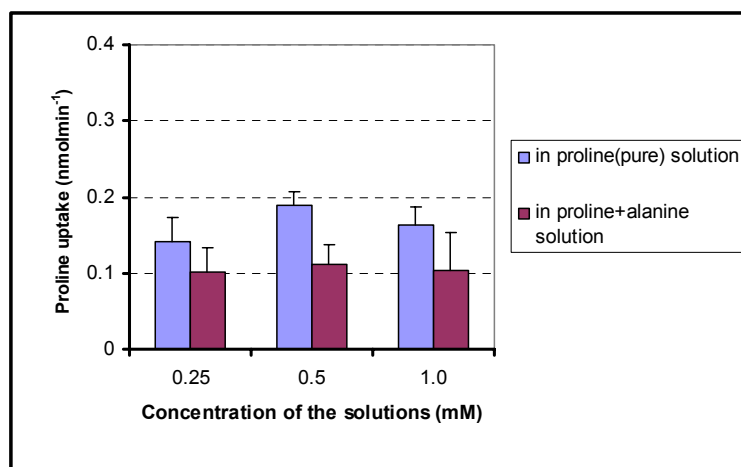μmoles/ min. In mixtures, concentrations of alanine, glucose and proline were equimolar)

**Fig. 2. Proline uptake rates nmoles/ min of dormant spores in proline(pure) and
in proline+alanine(mixture) solution for 120 min at 37 °C.**
(in the mixture of proline+alanine solution, the concentration of proline was equal to alanine)

DISCUSSION

Many combinations of amino acids and sugars with phosphate would probably stimulate spore germination of *R. oligosporus*. The present investigation is concerned with the ability of single substances, such as L-alanine, to stimulate germination of old spores in tempe starter. L-proline on the other hand, did not support germination and even inhibited the action of alanine.

Earlier researchers reported that germination was stimulated by L-alanine as well as L-proline. However, their experimental conditions, particularly the composition of the activation buffers used were inadequate. For example, the medium used by Medwid (Medwid and Grant, 1984) as well as the solutions used by Yanagita (Yanagita, 1957) and Miller (Miller, 1962) contained glucose. Our data in Table 2 show that a glucose containing buffer will allow considerable colony formation, irrespective of amino acids present.

In our investigation alanine is the best stimulator; even no glucose is required. Amino acids may serve as a source of carbon, nitrogen or both (Garraway and Evans, 1984; Carlile et al., 2001). We found that alanine fulfills this function in *R. oligosporus*. In addition, a source of phosphate has been reported necessary for germination of *R. arrhizus* (Ekundayo and Carlile, 1964; Carlile et al., 2001).

Our finding about the role of L-alanine for (dormant) spores of *R. oligosporus* is of interest because it was reported (Orlowski and Sypherd, 1978) that alanine was present at very high concentration in dormant spores but was rapidly depleted during hyphal germ tube emergence, while intracellular levels of free L-leucine and L-isoleucine remained relatively constant. These observations support our data and may also explain why we found that alanine uptake was much stronger, to compensate for its assimilation, whereas leucine and isoleucine remained relatively constant during spore germination.

It should be noted that we observed only a slight uptake of L-leucine (data not shown), which is in good agreement with earlier publications (Tripp and Paznokas, 1982). One more interesting result was found, namely that L-proline did not support germination but instead inhibits L-alanine uptake in a mixture of alanine and proline, so no colony formation on minimal agar medium took place, in contrast to alanine alone and the mixture of alanine-leucine-isoleucine. Although it was found earlier (Medwid and Grant, 1984) that when L-proline was used in combination with equimolar concentration of L-alanine, the effectiveness of germination was diminished, this effect could not be explained. Our data suggest that the alanine uptake system can be congested by L-proline when used in combination, and that higher concentrations of proline cause more transporters or cell (spore) membrane channels to be occupied (Fig. 2). This would hinder alanine uptake and colony formation on minimal agar medium. Proline uptake rate was very low (0.2 nmoles/min, a thousand times lower than alanine and glucose uptake); a phenomenon that is well known in brewers' yeast (MacLeod, 1977). Neither uptake nor metabolism of L-proline were necessary to trigger germination in *B. megaterium* (Rossignol and Vary, 1979). It has been shown earlier that e.g., *Arthrobotrys conoide* spores have a single carrier

for all amino acids (Gupta and Pramer, 1970) and that when several amino acids are present in the growth medium, competition for this same carrier is likely (Garraway and Evans, 1984). Alanine being a rather simple amino acid compared to the imino acid proline (Stryer, 1995) may give it the advantage of easy transport. However, proline may occupy the carrier which in turn is unable to deliver it inside the cell (Hunter and Segel, 1971).

It is important to note the role of phosphate in minimal medium. In the present data, the energizing role of glucose dissolved in minimal mineral medium was evident. In contrast, glucose dissolved in distilled water did not give rise to any colonies as shown in Table 1 and our previous experiments (Thanh and Nout, 2004). Other research also showed that the absence of either KH_2PO_4 or $MgSO_4$ lead to a substantial retardation of germination (Farach et al., 1979). It was found (Ekundayo, 1966) that in a glucose solution spores swell and that some produce germ tubes, but that maximum germination only occurs in the presence of utilizable carbon and nitrogen sources and suitable compounds containing phosphate, sulphate, potassium and magnesium ions that were also present in the minimal medium used in our experiments.

The uptake of glucose rate was 3-fold higher than of alanine. As nitrogen as well as carbon are needed for anabolism, and the latter is also required as a source of energy (Carlile et al., 2001), a balanced medium must have a C/N ratio of about 10. The uptake of amino acids is by active transport, as shown in *Neurospora crassa* which transports amino acids by proton-motive force, with two protons entering per molecule of amino acid. At low concentrations, glucose uptake is by active transport, with a K_m of approximately 10^{-5}M in *Saccharomyces* and *Neurospora*; at high glucose concentrations active transport is repressed and glucose enters the cell by a constitutive facilitated diffusion system with a K_m of approximately 10^{-2}M; most fungi have a constitutive transport system for glucose. Our results in Fig.1 agree with this concentration dependent scenario (Carlile et al., 2001). Alanine uptake was by active transport only, and was not much influenced by alanine concentration, but glucose uptake was concentration dependent: active transport occurred up to its maximum uptake rate at the concentration of 0.25 mM; from 1.25 mM upwards, active transport was repressed, leaving only the facilitated diffusion with concomitantly decreased uptake rate.

Data presented in Table 3 show that nutrient leaching from soybeans, which is common practice in tempe manufacture, leads to reduced fungal growth, indicating that there is scope for optimization of the bean pretreatment process in view of the physiological requirements of the starter.

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REFERENCES

Breeuwer, P., De Reu, J. C., Drocourt, J.-L., Rombouts, F. M., and Abee, T. (1997) Nonanoic acid, a fungal self-inhibitor, prevents germination of *Rhizopus oligosporus* sporangiospores by dissipation of the pH gradient. *Applied and Environmental Microbiology* **63**, 178-185.

Carlile, M. J., Watkinson, S. C., and Gooday, G. W. (2001) Fungal cells and vegetative growth. In *The Fungi* ed. G. W. Gooday, pp. 85-184. San Diego: Academic Press.

Ekundayo, J. A. (1966) Further studies on germination of sporangiospores of *Rhizopus arrhizus*. *Journal of General Microbiology* **42**, 288-291.

Ekundayo, J. A., and Carlile, M. J. (1964) The germination of sporangiospores of *Rhizopus arrhizus*: spore swelling and germ-tube emergence. *Journal of General Microbiology* **35**, 261-269.

Farach, M. C., Farach, H., and Mirkes (1979) Control of development in *Neurospora crassa*: Nutrient requirements for conidial germ tube emergence, elongation, and metabolic activation. *Experimental Mycology* **3**, 240-248.

Garraway, M. O., and Evans, R. C. (1984) Carbon nutrition and Nitrogen nutrition. In *Fungal Nutrition and Physiology* ed. R. C. Evans, pp. 71-230. New York: John Wiley & Sons.

Gottlieb, D. (1978) Introduction: germination. In *The germination of fungus spores* ed. D. Gottlieb, pp. 1-10. Durham, England: Meadowfield Press Ltd, Patterns of Progress Microbiology.

Griffin, G. J. (1970a) Carbon and nitrogen requirements for macroconidial gemination of *Fusarium solani*: dependence on conidial density. *Canadian Journal of Microbiology* **16**, 733-740.

Griffin, G. J. (1970b) Exogenous carbon and nitrogen requirements for chlamydospore germination by *Fusarium solani*: dependence on spore density. *Canadian Journal of Microbiology* **16**, 1366-1368.

Gupta, R. K., and Pramer, D. (1970) Amino acid transport by the filamentous fungus *Arthrobotrys conoides*. *Journal of Bacteriology* **103**, 120-130.

Hashimoto, C. D., Wu, C. D. R., and Blumenthal, H. J. (1972) Characterization of L-Leucine-induced germination of *Trichophyton mentagrophytes* microconidia. *Journal of Bacteriology* **112**, 967-976.

Hunter, D. R., and Segel, I. H. (1971) Acidic and basic amino acid transport systems of *Penicillium chrysogenum*. *Archives of Biochemistry and Biophysics* **144**, 168-183.

Kiers, J. L., Nout, M. J. R., and 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.

Lie, S. (1973) The ECB-ninhydrin method for determination of free alpha amio nitrogen. *Journal of The Institute of Brewing* **79**, 37-41.

MacLeod, A. M. (1977) Beer. In *Economic microbiology, Vol.1, Alcoholic Beverages* ed. A. H. Rose, Vol. 1, pp. 43-137. London: Academic Press.

Magné, C. M., and Larher, F. (1992) High sugar content of extracts interferes with colorimetric determination of amino acids and free proline. *Analytical Biochemistry* **200**, 115-118.

Medwid, R. D. (1984) Survival and germination of *Rhizopus oligosporus* sporangiospores. Ph.D. Thesis, Colorado State University.

Medwid, R. D., and Grant, D. W. (1984) Germination of *Rhizopus oligosporus* sporangiospores. *Applied and Environmental Microbiology* **48**, 1067-1071.

Miller, L. P. (1962) L-proline and L-alanine as substitutes for higher temperatures in the germination of fungus spores. *Phytopathology* **52**, 743 (Abstr.).

Nout, M. J. R., and Rombouts, F. M. (1990) Recent developments in tempe research. *Journal of Applied Bacteriology* **69**, 609-633.

Orlowski, M., and Sypherd, P. S. (1978) Regulation of macromolecular synthesis during hyphal germ tube emergence from *Mucor racemosus* sporangiospores. *Journal of Bacteriology* **134**, 76-83.

Pass, T., and Griffin, G. J. (1972) Exogenous carbon and nitrogen requirements for conidial germination by *Aspergillus flavus*. *Canadian Journal of Microbiology* **18**, 1453-1461.

Rossignol, D. P., and Vary, J. C. (1979) Biochemistry of L-Proline-triggered germination of *Bacillus megaterium* spores. *Journal of Bacteriology* **138**, 431-441.

Somogyi, M. (1952) Notes on sugar determination. *Journal of Biological Chemistry* **195**, 19-25.

Sorenson, W. G., and Hesseltine, C. W. (1966) Carbon and nitrogen utilization by *Rhizopus oligosporus*. *Mycologia* **LVIII**, 681-689.

Stryer, L. (1995) Protein structure and function. In *Biochemistry* ed. L. Stryer, pp. 17-44. New York: W.H.Freeman and Company.

Thanh, N. V., and Nout, M. J. R. (2002) Rhizopus oligosporus biomass, sporangiospore yield and viability as influenced by harvesting age and processing conditions. *Food Microbiology* **19**, 91-96.

Thanh, N. V., and Nout, M. J. R. (2004) Dormancy, activation and viability of *Rhizopus oligosporus* sporangiospores. *International Journal of Food Microbiology* **92**, 171-179.

Thevelein, J. M., Van Laere, A. J., Beullens, M., Van Assche, J. A., and Carlier, A. R. (1983) Glucose-induced trehalase activation and trehalose mobilization during early germination of *Phycomyces blakesleeanus* spores. *Journal of General Microbiology* **129**, 719-726.

Tripp, M. L., and Paznokas, J. L. (1981) Relationship between sporulation medium and germination ability of *Mucor racemosus* sporangiospores. *Journal of General Microbiology* **127**, 35-43.

Tripp, M. L., and Paznokas, J. L. (1982) Role of amino acids and endogenous protein in the germination of *Mucor racemosus* sporangiospores. *Journal of General Microbiology* **128**, 485-495.

Wax, R., and Freese, E. (1968) Initiation of the germination of *Bacillus subtilis* spores by a combination of compounds in place of L-Alanine. *Journal of Bacteriology* **95**, 433-438.

Weber, D. J. (1962) The role of proline in the germination of *Rhizopus stolonifer* spores. *Phytopathology* **52**, 756. (Abstr.).

Weber, D. J., and Ogawa, J. M. (1965) The specificity of proline in the germination of spores of *Rhizopus arrhizus*. *Phytopathology* **55**, 262-266.

Yanagita, T. (1957) Biochemical aspects on the germination of conidiospores of *Aspergillus niger*. *Archiven der Mikrobiologie* **26**, 329-344.

Chapter 5

Characterization of viability and physiological state transitions of *Rhizopus oligosporus* sporangiospores in tempe starter culture by flow cytometry

Abstract

The potential of using flow cytometry (FCM) in combination with fluorescent dyes to analyse the viability, measure various physiological characteristics of individual spore-cells in tempe starter cultures that had been stored for 8, 10, 16, and 30 months was examined. Results indicate that the shelf-life of tempe starter was not limited by the death of spores, but by sublethal damage to spores as well as dormancy of spores. Sublethally damaged and dormant spores can be resuscitated into activated (live) spores in a suitable activation medium such as malt extract broth.

During storage, the number of dormant and sublethally damaged spores increased while the number of metabolically active spores decreased. The longer the spores have been stored, the less dormant spores can still be activated; in contrast, the transition from sublethally damaged to activated spores did not decrease with longer storage. However, after a very long (30 months) storage, sublethally damaged spores could still be activated but could not germinate anymore, whereas 30 months old dormant spores would not be activated anymore. Thus, besides live, dead, and dormant spores we distinguished a category of sublethally damaged spores. The shelf-life of spores in tempe starter is related to the physiological state of spores being sublethally damaged; a mechanism of physiological state transitions of *R. oligosporus* sporangiospores is proposed.

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INTRODUCTION

Dormancy can be defined as a reversible state of metabolic shutdown (Kaprelyants and Kell, 1993) or low metabolic activity (Kaprelyants et al., 1993). In microbiology this often corresponds to a state in which cells are not alive in the sense of being able to form a colony when plated on suitable solid medium, but in which they are not dead in that when conditions are more favourable they can revert, by a process known as resuscitation, to a state of aliveness as so defined (Kaprelyants et al., 1993). Resuscitation denotes a transition of cells from nonculturable to culturable states with respect to a given medium. Dormant cells may be nonculturable in that they require specific stimuli before they become active and culturable (Kell et al., 1998).

Different phenomena are cell injury and repair. Some cells may respond to specific forms of damage by entering a physiological state in which specific reparative processes are necessary before (re)growth on their usual range of media is initiated. Regrowth is the return to an actively growing state of cells that had ceased growth but had not lost culturability (Kell et al., 1998).

It is widely recognized that classic culture techniques may underestimate the numbers of truly viable microorganisms, especially when cells have been damaged by physical treatments. Fluorescent staining and detection by flow cytometry (FCM) are widely used (Ritz et al., 2001) as rapid, sensitive and quantitative techniques to detect microorganisms and assess their viability in the field of food microbiology, veterinary science and medical research (Chitarra and Van den Bulk, 2003).

The advantages of using FCM in microscopy lie in the possibility of analyzing cells according to a multiplicity of parameters. Most cytometers feature lateral and forward light scatters and two or more fluorescent detectors. Therefore, cellular size, cytoplasm content, and physiological state can be simultaneously and swiftly documented and/or recorded for any given bacterial population (Ritz et al., 2001). This technique has demonstrated its potential as a means to assess the physiological state of damaged cells (Ueckert et al., 1997). FCM has been successfully used in several studies to assess the viability of microbial cells in probiotic products and dairy starters (Bunthof and Abee, 2002), starved cultures (Kaprelyants and Kell, 1993; Kaprelyants et al., 1996), or cells having been exposed to antibiotics (Novo et al., 2000) or high hydrostatic pressure (Ritz et al., 2001).

The method is useful in food applications to determine cell numbers and measure various physiological characteristics of individual cells, detect and distinguish between viable and non-viable bacteria, as well as for the detection and quantification of fungal spores (Roth et al., 1997; Bunthof and Abee, 2002; Chitarra and Van den Bulk, 2003). A wide range of fluorescent dyes are available which aim at specific cellular targets, such as DNA, enzyme activities, internal pH, or the cytoplasmic membrane (Ueckert et al., 1995). Fluorescent probes can not only distinguish between live and dead, but also between vigorous, frail, or injured organisms (Mason et al., 1994; Jepras et al., 1995).

Cell viability can be assessed using fluorescein derivatives, such as carboxyfluorescein diacetate (cFDA) or carboxyfluorescein succinimidyl ester. Membrane

integrity and activity have also been described as good indicators of viability; these properties can be studied with fluorescent markers, including propidium iodide (PI) or TOTO-1 {1'-(4,4,7,7-tetramethyl-4,7-diazaundecamethylene)-bis-4-[3-methyl-2,3dihydro (benzo-1,3-oxazole)-2-methylidene]-1-(3'-trimethylammoniumpropyl)-pyridinium tetraiodide}.

cFDA is commonly used as a live stain (Shapiro, 1995; Davey and Kell, 1996; Breeuwer and Abee, 2000). It is a nonfluorescent esterase substrate that enters the cell by diffusion. Once the compound is inside, the acetate groups are split off by enzymatic hydrolysis, releasing the green fluorescent compound carboxyfluorescein (cF). PI is a nucleic acid dye that generally does not cross intact cell plasma membranes and, hence, only enters into non-viable cells that lost their membrane integrity. TOTO-1 is a nucleic acid dye that is excluded by intact cells and thus stains a cell only when the membrane is damaged. TOTO-1 is often preferred over PI because the excitation and emission spectra are suitable for FCM, it has a high fluorescence enhancement, and its molecular mass is approximately twice as high as that of PI (Hirons et al., 1994; Bunthof et al., 2001; Chitarra and Van den Bulk, 2003).

The purpose of this study was to characterize the viability and physiological states of spores (spore subpopulations) in tempe starter cultures that were stored for 8, 10, 16, and 30 months. Flow cytometry in combination with cFDA, PI and TOTO-1 was used to assess the viability, membrane integrity and enzymatic activities of *Rhizopus oligosporus* spores before and after activation.

MATERIALS AND METHODS

Preparation of tempe starter

Tempe starter was prepared as described earlier (Thanh and Nout, 2002). Briefly, the procedure was as follows: *Rhizopus oligosporus* LU 575 (NRRL 5905) was grown on malt extract agar (MEA, Oxoid CM59) plates during 1 week at 30 °C, and spores were harvested by washing with sterile water. The spore suspension was diluted to approximately 10^5 spores per ml. This suspension "A" was used as inoculum for substrate used to prepare tempe starter. Polished broken rice was steam-sterilized, allowed to cool and sterilized ammonium sulfate and sterilized H_2SO_4 were added to adjust the pH to 4, being the optimum pH for spore germination of *Rhizopus oligosporus* (Medwid and Grant, 1984; Breeuwer et al., 1997). The sterile broken rice (50 g) was inoculated and thoroughly mixed with 0.4 ml of spore suspension "A". The inoculated rice was incubated at 40ºC for 2 hours, followed by incubation for 4 days at 30 °C. The mouldy mass was broken up and the entire mass of substrate, mycelium and spores was dried at 42 °C for 48 h in a forced-air drying cabinet. Only crushing by sterile pestle and mortar, but no fine grinding was used in order to avoid misjudging between spores and rice particles with similar size and

shape. The starter powder was stored in screw-cap glass tubes, protected from light, in a silicagel desiccator at 25 °C, as these conditions were considered to be more representative of practical usage than storage at low temperature. Moreover, it was shown that spores survive better at ambient (25-30 °C) temperature than at 5 °C (Thanh and Nout, 2002).

Preparation of spore suspensions

Sporangiospores in crushed rice powder were added and diluted with sterile water to suspensions of approximately 10^7 spores/ml, by vigorous agitation and filtration with a coarse Millipore membrane filter [(fluorassure); Chem filter 15, REF: 100-C2003-01; Chemunex] to remove mycelium and other debris. Spore suspensions were washed twice by centrifugation at 13000 x g for 3 min in an Eppendorf centrifuge (Greiner-labortechnik, 500 PP- Microcentrifuge tube 1.5 ml, Cat. No. 616201). After decanting, the spores in pellets were re-suspended to experimental suspensions according to the respective aims of studies: either in (a) phosphate buffer 50 mM of pH 7 for untreated and heat-killed samples or (b) in malt extract broth (MEB) for activation.

Heat-killed samples were made by heating spores (10^6/ml, in 50 mM KPi pH 7) at 100 °C for 30 min in a water bath, in covered glass tubes.

Activation samples were made by incubating spores (10^6/ml, in MEB) in a water bath at 37 °C for 60 min in Eppendorf tubes.

Fluorescent labeling

Single staining with cFDA, PI and TOTO-1 to be used as controls for calibration of the Flow Cytometer (FCM)

PI or TOTO-1 staining

PI was supplied by the manufacturer as a 1 mg/ml solution in distilled water. This was used as a working solution and stored in the refrigerator in the dark. TOTO-1 was supplied by the manufacturer as a 100 mM solution in dimethyl sulphoxide. The stock solution was further diluted in dimethyl sulphoxide to 100 μM to serve as the working solution. Heat-killed cell suspensions were exposed to 7.5 μM of PI or 1μM of TOTO-1. The mixtures were incubated for 30 min at 40 °C in a water bath to stain the spores. Stained samples were kept on ice in the dark and used within 1 h for FCM analysis.

cFDA staining

A stock solution (10 mM) of cFDA was prepared by dissolving 4.6 mg of cFDA/ml in acetone and was stored at -20 °C in the dark. The stock solution was diluted further in acetone to 1 mM to serve as the working solution. Samples containing 10^7 cells/ml were incubated in potassium phosphate buffer (50 mM, pH 7) in the presence of 10 μM cFDA for 30 min at 40 °C in a water bath. Stained samples were kept on ice in the dark and used within 1 h for FCM analysis.

Double staining

When dual labeling was performed, we used the same dye concentrations and incubation times as described above. Double staining was applied for untreated spore suspensions and activated spore suspensions of spores stored for 8, 10, 16, and 30 months. Each spore age sample was stained either with cFDA and PI or with cFDA and TOTO-1.

For multiparameter FCM analysis of double stained samples the following controls were used: nonstained, cF-stained, heat-killed-PI stained and heat-killed-TOTO-1- stained spore suspensions.

cFDA [5-(and-6)carboxyfluorescein diacetate], PI (propidium iodide), and TOTO-1 were purchased from Molecular Probes Europe, Leiden, The Netherlands. cFDA is an esterase substrate yielding the fluorescent carboxyfluorescein (cF) upon hydrolysis. cF has an excitation maximum (λ_{ex}) of 492 nm and an emission maximum (λ_{em}) of 517 nm. PI and TOTO-1 bind to DNA. PI has a molecular mass of 668 g per mol and a fluorescence enhancement of 20- to 30-fold upon binding. The PI-DNA complex has a λ_{ex} of 535 nm and a λ_{em} of 617 nm. TOTO-1 has a molecular mass of 1303 g per mol and a very high fluorescence enhancement of 1400-fold. The TOTO-1-DNA complex has a λ_{ex} of 514 nm and a λ_{em} of 533 nm (Bunthof et al., 2001)

Flow cytometry

Flow cytometric analyses were performed as described recently (Bunthof et al., 2001; Chitarra et al., 2003) on a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, California) equipped with an air-cooled argon ion laser at 15 mW (excitation wavelength 488 nm). Sporangiospores of *R. oligosporus* at a concentration of 10^6 spores/ml were delivered at the low flow rate, corresponding to 150 to 500 spores/second. FSC (forward-angle light scatter), SSC (side-angle light scatter), and three fluorescence signals were measured. A band pass filter of 530 nm (515 to 545 nm) was used to collect the green fluorescence (FL1), a band pass filter of 585 nm (564 to 606 nm) was used to collect the yellow-orange fluorescence (FL2), and a long pass filter of 670 nm was used to collect the red fluorescence (FL3). FSC was collected with a diode detector. SSC and the three fluorescence signals were collected with photomultiplier tubes. All signals were collected using the logarithmic mode. A combination of FSC and SSC was used to discriminate the spores from their background. Data were analyzed with the CELLQuest program (version 3.1f; Becton Dickinson) and the WinMDI program (version 2.8; Joseph Trotter, John Curtin School of Medical Research, Canberra, Australia).
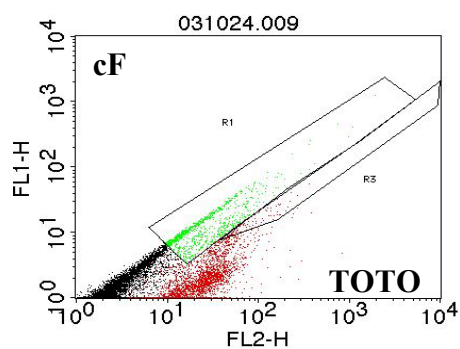
Viable spores

Colony formation was measured after FCM analysis on samples that were activated and non-activated. The spores were spread-plated on malt extract agar (MEA, malt extract
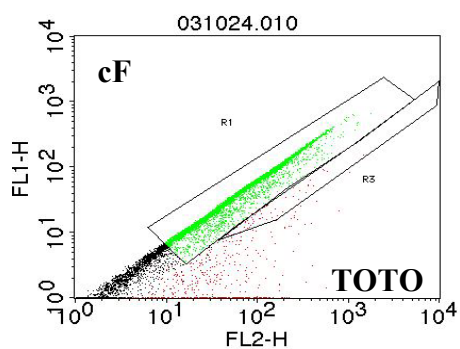
Not activated   **Activated**

8 month spores   **8 month spores**

a)   b)

c)   d)

Not activated   **Activated**

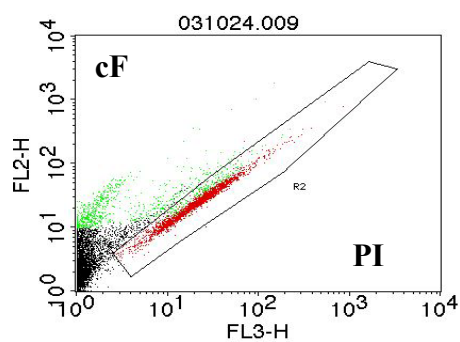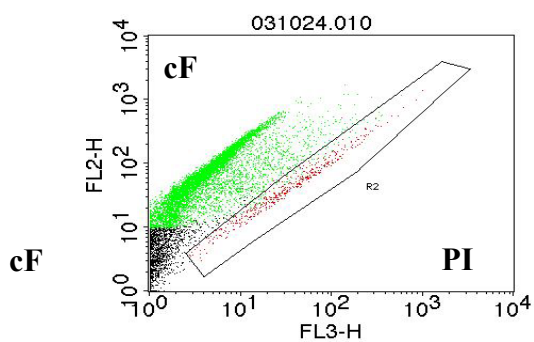**30-month spores**   **30-month spores**

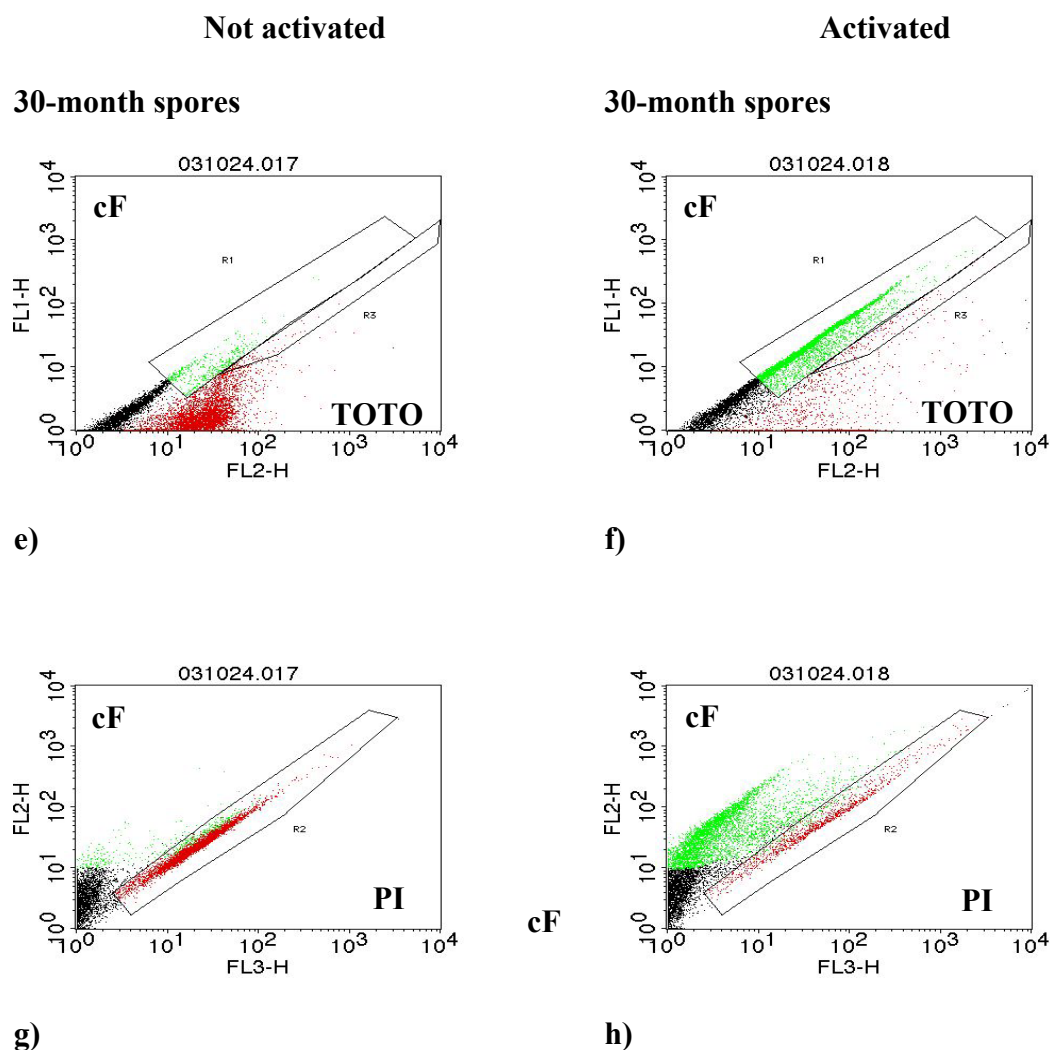e)   f)

g)   h)

Figure 1. FCM multiparameter analysis of staining with cFDA, PI, TOTO-1 of 8-months stored spores (a, b, c, d) and 30-months stored spores (e, f, g, h) before and after activation in MEB at 37 °C, 60 min.

R1: cF stained population; R2: PI stained population; R3: TOTO-1 stained population.

30 g/l, mycological peptone 5 g/l, agar 15 g/l, Oxoid, CM 59). Colonies were counted after incubation at 37 °C for 12 h.

Total spores

Spore suspensions were diluted as appropriate and counted using a Bürker-Türk counting chamber. Microscopic counts were also made similarly to determine fluorescent spores, as described previously (Thanh and Nout, 2004).

RESULTS

Differential staining of live and dead spores by cFDA, PI, and TOTO-1 was validated by testing the probes on treatments: spores (stored for 8, 10, 16, and 30 months) that were not treated and spores that were activated for 60 min in MEB at 37 °C, and also spores that were heat-killed at 100 °C for 30 min (as a control for PI and/ or TOTO-1 stained dead spores). FCM analysis of non-activated, unstained spores (stored for 8 months) showed less than 1% of cF-, PI- or TOTO-1- single-stained cells (Table 1). Single staining with cFDA of spores activated in MEB at 37 °C for 60 min obtained 59.6% cF-stained spores. In the heat killed treatment (100 °C, 30 min), single stained with PI or TOTO-1, we did not obtain 100% fluorescent spores as expected, but only 86.5% PI-stained or 84% TOTO-1-stained spores.

Double stainings with cFDA and PI, or with cFDA and TOTO-1 were used to differentiate viable (active), dead and dormant spores in non-activated and activated suspensions of spores that had been stored for 8, 10, 16, and 30 months. Retention of cF by cells indicates enzymatic activity; retention of PI or TOTO-1 indicates loss of membrane integrity; unstained cells indicate dormancy. In Fig. 1, the dot plots show the unstained cells in the lower left corner. Spores stored for 8, 10, 16, and 30-months were exposed to phosphate buffer pH 7 (for non-activated) or MEB (for activation at 37 °C for 1 h) and then stained simultaneously with cFDA and PI, or with cFDA and TOTO-1. FCM analysis showed that the cF- and the TOTO-1-labeled populations could be spatially resolved in dot plots of FL1 and FL2 as illustrated by double stained spores that were stored for 8 and 30 months (Fig.1 a,b,e,f). R1 and R3 were slightly separated subpopulations that had been calibrated using single stained controls with cFDA or TOTO-1. R2 (PI stained population) was rather well separated from others. It was shown earlier with lactic acid bacteria (Bunthof et al., 2001), that subpopulations can be resolved in dot plots of FL1 and FL2. PI-stained red fluorescent events could be detected by FL3 detector (Amor et al., 2002). Our plots indicate that this is applicable to *Rhizopus oligosporus* sporangiospores as well.

In general, the dual-parameter dot plots of Fig.1 indicate the existence of four main subpopulations: (1) R1: cF-stained (live) green population, (2) R2: PI-stained red population (3) R3: TOTO-1-stained (dead) yellow population and (4) non-stained (dormant) black population. Due to activation, R1: the number of cF-stained spores (Fig.1d) increased enormously as compared to that in the non-activated sample (Fig.1a); and R2: the number of PI-stained spores (Fig.1d) decreased strongly in comparison to that in the non-activated sample (Fig.1c), while R3: TOTO-1 stained spore populations in Fig.1a and 1b seem rather similar in size. Similar results of FCM were found with spores stored for 30 months- (Fig.1e, f, g, h) and also in 10 and 16-months stored spores (not shown).
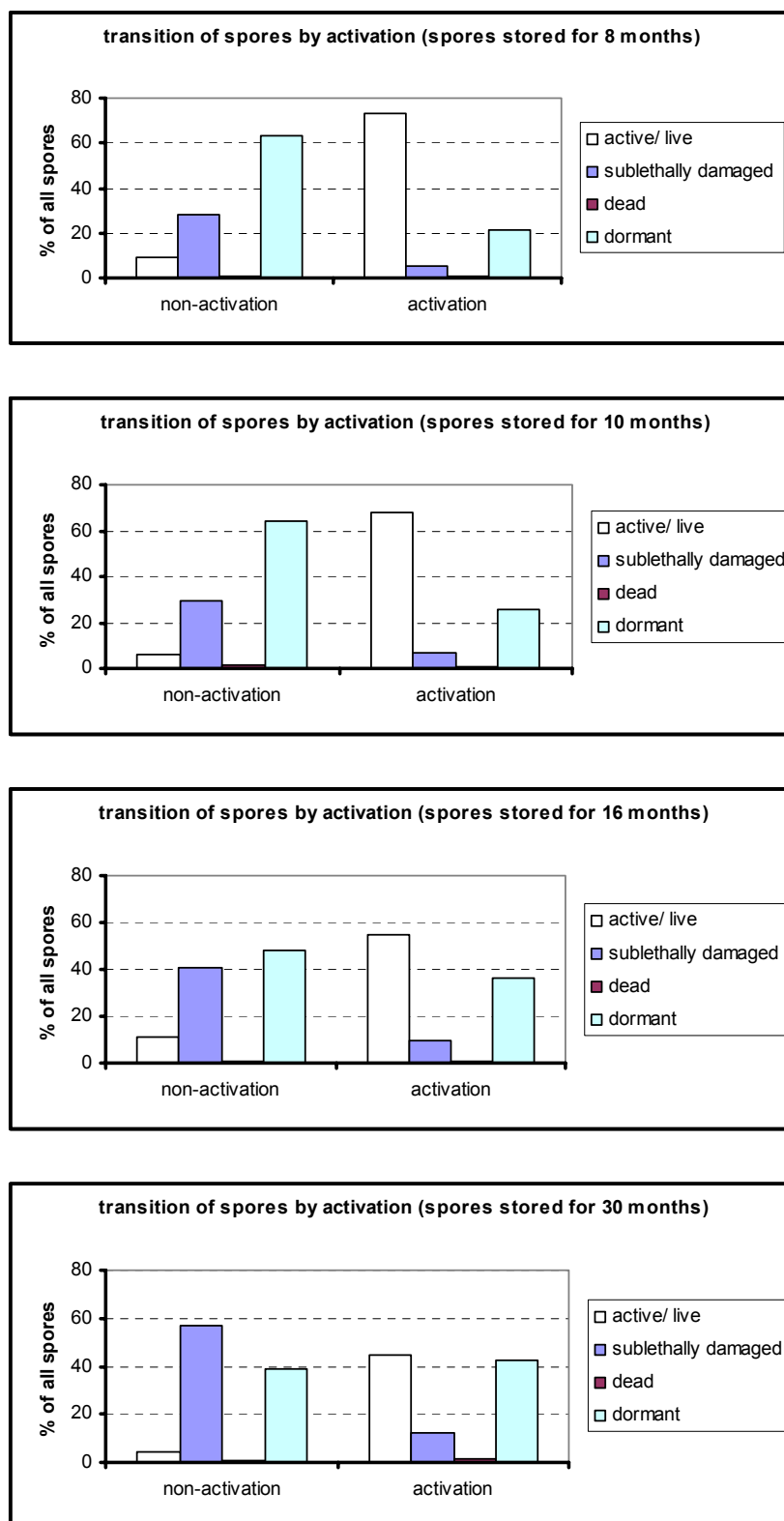
Figure 2. Transitions of viable, dormant, sublethally damaged, and dead spores by activation (in MEB at 37 °C for 60 min)

Table 1. FCM analysis of non-activated and activated spores in tempe starter preparations

PI + cFDA staining

Spore age (months)	Not activated				Activated			
	cF-stained: (active/viable) (%)	PI-stained (sublethally damaged) (%)	TOTO-stained (dead) (%)	Not stained (dormant) (%)	cF-stained: (active/viable) (%)	PI-stained (sublethally damaged + dead) (%)	TOTO-stained (dead) (%)	Not stained (dormant) (%)
8	8.9	28.0	0.9	63.1	73.2	5.2	0.6	21.6
10	6.0	29.6	1.2	64.5	67.8	6.6	0.9	25.6
16	11.0	40.7	1.1	48.3	54.8	9.3	1.1	35.9
30	4.3	56.9	1.0	38.8	44.8	12.5	1.6	42.8

TOTO-1 + cFDA staining

8	8.6	0.1	0.7	91.4	70.5	0.0	2.3	29.5
10	8.8	0.0	0.3	91.2	67.2	0.1	1.8	32.7
16	9.4	0	0.3	90.7	58.2	0.5	1.7	41.3
30	8.5	0.1	1.7	91.5	45.3	0.1	6.5	54.7

Single staining for setting FCM

Spore-suspension[1] (no staining)	0.1	0.1	0.1	99.8				
activated+cFDA staining					59.6	0.2	0.1	40.2
Heat killed + PI staining	0	86.5	0.1	13.4				
Heat-killed+TOTO staining	0.9	0.3	84.0	14.9				

[1] 8-month-stored spores were used.

In Table 1 and Fig. 2, double staining with cFDA and PI was performed on non-activated and activated samples of 8-months stored spores and they were analyzed by FCM. Transitions of spores occurred once activation was applied: cF-stained spores increased from 8.9% to 73.2%, while unstained spores decreased from 63.1% to 21.6% and PI-stained spores decreased from 28% to 5.2%. These findings agree with our previous results (Thanh and Nout, 2004).

Results in Table 1 show that the increase of metabolic active (cF-stained) spores represents approximately the sum of the decrease of PI stained spores and dormant (unstained) spores. Only the number of TOTO-1-stained spores remained rather unchanged (0.7-2.3%). Using the same samples and same treatment, we observe that the number of PI stained spores strongly decreased after activation, while the TOTO-1 stained spores remained similar before or after activation.

Transitions of 10 months old spores were observed as shown in Table 3. cF-stained (metabolically active) spores increased from 6.0% to 67.8% by activation, while unstained (dormant) spores decreased from 64.5% to 25.6% (Table 1); PI-stained spores decreased from 29.6% to 6.6% (this means that 22.9% were sublethally damaged spores; see Discussion) and 0.9% TOTO-1-stained (dead) spores (Table 1 & Fig.2). Similar trends were observed for spores stored during 16 and 30 months, as shown in Table 3 and Fig. 2.

Our activation and FCM experiments resulted in a pattern of transition of physiological states. These transitions were associated with spore ages (8, 10, 16, and 30 months). With longer storage, numbers of active spores decreased from 73.2 to 44.8%, and dormant and sublethally damaged spores increased from 21.6 to 42.8% and from 22.8 to 44.5%, respectively.

DISCUSSION

In a previous study (Thanh and Nout, 2004) we showed that malt extract broth is a good activation medium. During activation with this medium, some interesting transitions took place: the proportion of dormant spores very rapidly decreased, with a concomitant increase of metabolically active spores as measured by fluorescent microscopy using fluorescent markers (Thanh and Nout, 2004).

We now examined the usefulness of flow cytometry for viability assessment of *R. oligosporus* sporangiospores. FCM was chosen for its capacity of multiparameter analysis and the possibility of using several fluorescent viability markers, to determine cell numbers, to measure various physiological characteristics of individual cells, and to distinguish between viable and non-viable cells.

For the staining protocol, several trials were carried out for single and double probes to achieve the optimum staining conditions for FCM analysis (Materials and Methods). As a result of staining, labeled and non-labeled spores were distinguished.

Table 2. Proposed definitions of physiological states of spores based on uptake or exclusion of cFDA, PI and TOTO-1

Result with probe:			Cell status
cFDA	TOTO-1	PI	
negative	negative	negative	Dormant (cell is inactive but living and cannot be detected by probes)
positive	negative	negative	Active (cell is living)
negative	negative	positive	Sub-lethally damaged (cell is gradually dying but can still be resuscitated) (reversible)
negative	positive	positive	Dead (irreversible)

Table 3. Transitions of spores by activation spores in MEB, 37 °C for 60 min, active spores emitted fluorescence and were detected by FCM

Spore-samples (months)	Activated spores (%)			Active before activation (%)		Active after activation (%)	
	transited from		total				
	Sub-lethally damaged	Dormant					
	(1)	(2)	(3) = (1)+(2)	(4)		(5)=(3)+(4)	
8	22.8	41.6	64.3	8.9	(7.9)[*]	73.2	(9.3)[*]
10	22.9	38.9	61.8	5.9	(8.7)	67.8	(10.7)
16	31.4	12.3	43.8	11.0	(7.4)	54.8	(10.0)
30	44.5	0	40.5	4.3	(4.3)	44.8	(4.4)

* ( ) % of CFU/g of non-and activated samples that were used in FCM analysis

successfully and reported in figures and in region statistics tables by FCM. Labeled spore suspensions were also checked visually by fluorescence microscopy.

In order to validate the multi-parameter assay, it was necessary to establish adequate controls. Unstained, single cF-stained, PI-stained and TOTO-1-stained cells served as controls to show their distributions by which to set the FCM detectors (and compensation settings).

cFDA was tested as a viable cell stain for *R. oligosporus* sporangiospores. cFDA is an esterase substrate that needs both enzyme activity to yield the fluorescent compound, and membrane integrity to retain the compound in the cell. Esterase activity is an indicator of metabolic activity, and comparing this activity before and after activation can help to assess the physiological state of spore cells.

PI is a red fluorescent phenanthridinium dye intercalating nucleic acid, that generally does not traverse intact cell membranes and, hence, only enters into non-viable cells with damaged membranes, or into dead cells (Lopez-Amoros et al., 1995; Mortimer et al., 2000; Chitarra and Van den Bulk, 2003). Also, TOTO-1, a yellow fluorescent dimeric cyanine dye was used because the excitation and emission spectra are suitable for FCM, it has a high fluorescence enhancement, and its molecular mass is approximately twice as high as that of PI (Hirons et al., 1994; Bunthof et al., 2001).

The low levels of fluorescence in unstained spores (Table 1) may be caused by autofluorescence of a minority of the spores, as observed earlier elsewhere (Wu and Warren, 1984).

The seemingly low levels of cF-stained spores after activation (Table 1) should be considered in the light of their storage history (8 months); values obtained are in good agreement with our previous results: about 60% fluorescent spores of 10 months old sample in comparison with 93.3% in freshly harvested spores (Thanh and Nout, 2004).

Heat treatment did not result in 100% PI or TOTO-1 labeled spores as might be expected. Whereas this phenomenon remains unexplained, it may be due to heat resistance occurring in some spores in the starter culture. Whereas it was shown earlier (Brul et al., 1997) that upon a 10 minute treatment at 65 °C *Aspergillus niger* spores were all PI positive, *Paecilomyces variotii* spores to the contrary were only 45% PI positive and remarkably 55% CDFDA ([5-(and-6)-carboxy-2',7'-dichlorofluorescein-diacetate); CDFDA is converted to a fluorescent product similar to cFDA after acetate hydrolysis] positive. Indeed heat treatment can activate the metabolism of fungal spores (Griffin, 1994).

As we considered PI-stained spores representing dead spores, their number would be expected to remain independent of activation treatment. Thus, the decrease of the number of PI-stained spores after activation posed a question in need of discovery. Therefore, instead of PI, TOTO-1 was used together with cFDA for double staining of the same (8-month stored spores) sample (Table 1). Remarkably, the number of TOTO-1 stained cells was of the same order of that of PI stained cells after activation (2.3% and 1.6% respectively). We deduce that TOTO-1 is superior to PI in discriminating intact and dead *Rhizopus* spores, as was reported earlier (Bunthof et al., 2001) for lactic acid bacteria.

This may be explained because TOTO-1 is larger than PI; the molecular masses are 1303 and 668g per mol, respectively. Furthermore, the very high fluorescence enhancement of TOTO-1 enables good distinction of nonlabeled and labeled cells in the FCM. The labeling with TOTO-1 gave clear discrimination between live and dead cells, whereas PI gave less contrast. The decrease of the number of PI-stained spores probably was caused by a transition from sublethally damaged cells with reversible membrane permeability into active cells, disabling entry of PI. Seriously damaged spores (with bigger and irreversible membrane damage) were stained with TOTO-1 (as well as with PI) and could be considered as truly dead spores. These could not be resuscitated anymore, the number of TOTO-1 stained spores remained almost similar before or after activation.

It is probable that the simultaneous decrease of PI-stained spores and increase of cF-stained spores is caused by a restoration of the membrane barrier due to activation in malt extract broth. This would be in agreement with the resuscitation behaviour of *Micrococcus luteus* in activation medium (Kaprelyants and Kell, 1993) that was associated with a restoration of their membrane barriers (Kaprelyants et al., 1996). It was concluded (Kaprelyants et al., 1994; Votyakova et al., 1994) that viable cells might produce factors that stimulate the resuscitation of dormant cells, promote the transition of cells from a state in which they are incapable of growth and division to one in which they are capable of colony formation. However, it is unknown whether this happens as well in fungal spores.

In the present study we considered the resuscitated spores to have been sublethally damaged. Thus, besides viable, dead, and dormant spores in tempe starter culture we were able to distinguish one more subpopulation, namely sublethally damaged spores. The number of sublethally damaged spores could be quantified by FCM by comparing responses to PI and TOTO-1.

Earlier results with lactic acid bacteria (Bunthof et al., 2001) showed that cFDA was an accurate stain for live cells, but that PI did not give a clear distinction between live and dead cells. TOTO-1 on the other hand, gave a clear discrimination of live and dead cells. The combination of cFDA and TOTO-1 makes an excellent live/dead assay with versatile applications. Our cF and PI double stains (before and after activation) showed a combination for live, dead and sublethally damaged assay with cF and TOTO-1 double stains as a comparable treatment. As a result, proposed definitions of physiological state of spores based on uptake or exclusion of cFDA, PI and TOTO-1 are presented in Table 2.

It is important to note (Table 3) that the increased number of activated 8 months old spores (64.3%) is the sum of disappeared dormant (41.6%) and sub-lethally damaged spores (22.8%) and likewise for the spores of other ages. This proved that not only dormant, but also sublethally damaged spores may transit into viable spores. The number of sublethally damaged spores, similar to dormant spores, increased during storage (Table 1).

It was observed that starved *Micrococcus luteus* cells may contain a mixture of dormant and injured cells; moreover, dormant cells could have injuries as well. The resuscitation may actually represent their recovery from injury, or the repair of the membrane barrier (Kaprelyants et al., 1996; Kell et al., 1998). Our data indicate a similar situation for *Rhizopus oligosporus*.

It is remarkable that even after a long time of storage, the spores of the tempe starter were not dead but merely sublethally damaged. By activation, dormant as well as sublethally damaged spores can recover and become active spores as illustrated in Table 3.

The decrease of active spores during storage (Table 3) was mainly due to the decreased transition from dormant to activated spores. In contrast, transitions from sublethally damaged to activated spores did slightly increase. In particular, of the 30 months old starter, 0% of dormant spores made the transition but sublethally damaged spores made even two transitions: 40.5% to active spores and about 4% to dormant spores. This indicates that the transition from dormant to activated spores depends on spore age, being gradually disabled in very old (30 months) spores. On the contrary, the transition of sublethally damaged spores occurred at all ages and even increased with higher age (Table 3).

In the proposed model (Figure 3) transitions may simultaneously take place during activation, from dormant spores to active spores, as well as from sublethally damaged spores (a) to dormant and further to active spores, or from sublethally damaged spores (b) directly to active spores. Our data show that depending on age, either all sublethally damaged spores transit to active spores (8-16 months), or most of them transit to active and a minority to dormant spores (30 months).

The effect of storage on the viability of spores was reported by other investigators. At room temperature, a significant decrease in viability of *R. oligosporus* spores was noted already after 2 months (Wang et al., 1975); germination percentages decreased rapidly at first, followed by a leveling off of the germination percentage, and subsequently declined to very low levels (Rusmin and Ko, 1974).

It is interesting that while the numbers of dead spores was quite low (6.5%) after 30 months of storage, and activation is still effective (44.8% of spores activated), only 4.4% of spores are still able to form colonies, a level that is similar to 4.3% of non-activated spores.

It can be concluded that after 30 months, spores in starter culture could still be activated but that these active spores have lost the ability to germinate, which may be due to the fact that the activated spores originated from sublethally damaged ones, unlike other younger spores that were activated from dormant spores.

Although we did not attempt the sorting of spores by FCM, plate counts on non-and activated samples showed that there was no correlation between the number of activated spores (very high) and colony forming units (low) as shown in Table 3. This shows agreement with experiments reported with starved *M. luteus* cells of which the resuscitation was not immediately followed by an increase in their colony forming ability (Kaprelyants et al., 1996). From a theoretical point of view, the time (60 min) of activation in MEB at 37 °C would also be too short to enable adequate uptake of N and C by activated spores to allow them to produce colonies. However, all activation treatments resulted in slightly improved culturability. This aspect is also supported by our earlier finding (Thanh and Nout, 2004) that in stored spores, maximum colony formation (56.2%) could be obtained only after 4 h of activation.
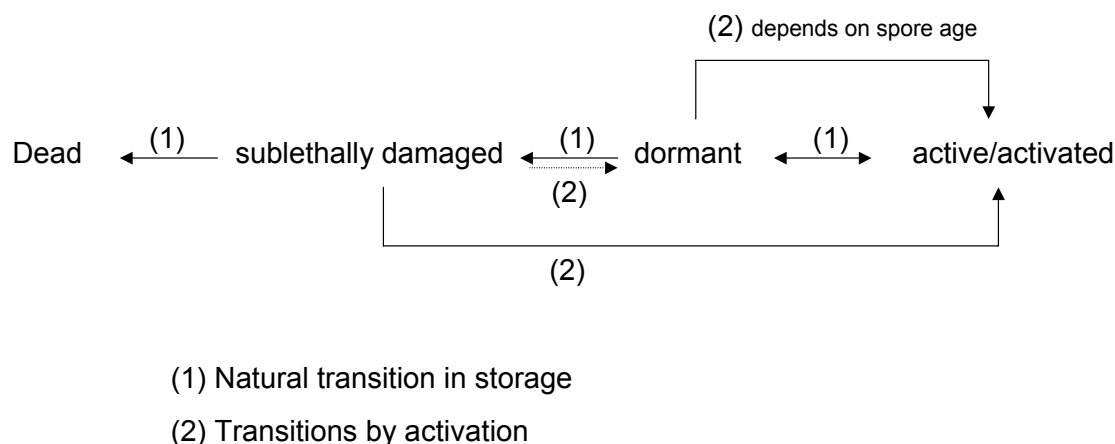
(2) depends on spore age

Dead ←(1)— sublethally damaged ←(1)→ dormant ←(1)→ active/activated
(2)
(2)

(1) Natural transition in storage

(2) Transitions by activation

Figure 3. Model of transitions of spore populations

In conclusion, sublethally damaged spores were found in tempe starter culture whereas the number of dead spores was not high, even after 30 months of storage. The shelf-life of spores in tempe starter is related to the physiological state of sublethally damaged and dormant spores. Both dormant and sublethally damaged spores can be resuscitated and become active spores. Our previous results (Thanh and Nout, 2004) and data in this paper showed that long-stored dormant spores can be activated and can give rise to high numbers of colonies. Dormancy is a resting period in the life cycle of a fungus, a reversible interruption in the phenotypic development of the organism (Sussman and Halvorson, 1966b). Some spores are exogenously dormant; their failure to geminate is due to unfavourable environmental conditions. In this case germination will proceed as soon as environmental conditions are satisfactory. In contrast, endogenously dormant spores do not germinate even under ideal environmental conditions. Spores of this type require either a period of aging or specific treatments to activate the germination process (Sussman and Halvorson, 1966a; Garraway and Evans, 1984; Griffin, 1994). Therefore, due to our present and earlier results, we can conclude that the dormancy of *R. oligosporus* sporangiospores is not endogenous but of an exogenous character.

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REFERENCES

Amor, K. B., Breeuwer, P., Verbaarschot, P., Rombouts, F. M., Akkermans, A. D. L., De Vos, W. M., and Abee, T. (2002) Multiparametric flow cytometry and cell sorting for the assessment of viable, injured, and dead *Bifidobacterium* cells during bile salt stress. *Applied and Environmental Microbiology* **68**, 5209-5216.

Breeuwer, P., and Abee, T. (2000) Assessment of viability of microorganisms employing fluorescence techniques. *International Journal of Food Microbiology* **55**, 193-200.

Breeuwer, P., De Reu, J. C., Drocourt, J.-L., Rombouts, F. M., and Abee, T. (1997) Nonanoic acid, a fungal self-inhibitor, prevents germination of *Rhizopus oligosporus* sporangiospores by dissipation of the pH gradient. *Applied and Environmental Microbiology* **63**, 178-185.

Brul, S., Nussbaum, J., and Dielbandhoesing, S. K. (1997) Fluorescent probes for wall porosity and membrane integrity in filamentous fungi. *Journal of Microbiological Methods* **28**, 169-178.

Bunthof, C. J., and Abee, T. (2002) Development of a flow cytometric method to analyze subpopulations of bacteria in probiotic products and dairy starters. *Applied and Environmental Microbiology* **68**, 2934-2942.

Bunthof, C. J., Bloemen, K., Breeuwer, P., Rombouts, F. M., and Abee, T. (2001) Flow cytometric assessment of viability of lactic acid bacteria. *Applied Environmental Microbiology* **67**, 2326-2335.

Chitarra, G. S., Breeuwer, P., Nout, M. J. R., van Aelst, A. C., Rombouts, F. M., and Abee, T. (2003) An antifungal compound by *Bacillus subtilis* YM 10-20 inhibits germination of *Penicillium roqueforti* conidiospores. *Journal of Applied Microbiology* **94**, 159-166.

Chitarra, L. G., and Van den Bulk, R. W. (2003) The application of flow cytometry and fluorescent probe technology for detection and assessment of viability of plant pathogenicbacteria. *European Journal of Plant Pathology* **109**, 407-417.

Davey, H. M., and Kell, D. B. (1996) Flow cytometry and cell sorting of heterogeneous microbial populations: the importance of single-cell analyses. *Microbiology Reviews* **60**, 641-696.

Garraway, M. O., and Evans, R. C. (1984) Spore dormancy, activation, and germination. In *Fungal Nutrition and Physiology* ed. M. O. Garraway and R. C. Evans, pp. 212-230. New York: John Wiley & Sons.

Griffin, D. H. (1994) *Spore dormancy and germination. In: Griffin, D. H.(Ed.), Fungal physiology*, New York: John Wiley & Sons, Inc., New York.

Hirons, G. T., Fawcett, J. J., and Crissman, H. A. (1994) TOTO and YOYO: new very bright fluorochromes for DNA content analyses by flow cytometry. *Cytometry* **15**, 129-140.

Jepras, R. I., Carter, J., Pearson, C. S., Paul, F. E., and Wilkinson, M. J. (1995) Development of a robust flow cytometric method for determining viable numbers of bacteria. *Applied and Environmental Microbiology* **61**, 2696-2701.

Kaprelyants, A. S., Gottschal, J. C., and Kell, D. B. (1993) Dormancy in nonsporulating bacteria. *FEMS Microbiology Reviews* **104**, 271-286.

Kaprelyants, A. S., and Kell, D. B. (1993) Dormancy in stationary-phase cultures of Micrococcus luteus: flow cytometric analysis of starvation and resuscitation. *Applied and Environmental Microbiology* **59**, 3187-3196.

Kaprelyants, A. S., Mukamolova, G. V., Davey, H. M., and Kell, D. B. (1996) Quantitative analysis of the physiological heterogeneity within starved cultures of *Micrococcus luteus* by flow cytometry and cell sorting. *Applied and Environmental Microbiology* **62**, 1311-1316.

Kaprelyants, A. S., Mukamolova, G. V., and Kell, D. B. (1994) Estimation of dormant Micrococcus luteus cells by penicillin lysis and by resuscitation in cell-free spent medium at high dilution. *FEMS Microbiology Letters* **115**, 347-352.

Kell, D. B., Kaprelyants, A. S., Weichart, D. H., Harwood, C. R., and Barer, M. R. (1998) Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. *Antonie van Leeuwenhoek* **73**, 169-187.

Lopez-Amoros, R., Comas, J., and Vives-Rego, J. (1995) Flow cytometric assessment of *Escherichia coli* and *Samonella typhimurium* starvation-survival in seawater using rhodamine 123, propidium iodide, and oxonol. *Applied and Environmental Microbiology* **61**, 2521-2526.

Mason, D. J., Allman, R., Stark, J. M., and Lloyd, D. (1994) Rapid estimation of bacterial antibiotic susceptibility with flow cytometry. *Journal of Microscience* **176**, 8-16.

Medwid, R. D., and Grant, D. W. (1984) Germination of *Rhizopus oligosporus* sporangiospores. *Applied and Environmental Microbiology* **48**, 1067-1071.

Mortimer, F. C., Mason, D. J., and Grant, V. A. (2000) Flow cytometric monitoring of antibiotic-induced injury in *Escherichia coli* using cell-impermeant fluorescent probes. *Antimicrobial Agents and Chemotherapy* **44**, 676-681.

Novo, D. J., Perlmutter, N. G., Hunt, R. H., and Shapiro, H. M. (2000) Multiparameter flow cytometric analysis of antibiotic effects on membrane potential, membrane permeability, and bacterial counts of *Staphylococcus aureus* and *Micrococcus luteus*. *Antimicrobial Agents and Chemotherapy* **44**, 827-834.

Ritz, M., Tholozan, J. L., Federighi, M., and Pilet, M. F. (2001) Morphological and physiological characterization of *Listeria monocytogenes* subjected to high hydrostatic pressure. *Applied and Environmental Microbiology* **67**, 2240-2247.

Roth, B. L., Poot, M., Yue, S. T., and Millard, P. J. (1997) Bacterial viability and antibiotic susceptibility testing with SYNTOX green nucleic acid stain. *Applied and Environonmental Microbiology* **63**, 2421-2431.

Rusmin, S., and Ko, S. D. (1974) Rice-grown *Rhizopus oligosporus* inoculum for tempeh fermentation. *Applied and Environonmental Microbiology* **28**, 347-350.

Shapiro, H. M. (1995) *Practical flow cytometry*, 3rd/Ed.: Wiley-Liss, Inc., New York, N.Y.

Sussman, A. S., and Halvorson, H. O. (1966a) Mechanisms of dormancy. In *Spores, their dormancy and germination* ed. A. S. Sussman and H. O. Halvorson, pp. 270-315. New York: Harper & Row Publishers.

Sussman, A. S., and Halvorson, H. O. (1966b) *Spores, their dormancy and germination*, New York: Harper & Row Publishers.

Thanh, N. V., and Nout, M. J. R. (2002) Rhizopus oligosporus biomass, sporangiospore yield and viability as influenced by harvesting age and processing conditions. *Food Microbiology* **19**, 91-96.

Thanh, N. V., and Nout, M. J. R. (2004) Dormancy, activation and viability of *Rhizopus oligosporus* sporangiospores. *International Journal of Food Microbiology* **92**, 171-179.

Ueckert, J. E., Breeuwer, P., Abee, T., and Stephens, P. (1995) Flow cytometry applications in physiological study and detection of food-borne microorganisms. *International Journal of Food Microbiology* **28**, 317-326.

Ueckert, J. E., Nebe von-Caron, G., Bos, A. P., and ter Steeg, P. F. (1997) Flow cytometric analysis of Lactobacillus plantarum to monitor lag times, cell division and injury. *Letters in Applied Microbiology* **25**, 295-299.

Votyakova, T. V., Kaprelyants, A. S., and Kell, D. B. (1994) Influence of viable cells on the rescuscitation of dormant cells in *Micrococcus luteus* cultures held in an extended stationary phase; the population effect. *Applied and Environmental Microbiology* **60**, 3284-3291.

Wang, H. L., Swain, E. W., and Hesseltine, C. W. (1975) Mass production of *Rhizopus oligosporus* spores and their application in tempeh fermentation. *Journal of Food Science* **40**, 168-170.

Wu, C. H., and Warren, H. L. (1984) Natural autofluorescence in fungi, and its correlation with viability. *Mycologia* **76**, 1049-1058.

Chapter 6

Protein profiles of dormant and activated sporangiospores of

Rhizopus oligosporus detected by 2-D electrophoresis

Abstract

The profile of proteins in dormant sporangiospores of *Rhizopus oligosporus* was compared with those of spores activated during 2 and 4 h in malt extract broth. In dormant sporangiospores 18 proteins were detected that disappeared during activation; these may be involved in keeping the spores from germination (i.e. to maintain dormancy). After 2 h, the first signs of 4 newly synthesized proteins could be detected; these proteins could be germination-specific proteins in dormant spores. Maximum levels of 17 newly synthesized proteins were observed during the period of germ tube emergence. In addition, 9 proteins were detected in the dormant spores that gradually increased in concentration. The possible functionality of the groups of proteins observed is discussed.

INTRODUCTION

Fungal spore germination provides a useful experimental system for investigating the concept that the cellular development and resumption of growth from a dormant or quiescent state involves unique macromolecular biosynthetic activities. One of the impressive morphological developments in the life cycle of a mycelial fungus is the conversion of a dormant spore into an actively growing mycelium. The initiation of spore germination leads to rapid increases in the rates of respiration and in protein and nucleic acid synthesis (Van Etten et al., 1974). Early investigations showed that protein synthesis is required for germ tube formation in all fungi, and dealt with the nature and function of the earliest proteins synthesized, regulation of protein synthesis in the spore (Brambl et al., 1978), the differences in proteins synthesized throughout the germination process (Van Etten et al., 1972; Wenzler and Brambl, 1978) and the specificity of proteins essentially for germ tube emergence, or for continued germ tube formation and vegetative growth. Also in yeasts - *Saccharomyces cerevisiae* - protein synthesis was required for early events in the germination program (Herman and Rine, 1997). In accordance with their functionality, protein dynamics are time-dependent: during the germination of *Rhizopus stolonifer* sporangiospores ribonucleic acid (RNA) and protein syntheses begin within a few minutes after the spores are placed under conditions which are favourable for germination (Van Etten et al., 1974). However, cellular proteins were not synthesized by germinating ascospores of *Neurospora tetrasperma* until 90 min after spore activation. At 90 min the respiratory rates accelerated rapidly, protein synthesis and transcripts for a subunit of the mitochrondrial ATPase (Hill et al., 1992) were initiated. Non-germinated spores – such as the conidiospores of *Botryodiplodia theobromae* - also contain several proteins such as ribosomes, aminoacyl-transfer RNA (tRNA) synthetases and transfer enzymes (Van Etten and Brambl, 1969).

The resolution and sensitivity of the separation of proteins by two-dimensional polyacrylamide gel electrophoresis makes it a powerful tool for the analysis and detection of proteins from complex biological sources. Proteins are separated according to their isoelectric point by isoelectric focusing in the first dimension, and according to their molecular weight by sodium dodecyl sulfate electrophoresis in the second dimension (O'Farrell, 1975). In combination with silver staining, a high sensitivity for proteins, RNA and DNA in the nanogram range on a colourless, transparent background (Blum et al., 1987) is achieved.

Rhizopus oligosporus is a fungus of industrial importance, being used in food fermentations (Nout and Aidoo, 2002) particularly to produce tempe. We previously showed that the efficiency of *R. oligosporus* starter powders is limited by dormancy of the sporangiospores (Thanh and Nout, 2002). We demonstrated that this dormancy can be overcome by activation and that certain amino acids play a crucial role in this process (Thanh and Nout, 2004).

In the present paper we use the 2-D electrophoresis approach to compare protein profiles of sporangiospores of *R. oligosporus* in the dormant stage (non-germinated) with those of spores during activation treatments.

MATERIALS AND METHODS

Preparation of tempe starter

Tempe starter was prepared as described earlier (Thanh and Nout, 2002). Briefly, the procedure was as follows: *Rhizopus oligosporus* LU 575 (NRRL 5905) was grown on Malt Extract Agar [MEA (malt extract 30 g/l, mycological peptone 5 g/l, agar 15 g/l), Oxoid, CM 59] plates during 1 week at 30 °C, and spores were harvested by washing with sterile water. The spore suspension was diluted to approximately 10^5 spores/ml. This suspension "A" was used as inoculum for substrate used to prepare tempe starter. Polished broken rice was steam-sterilized, allowed to cool and sterilized ammonium sulfate and sterilized 0.5 M H_2SO_4 were added to adjust the pH to 4, which is the optimum pH for spore germination of *Rhizopus oligosporus* (Breeuwer et al., 1997). The sterile broken rice (50 g) was inoculated and thoroughly mixed with 0.4 ml of spore suspension "A". The inoculated rice was incubated at 40 °C for 2 hours, followed by incubation for 4 days at 30 °C. The mouldy mass was broken up and the entire mass of substrate, mycelium and spores was dried at 42 °C for 48 h in a forced-air drying cabinet. Only crushing by sterile pestle and mortar, but no fine grinding was used in order to avoid misjudging during microscopic counting between spores and rice particles of similar size and shape. The starter powder was stored in closed screw-cap glass tubes, protected from light, in a silicagel desiccator at 25 °C, as these conditions were considered to be more representative of practical usage than storage at low temperature. Moreover, it was shown that spores survive better at ambient (25-30 °C) temperature than at 5 °C (Thanh and Nout, 2002).

Preparation of spore suspensions

Sporangiospores in crushed rice powder were diluted with sterile water to suspensions (approximately 10^7 spores/ml) by vigorous agitation and coarse filtration with a Millipore membrane filter (Fluorassure, Chem filter 15, Ref: 100-C2003-01, Chemunex, France) to remove mycelium and other debris. The filtrate containing the spores was washed twice by centrifugation at 13000 x *g* for 3 min in an Eppendorf centrifuge. After decanting, the spores in pellets were re-suspended in KPi pH 7 (for non-activated spores) and in MEB (for activation during 2 and 4 h) in which several pellets were pooled to obtain approximately 10^9 spores/ml.

Protein extraction

A sporangiospore suspension (3 ml) with a density of 10^9 spores/ml was obtained from 18 g of tempe starter culture of 6 months of age. For activation, harvested spores were

incubated in malt extract broth (MEB) for 2 and 4 h at 37 °C, with shaking at 140 rpm followed by centrifugation at 4000 x *g* for 4 min at 25 °C. The pellets were resuspended in potassium phosphate buffer, KPi pH 7.0, containing protease inhibitor at 0.2 g/ml as well as some zirconium beads (diameter, 1mm; Biospec Products). Total cellular proteins were extracted from the sporangiospore suspensions using a bead beater (B. Braun Biotech International, Melsungen, Germany), by four treatments of 3 min, and cooling on ice (Wouters et al., 1999). The zirconium beads were allowed to sediment by gravity, and remaining preparations were centrifuged twice at 13000 x *g* for 5 min at 4 °C. The supernatant containing the cellular proteins was analyzed by two-dimensional gel electrophoresis (2D-E). The protein content of the sporangiospore extract was determined using the bicinchoninic acid assay (Sigma, St. Louis, MO) with bovine serum albumin as the standard; equal amounts of protein were applied on the gel.

Protein analysis by 2D-Electrophoresis

2D-E was performed using a Pharmacia 2D-E system (Pharmacia Biotech, Uppsala, Sweden) (Wouters et al., 1999). Equivalent amounts of protein (50 µg of protein) extracted from non-activated spores, as well as from spores activated for 2h and 4h in MEB, were used. Each sample solution containing 50 µg of protein (of 0, 2, and 4 h activated spores, respectively) was treated with an equal volume of lysis solution (9 M urea, 2% 2-mercaptoethanol, 2% IPG buffer 3-10L (Pharmacia Biotech, Sweden), 2% Triton X-100, 6 mM Pefabloc SC (Merk, Darmstadt, Germany)). These preparations were kept on ice, and 2 volumes of sample solution (8 M urea, 2% 2-mercaptoethanol, 2% IPG buffer 3-10L, 0.5% Triton X-100, a few grains of bromophenol blue) were added to the protein extract. The total volume was loaded on the acidic end of a first-dimension Iso-Electric Focussing (IEF) gel with iso-electric points (pI) ranging from 4 to 7 (Immobiline Dry Strips; Pharmacia Biotech, Sweden). For the second dimension, Criterion Precast gels 8-16% Tris-HCl (BIO-RAD) were used. A molecular mass marker (Bio-rad) indicating 75, 50, 37, 25, 20, 15, and 10 kDa was used. The gels were silver stained (Blum et al., 1987) and were analyzed using PDQuest software (BioRad, Richmond, USA). Representative gels of duplicate experiments are depicted in Figure 1.

RESULTS

Sporangiospores that were stored for 6 months were used for analysis of proteins. Figure 1 shows the 2-D electrophoresis gels, and the categories of detected proteins are summarized in Table 1. Eighteen proteins - a, b, c, d, e, f, g, h, i, j, k, l, m, n, o, p, q, r - were identified on the gels as proteins present in dormant spores that disappear after 2 h of activation. Conversely, seventeen proteins – 1 to 17 - were newly detected after 4 h (and some – 1,2,4,5 - already after 2 h) of activation. Besides, nine proteins - A, B, C, D, E, F, G, H, I - were encountered in all three spore samples, with low intensity in dormant and with increasing intensity during successive activation treatment. The proteins on gels are numbered or marked consecutively from the highest molecular weight to the lowest.
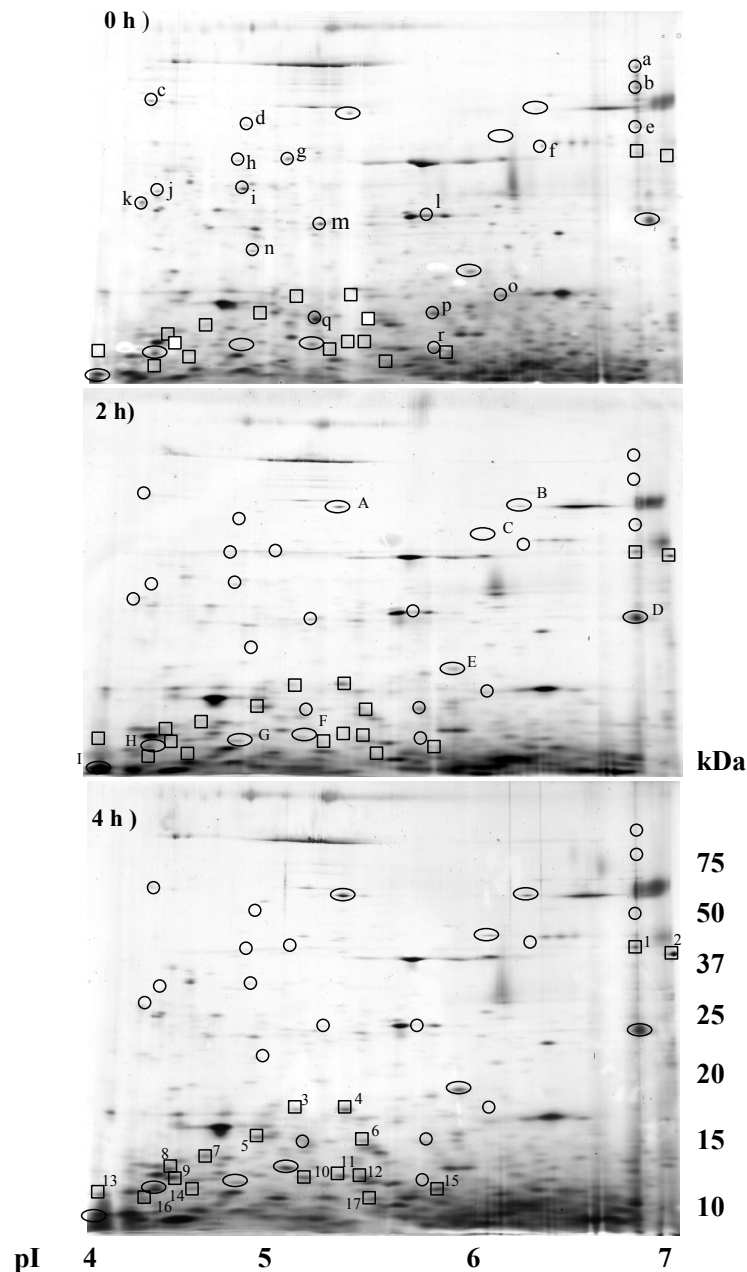
Figure 1. Effect of activation on protein profiles of *R. oligosporus* sporangiospores.
Two-dimensional gel electrophoresis of cell free extracts of non-activated spores (0 h), and activated spores incubated 2 h at 37 °C, and 4 h at 37 °C, using a pI ranging from 4 to 7. ○ = proteins that disappear from dormant spores, ⬭ = proteins present in dormant spores but that increase during activation, □ = proteins that appear as a result of activation.

Four hours after the activation of germination, the emergence of germ tubes had occurred (Thanh and Nout, 2004) simultaneously with the synthesis of new proteins (3, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17) in addition to the four new proteins detected after 2 h. Proteins found only in dormant spores had completely disappeared after 2 h.

Table 1. Comparison of protein patterns between non-activated (0h), activated (2h) and activated (4h) sporangiospores of *Rhizopus oligosporus* in MEB at 37 °C

Proteins	Non-activated spores (0 h)	Activated spores	
		2 h	4 h
a, b, c, d, e, f, g, h, i, j, k, l, m, n, o, p, q, r	+	-	-
A, B, C, D, E, F, G, H, I	+*	+*	+*
1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17	-	+	+

*proteins detected in dormant spores, with increasing levels in activated spores after 2h and 4h.

DISCUSSION

The purpose of this study was to distinguish proteins in dormant and activated and/or germinated sporangiospores of *R. oligosporus* by high resolution two-dimensional polyacrylamide gel electrophoresis.

While interpreting the protein profiles of the 3 situations studied, it should be noted that the non-germinated spores did not consist entirely of dormant spores (about 90% are dormant), whereas in activated spores after 2 h about 67% are activated and after 4 h this would have increased to 70%. If it would be possible to separate large quantities of purely dormant or activated spores, an optimum distinction between their protein profiles could be made. However, the throughput capacity of flowcytometer sorting devices do not allow this as yet. Notwithstanding these limitations, based on our results we can make a distinction of 3 categories of proteins.

The first category are proteins (18 were detected) that are present in dormant sporangiospores, but that disappear during activation for 2 h at 37 °C in MEB. These proteins may be involved in the maintenance of the dormant state. Dormant conidia contain a pre-existing pool of mRNA and ribosomes, primed for rapid activation and translation in the presence of nutrients (Osherov and May, 2001). In *R. stolonifer,* dormant spores contain preformed functional mRNAs and during germination, the majority of the changes in the mRNA population occur just prior to or simultaneously with changes in the proteins labeled in vivo; thus the majority of the proteins synthesized during *R. stolonifer* spore

germination appear to be regulated at the transcriptional level (Freer and Van Etten, 1978). In non-germinated ascospores transcription and translation were reported to play a role in ascospore survival (Brengues et al., 2002). The proteins detected in our dormant sporangiospores which disappeared during activation, presumably played a role in keeping the spores from germination (dormancy maintaining) and could be named 'dormant spore proteins'. Those dormant spore proteins may be different from proteins in activated spores, as was suggested by early experiments in yeast ascospores (West et al., 1985), in which the proteins of resting ascospores differed significantly from those found 15 min after the initiation of germination. An immediate transition from ascospore proteins to proteins required for ascospore germination, appears likely. Another indication for fundamental differences between proteins before and after germination is provided in *Bacillus megaterium* by the occurrence of two distinct proteolytic systems (Setlow, 1975): one degrading a unique class of dormant spore proteins and the other degrading primarily protein synthesized during germination. Proteolysis of dormant spore protein began by the 3rd min of germination and by 25 min it had degraded 15 to 20% of the pre-existing protein to free amino acids. Proteins synthesized early in germination (0 to 12 min) were also degraded rapidly (20% per hour). However, proteins synthesized later in germination (90 to 100 min) were degraded more slowly (~ 4% per hour). For dormant spores of *R. oligosporus*, it is not known whether the degradation of germination proteins occurred or not, but dormant spore proteins were degraded and disappeared from the onset of germination.

So far, it has not been demonstrated that the products translated from spore mRNA resemble authentic proteins (Freer and Van Etten, 1978) except it was shown (Horikoshi and Ikeda, 1968; Lovett, 1976) that dormant fungal spores carry a virtually complete system for protein synthesis, including 80S ribosomes, aminoacyl-tRNA synthetases, the initiation, elongation and termination proteins, tRNA, and mRNA in an inactive form. We presume that these can also be found in dormant *R. oligosporus* sporangiospores.

The second category involves proteins (17 were detected) that are newly synthesized during the 4h activation treatment resulting in the germination of spores. The time-frame of this synthesis corresponds with changes of physiological state, i.e. 0 h corresponds with the dormant state, 2 h with swollen spores, and 4 h with the emergence of germ tubes. It was reported (Freer and Van Etten, 1978) that a few proteins were synthesized only at discrete time periods during the germination process and, thus, may be germination-specific proteins. Our data show that only 4 proteins appeared at 2 h; these might be germination-specific proteins. However, in view of the large variety of proteins assembled at very early stages of fungal spore germination, further research will be required to ascertain the function of these proteins. The moment in time (2 h) that we detected the first newly synthesized proteins is of comparable order as has been reported for *A. nidulans* (50 min) (Bainbridge, 1971), *Rhizopus stolonifer* and *Phycomyces blakesleanus* spores (2h) (Van Assche and Carlier, 1973). It was also reported (Hill et al., 1992; Plesofsky-Vig et al., 1992) that cellular proteins were not synthesized by germination ascospores of *Neurospora tetrasperma* until 90 min after spore activation. After 90 min the respiratory rates accelerated rapidly, and protein synthesis was initiated.

In *Dictyostelium discoideum,* RasG protein levels were very low in dormant spores, remained low during the lag period, but only increased late during spore swelling just prior to the emergence stage of germination (Khosla et al., 1994). Our data show that many more new proteins were synthesized during the period of germ tube emergence (4 h) which may be accounted for by the activation of ribosomes and increasing capacity of the protein synthesizing system (Horikoshi and Ikeda, 1968). Proteins synthesized early (2 h) remained stable up to 4 h of germination, implying that 4 germination-specific proteins still exist and integrate into differential proteins that were synthesized later. Their existence also means they may participate in normal functions during spore germination. Protein synthesis occurs in all regions of the cell during germ tube emergence (Orlowski and Sypherd, 1978) and the latter might depend on the rapid synthesis of such hypothetical structures or areas, which could include mitochondria or cell wall proteins (Bartnicki-Garcia and Lippman, 1978).

Finally, the third category of proteins (9 were detected) are detectable in all physiological stages tested, but with gradually increasing density of the spots (increasing levels of concentration). It is most likely that these represent enzymes and/or ribosomes and that the increase of their intensity in the gels is due to the increase of their activity. This hyphothesis is supported by abundant evidence for the increase of specific activities of various enzymes such as glutamate dehydrogenase (Tuveson et al., 1967), trehalase (Sussman, 1969) and ribosomes (Horikoshi and Ikeda, 1968) during spore germination. Ribosomes occur as monomers, dimers, trimers, etc., even as hexamers. When present as polysomes, the ribosomes are active whereas they are not naturally active as monosomes. For example, in dry resting conidia of *Neurospora crassa* only 3% of the ribosomes existed as polysomes, the number increased to 30% in hydrated spores, and during the first 15 to 30 minutes of germination this value further increased to 75%; this increase would be consistent with the function of polysomes in protein synthesis (Mirkes, 1974).

In conclusion, we were able to distinguish 3 categories of proteins that undergo changes during the activation and germination of *R. oligosporus* sporangiospores. Further research will be required to study the nature and function of these proteins, particularly those that are newly synthesized or increasing during the activation of dormant sporangiospores of *R. oligosporus* in tempe starter cultures.

REFERENCES

Bainbridge, B. W. (1971) Macromolecular composition and nuclear division during spore germination in *Aspergillus nidulans*. *Journal of General Microbiology* **66**, 319-325.

Bartnicki-Garcia, S., and Lippman, E. (1978) Fundamental aspects of hyphal morphogenesis. In *Microbial differentiation* ed. J. M. Ashworth and J. E. Smith, Vol. 23. Symposium of the Society for Microbiology.

Blum, H., Beier, H., and Gross, H. J. (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **8**, 93-99.

Brambl, R., Dunkle, L. D., and Van Etten, J. L. (1978) Nucleic acid and protein synthesis during fungal spore germination. In *The Filamentous Fungi* ed. J. E. Smith and D. R. Berry, Vol. 3, pp. 94-118. London: Edward Arnold Limited.

Breeuwer, P., De Reu, J. C., Drocourt, J.-L., Rombouts, F. M., and Abee, T. (1997) Nonanoic acid, a fungal self-inhibitor, prevents germination of *Rhizopus oligosporus* sporangiospores by dissipation of the pH gradient. *Applied and Environmental Microbiology* **63**, 178-185.

Brengues, M., Pintard, L., and Lapeyre, B. (2002) mRNA decay is rapidly induced after spore germination of *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **277**, 40505–40512.

Freer, S. N., and Van Etten, J. L. (1978) Changes in messenger RNAs and protein synthesis during germination of *Rhizopus stolonifer* sporangiospores. *Experimental Mycology* **2**, 313-325.

Herman, P. K., and Rine, J. (1997) Yeast spore germination: a requirement for Ras protein activity during re-entry into the cell cycle. *The EMBO Journal* **16**, 6171–6181.

Hill, E. P., Plesofsky-Vig, N., Paulson, A., and Brambl, R. (1992) Respiration and gene expression in germinating ascospores of *Neurospora tetrasperma*. *FEMS Microbiology Letters* **90**, 111-115.

Horikoshi, K., and Ikeda, Y. (1968) Studies on the conidia of *Aspergillus oryzae*. VII. Development of protein synthesizing activity during germination. *Biochimica et Biophysica Acta* **166**, 505-511.

Khosla, M., Spiegelman, G. B., Weeks, G., Sands, T. W., Virdy, K. J., and Cotter, D. A. (1994) RasG protein accumulation occurs just prior to amoebae emergence during spore germination in *Dictyostelium discoideum*. *FEMS Microbiology Letters* **117**, 293-298.

Lovett, J. S. (1976) Regulation of protein metabolism during spore germination. In *The fungal spore* ed. D. J. Weber and W. M. Hess, pp. 189-242. Utah: John Wiley and Sons.

Mirkes, P. E. (1974) Polysomes, rhibonucleic acid, and protein synthesis during germination of *Neurospora crassa* conidia. *Journal of Bacteriology* **117**, 196-202.

Nout, M. J. R., and Aidoo, K. E. (2002) Asian fungal fermented food. In *Industrial applications.* ed. H. D. Osiewacz, Vol. Vol.X, pp. 23-47. Berlin-Heidelberg-New York: Springer-Verlag.

O'Farrell, P. (1975) High resolution two-dimensional electrophoresis of proteins. *The Journal of Biological Chemistry* **250**, 4007-4021.

Orlowski, M., and Sypherd, P. S. (1978) Location of protein synthesis during morphogenesis of *Mucor racemosus*. *Journal of Bacteriology* **133**, 399-400.

Osherov, N., and May, G. S. (2001) The molecular mechanisms of conidial germination. *FEMS Microbiology Letters* **199**, 153-160.

Plesofsky-Vig, N., Paulson, A., Hill, E. P., Glaser, L., and Brambl, R. (1992) Heat shock gene expression in germinating ascospores of *Neurospora tetrasperma*. *FEMS Microbiology Letters* **90**, 117-122.

Setlow, P. (1975) Protein metabolism during germination of *Bacillus megaterium* spores. II. Degradation of pre-existing and newly synthesized protein. *The Journal of Biological Chemistry* **250**, 631-637.

Sussman, A. S. (1969) The dormancy and germination of fungus spores. *Symposium of the Society of Experimental Biology* **23**, 99-121.

Thanh, N. V., and Nout, M. J. R. (2002) Rhizopus oligosporus biomass, sporangiospore yield and viability as influenced by harvesting age and processing conditions. *Food Microbiology* **19**, 91-96.

Thanh, N. V., and Nout, M. J. R. (2004) Dormancy, activation and viability of *Rhizopus oligosporus* sporangiospores. *International Journal of Food Microbiology* **92**, 171-179.

Tuveson, R. W., West, D. J., and Barrett, R. W. (1967) Glutamic acid dehydrogenases in quiescent and germinating conidia of *Neurospora crassa*. *Journal of General Microbiology* **48**, 235-248.

Van Assche, J. A., and Carlier, A. R. (1973) The pattern of protein and nucleic acid synthesis in germinating spores of *Phycomyces blakesleanus*. *Archive der Mikrobiologie* **93**, 129-136.

Van Etten, J. L., and Brambl, R. M. (1969) Protein synthesis during spore germination. III. Transfer activity during germination of *Botryodiplodia theobromae* spores. *Phytopathology* **59**, 1894-1902.

Van Etten, J. L., Bulla, L. A., and St. Julian, G. (1974) Physiological and morphological correlation of *Rhizopus stolonifer* spore germination. *Journal of Bacteriology* **117**, 882-887.

Van Etten, J. L., Roker, H. R., and Davies, E. (1972) Protein synthesis during fungal spore germination: differential protein synthesis during germination of *Botryodiplodia theobromae* spores. *Journal of Bacteriology* **112**, 1029-1031.

Wenzler, H., and Brambl, R. (1978) *In vitro* translation of polyadenylate-containing RNAs from dormant and germinating spores of the fungus *Botryodiplodia theobromae*. *Journal of Bacteriology* **135**, 1-9.

West, T. P., Armstrong, R. L., and Magee, P. T. (1985) Changes in *Saccharomyces cerevisae* ascospore proteins during early germination. *FEMS Microbiology Letters* **28**, 255-258.

Wouters, J. A., Jeynov, B., Rombouts, F. M., De Vos, W. M., Kuipers, O. P., and Abee, T. (1999) Analysis of the role of 7 KDa cold-shock proteins of *Lactococcus lactis* MG 1363 in cryoprotection. *Microbiology* **145**, 3185-3194.

Chapter 7

General discussion

Introduction

Soybean foods serve as a source of protein in the daily diet of Asian people. Tempe is a traditional Indonesian fermented food in which moulds particularly *Rhizopus oligosporus*, play an essential role. A crucial step in tempe fermentation is the germination of spores of the starter culture. Tempe starters are limited in their effectiveness by the shelf-life of their spores. The work described in this thesis highlights the nature of this shelf-life, in particular the physiological states such as dormancy, sublethal damage and death that limit the viability of spores.

Dormancy and activation of dormant spores

The life cycle of many organisms includes a period of dormancy or semi-dormancy characterized by reduced metabolic activity (Mirkes, 1974). Dormancy may be defined as "a reversible state of low metabolic activity, in which cells can persist for extended periods without division" (Kaprelyants et al., 1993); in microbiology this often corresponds to a state in which cells are not viable in the sense of being able to form a colony when plated on a suitable solid medium but in favourable conditions they can be activated into a state of viability.

In this thesis, dormancy of *R. oligosporus* sporangiospores was investigated. One approach used fluorescence markers (cFDA and PI) in combination with fluorescence microscopy to detect live, dead and dormant spores (Chapter 2), whereas the other approach used cFDA, PI and TOTO-1 in combination with flowcytometry (FCM) to detect not only live, dead and dormant but also to differentiate physical categories of individual spores (Chapter 5). Fluorescent probes can distinguish not only between live and dead but also between vigorous, frail, or injured organisms (Lloyd and Hayes, 1995). cFDA is commonly used as a live stain (Shapiro, 1995; Davey and Kell, 1996; Breeuwer and Abee, 2000). PI and TOTO-1 are nucleic acid dyes that generally do not cross intact cell plasma membranes and, hence, only enter into non-viable cells that lost their membrane integrity. TOTO-1 gave a better discrimination of live and dead bacterial cells than PI, and the combination of cFDA and TOTO-1 was reported earlier to make an excellent live/dead assay with versatile applications (Bunthof et al., 2001).

In this thesis, cFDA was tested as a viable cell stain for *R. oligosporus* sporangiospores, and the results showed that cFDA fluorescence gave a very good correlation with viability as measured by culturing. The number of spores that gave a fluorescent reaction with PI was quite low whereas a big amount of spores kept quiescent (neither staining with cFDA nor PI). This showed that the majority of sporangiospores in the starter culture are in a state of dormancy, having intact membranes but lacking enzyme

activity (Chapter 2). These findings are supported by those of Kaprelyants and Kell who reported that dormancy may be far more common than death in starving microbial cultures (Kaprelyants and Kell, 1993).

MEB (Malt Extract Broth) was used to study germination of several fungal spores, such as *R. oligosporus* sporangiospores (Breeuwer et al., 1997) and conidia of *Penicillum roqueforti, Fusarium culmorum,* and *P. paneum* (Chitarra, 2003). Whereas heat treatments were unsuccessful, we showed MEB to be a good activation medium for dormant spores of *R. oligosporus* with 80% of dormant spores being activated as compared with 11 % of the control. This is in contrast with other fungal spores such as *Phycomyces blakesleeanus* (Van Laere et al., 1980); *Neurospora tetrasperma, Mucor miehi* (Sussman, 1976), and ascospores of *Talaromyces macrosporus* (Kikoku, 2003) that could be activated by heat treatments. The reason for this different behaviour may be another dormancy mechanism of *R. oligosporus* sporangiospores. Constitutive dormancy has been reported in *Distyostelium discoideum* (Cotter and Raper, 1966) and *Phycomyces blakesleeanus* (Sussman and Douthit, 1973; Van Mulders and Van Laere, 1984). Supposing constitutive dormancy occurs in *N. tetrasperma* and *M. miehi spores,* because heat treatment is required for activation, it might indicate that the dormancy of *R. oligosporus* and *M. rouxii* (Dewerchin and Van Laere, 1984) could be termed exogenous, as nutrient supplementation initiates their germination.

Malt extract broth (MEB) contains peptone and maltose. MEB, peptone and yeast extract were used in our activation studies. Like MEB, peptone and yeast extract showed to be good activators for disruption of dormancy in spores of *R. oligosporus* (Chapter 3). The role of peptone in activation for spore germination was reported elsewhere, for instance in *Dictyostelium discoideum* spores (Cotter and Raper, 1966), *Mucor racemosus* sporangiospores (Tripp and Paznokas, 1981), and *Trichophyton mentagrophytes* microconidia (Hashimoto et al., 1972). Peptone can be prepared from various proteinaceous ingredients using different chemical or enzymatic means of hydrolysis. By consequence, its composition is complex and only partially defined.

L-alanine, an amino acid predominating in peptone played an important role (of the same order as that of peptone) in the stimulation of germination of our dormant spores. L-proline on the other hand, did not support germination but instead, inhibited L-alanine uptake from a mixture of alanine and proline. Since alanine is a rather simple amino acid compared to the imino acid proline (Stryer, 1995), it may have the advantage of easy transport into the spore. However, proline may occupy the transport carrier which in turn is unable to deliver it inside the cell (Hunter and Segel, 1971).

Many reports have been published about role of alanine and proline in the germination of fungal spores, but none of them in relation to dormant spores of *R. oligosporus*. L-alanine and L-proline were reportedly most effective in promoting germination of *R. oligosporus* sporangiospores (Medwid, 1984b), *Aspergillus niger* conidia (Yanagita, 1957; Miller, 1962), and *Bacillus cereus* spores (O'Connor and Halvorson, 1961). However, the activation buffers used in these experiments also contained glucose. Our data show that a buffer containing glucose will allow considerable colony formation,

irrespective of the amino acids present. We expect that this may have resulted in misinterpretations of the role of proline.

Our finding that germination on glucose is accelerated by the addition of phosphate confirms earlier reports (O'Connor and Halvorson, 1961). However, optimum germination induced by L-alanine can take place without additional phosphate.

Alanine is a good stimulator of spores; even no glucose is required because alanine can satisfy the requirements of carbon as well as nitrogen for spore germination (Chapter 4). The dual role played by L-alanine is understandable because it represents one of the most direct precursors of pyruvate. Alanine was reported to highly support the germination of *Aspergillus flavus* conidia (Pass and Griffin, 1972), and spores of *Bacillus subtilis* (Wax et al., 1967), *B. megaterium* (Levinson and Hyatt, 1962) and *B. licheniformis* (Halmann and Keynan, 1962). Interestingly, alanine was liberated to a large extent during the fermentation of soybeans with *Rhizopus* sp. (Kiers, 2001) whereas *Bacillus subtilis* soybean fermentation led to major increases in almost all amino acids (Sarkar et al., 1997). It will be of interest to further investigate the regulation of *R. oligosporus* alanine production in relation to its requirements.

Earlier investigations showed that glucose is a good activator of dormant spores, triggering germination of sporangiospores of *Rhizopus arrhizus* (Ekundayo and Carlier, 1964), *Mucor racemosus* (Tripp and Paznokas, 1982), *Mucor rouxii* (Dewerchin and Van Laere, 1984), *Pilobolus longipes* (Bourret, 1986), and yeast ascospores (Thevelein, 1984). Glucose was also reported to be essential for initiation of spore swelling and germ tube emergence in *Syncephalastrum racemosum* (Hobot and Gull, 1977).

However, our data show that glucose only is insufficient to allow colony formation from dormant spores of *R. oligosporus* (Chapter 3 and 4) except when using glucose dissolved in phosphate buffer (minimal medium), e.g. no colony formation was found when using glucose dissolved in distilled water. This explains the different conclusion regarding the role of glucose, namely that it can only serve as an activator in the presence of phosphate.

Transitions of *R. oligosporus* sporangiospores in tempe starter
Transitions are changes of physiological state taking place as a result of aging during storage, or due to activation of dormant spores. We observed physiological transitions taking place between metabolically active, dormant, viable, and dead spores.

Natural transitions of spore subpopulations in tempe starter by spores of various storage ages
Our studies of tempe starter indicated that freshly after harvest and mild drying, most spores (about 90%) were in the dormant (quiescent) state. During storage, the transition of dormant spores into active and dead spores occurred. Numbers of active spores corresponded well with the number of viable spores. Notably, the number of active spores still slightly increased until 16 months of storage; the maximum level of viable spores was

observed after a period of 1-3 months (Chapter 3, Fig. 2), which agrees with earlier studies (Tanuwidjaja, 1985; Tunçel et al., 1989).

Similar transitions still occurred up to 30 months of storage. At first, it seemed that the spores in this 30-months old tempe starter had died because only a very small proportion of metabolic active (4.3%) and very high portion of PI stained spores (57%) were found. This would have been the result of the excessively long period of storage, as compared with other studies that indicated shelf-life of rice-base starters of about 2.5 to 4 months. Later, we found that many of the "dead" PI-stained spores could still be resuscitated (Chapter 3), indicating that they had been sublethally damaged rather than dead, which was backed up by our experiments with TOTO-staining (Chapter 5).

It could be stated that our study partly fulfilled the previously formulated need (Hawker and Madelin, 1976) : "To study development of the spores and to look at spores of all ages until one has what is obviously the oldest, and one that can be shown to be physiologically a dormant spore."

Transitions of spore subpopulations in tempe starter by activation and resuscitation treatments

Dormant cells – as defined by Kell (Kell *et al.*, 1998) - may be non-culturable in that they require specific stimuli before they become active and culturable; activation denotes a transition of cells from non-culturable to culturable states with respect to a given medium. Different phenomena are cell injury and repair. Some cells may respond to specific forms of damage by entering a physiological state in which specific reparative processes are necessary before (re)growth on their usual range of media is initiated. Regrowth is the return to an actively growing state of cells that had ceased growth but had not lost culturability (Kell *et al.*, 1998). For example, it was observed that starved *Micrococcus luteus* cells may contain a mixture of dormant and injured cells; moreover, dormant cells could have injuries as well. Resuscitation may actually represent their recovery from injury, or the repair of the membrane barrier (Kaprelyants *et al.*, 1996; Kell *et al.*, 1998).

From several of our own consecutive experiments, MEB was found to be a very good activation medium for dormant spores of *R. oligosporus* (Chapter 3). To get a better insight into the transitions of stored spores, double staining with cFDA and PI/TOTO-1 was performed on non-activated and activated samples of 8, 10, 16, and 30 months-stored spores and they were analyzed by FCM. Transitions of spores occurred once activation was applied: numbers of cF-stained (live) spores increased strongly, while numbers of unstained (dormant) spores decreased and PI-stained (dead) spores decreased (Chapter 5, Table 1 and Fig. 2). The decreased number of PI–spores by activation prompted us to attempt TOTO-1 (instead of PI) together with cFDA. Results showed that the increase of the number of metabolic active (cF-stained) spores was of nearly the same magnitude and the decrease of dormant (unstained) spores was almost similar to that in the PI and cFDA staining before and after activation. Notably, the number of TOTO-1 stained spores was of the same order of that of PI stained spores after activation (Chapter 5, Table 1). It is probable that the simultaneous decrease of PI-stained spores and increase of cF-stained

spores is due to a restoration of the membrane barrier caused by the activation in MEB. This might therefore be a similar response to activation as that of *M. luteus* mentioned above.

Thus, one more subpopulation, namely sublethally damaged spores was distinguished besides viable, dead, and dormant spores in tempe starter culture. Sublethally damaged spores can be quantified by FCM by comparing responses to PI and TOTO-1.

We demonstrated that even after 30 months of storage, the spores of the tempe starter were not dead but merely sublethally damaged. By activation, both dormant and sublethally damaged spores can recover and become active spores as illustrated in Chapter 5, Table 3. Our results indicate that the ability to make the transition from dormant to activated spores depends on spore age, being gradually disabled in very old (30 months) spores. Conversely, the transition of sublethally damaged spores occurred at all ages and even increased with higher age (Chapter 5, Table 3). Although activation is still effective (44.8% of spores were activated), only 4.4% of spores were still able to form colonies while the numbers of dead spores was quite low (6.5%). This apparent discrepancy between numbers of activated and viable (culturable) spores may be due to the fact that the activated spores originated from sublethally damaged ones. A similar observation was made with *M. luteus* cells (Kaprelyants and Kell, 1993): if the cells were stored for 7 months, only 25% of the cells could be converted to a metabolically active state (as judged with FCM), but these cells did not form colonies anymore.

Based on results of the FCM experiments, it can be concluded that the shelf-life of spores in tempe starter is related to the physiological state, particularly of sublethally damaged and dormant spores; a model of physiological state transitions of *R. oligosporus* sporangiospores was proposed (Chapter 4, Figure 3). Transitions may take place simultaneously during activation, from dormant to active, as well as from sublethally damaged (a) to dormant and further to active, or from sublethally damaged (b) directly to active spores. Our data show that depending on age, either all sublethally damaged spores transit to active spores (8-16 months old), or most of them transit to active and a minority to dormant spores (30 months old). In principle, both dormant and sublethally damaged spores can be activated and resuscitated respectively, and become active spores. Our data presented in Chapters 3 and 4 show that long-stored dormant spores can be activated and can give rise to high numbers of colonies.

Dormancy of *R. oligosporus* sporangiospores is of exogenous nature

Dormancy is a resting period in the life cycle of a fungus, a reversible interruption in the phenotypic development of the organism (Sussman, 1966). Some spores are exogenously dormant; their failure to germinate is due to unfavorable environmental conditions. In this case germination will proceed as soon as environmental restrictions have been removed. In contrast, endogenously dormant spores do not germinate, even under ideal environmental conditions. Spores with this type of dormancy require either a period of aging or specific treatments to activate the germination process (Garraway and Evans, 1984; Griffin, 1994). Our accumulated data (Chapter 3, 4, 5) demonstrate that activation in MEB at 37 °C of all

stored spores tested, gave a significant increase of metabolically active spores. Likewise, it has been reported (Breeuwer et al., 1997) that exogenous factors such as nonanoic acid may contribute to the regulation of swelling and germination. We therefore conclude that the dormancy of *R. oligosporus* sporangiospores in tempe starter cultures is not of an endogenous, but of an exogenous character.

Soybean cook water promotes spore germination of *R. oligosporus*

It was reported earlier (Medwid, 1984a) that a heat-stable, dialyzable factor(s) present in aqueous soybean extract (soybean cook water) promoted more rapid and more efficient germination than was noted on other media. The factor(s) also resulted in increased diameter during phase I of germination (swelling) and multiple germ tubes in phase II (emergence of germ tube). Results suggested that soybean cook water may provide a useful medium for studies of spore germination and spore injury.

Similar results were found in our study, i.e. soybean cook water promoted more efficient germination of dormant spores of *R. oligosporus* in starter cultures. Considering the effect of amino acids presented in Chapter 4, the stimulatory effect of soybean cook water might be caused by free amino acids such as alanine. Our results indicate that nutrient leaching from soybeans which is a common consequence of traditional tempe manufacture, leads to reduced fungal growth (Chapter 4, Table 3). This indicates that there is scope for optimization of the bean pretreatment operations in tempe manufacture, in view of optimizing the environmental conditions in accordance with the physiological requirements of the starter.

Protein profile of *R. oligosporus* sporangiospores

The initiation of spore germination leads to rapid increases in the rates of respiration and in protein and nucleic acid synthesis (Van Etten et al., 1974). Several investigators have demonstrated that dormant fungal spores contain polysomes (Mirkes, 1974; Brambl, 1975): preformed functional mRNAs which code for the synthesis of proteins (Freer and Van Etten, 1978); 80S ribosomes, aminoacyl-tRNA synthetases, the initiation, elongation and termination proteins, tRNA, and mRNA (Lovett, 1976). Moreover, ongoing transcription and translation in non-germinating spores are part of the spore survival mechanism (Brengues et al., 2002). It was reported (Freer and Van Etten, 1978) that a few proteins were synthesized only at discrete time periods during the germination process and, thus, may be germination-specific proteins. Furthermore, swelling and germination of *R. oligosporus* sporangiospores require active protein synthesis and respiratory activity (Medwid, 1984a).

From these reports and our results, it can be implied that our dormant spores (Chapter 6, Fig. 1; 0 h gel) may contain a variety of preformed functional mRNAs, ribosomes, metabolic enzymes, and maybe 'survival proteins' or complex substances that keep dormant spores from germination; several proteins could be visualized by two dimensional electrophoresis. These "dormant proteins" may be different from "activated proteins", which would agree with the earlier findings (West et al., 1985) that proteins

synthesized in non-germinated yeast ascospores differed significantly from the proteins found 15 min after the initiation of germination in the ascospores.

It is evident from our work that proteins in dormant spores differ from proteins in activated spores. Eighteen proteins were detected in the dormant state which disappeared when activated 2 h at 37 °C in MEB. At the same time, 17 new proteins were synthesized in activated spores (after 4 h) and during germination of spores, of which 4 could already be detected after 2 h of activation.

The protein patterns of spores revealed that several proteins were synthesized at specific stages of germination; the majority of these changes occurred (1) after the initiation of germination and (2) just at the time of germ tube emergence (4 h). When the protein patterns of dormant spores (0 h), swollen spores (2 h), and spores with germ-tubes (4 h) were compared, results showed a good agreement with the emergence of new proteins on the electrophoresis gels.

Viability and heterogeneity

Heterogeneity of a cell population may arise from (1) cells at different stages of growth (i.e. in a population, cells may be in the lag phase, actively growing and dividing, in the stationary phase, or dead), (2) oscillatory intracellular dynamics such as protein turnover, glycolysis rate, and regulation or redox state, (3) difference in environmental conditions of individual cells, such as a dissimilar availability of limiting nutrients (Kell et al., 1991). With respect to viability, a population can be arbitrarily divided in dead, viable but non-culturable, resting (dormant), and active (dividing) subpopulations (Mason et al., 1986). Fluorescence labeling techniques allow analysis of individual cells by microscopy, flow cytometry (FCM) or image analysis. Using FCM, it was shown (Kaprelyants and Kell, 1992) that *M. luteus* cells were heterogeneous with respect to their ability to accumulate Rhodamine 123. Two subpopulations could be distinguished: viable and non-viable cells. However, when the cells were resuscitated by addition of suitable nutrients, a part of the cells significantly increased their ability to accumulate Rhodamine 123 representing 'non-viable but resuscitable' cells. Analysis of the germination of *R. oligosporus* sporangiospores indicated that spore populations are very heterogeneous (Breeuwer et al., 1997).

During our experiments we also observed that *R. oligosporus* sporangiospores are heterogeneous through fluorescence microscopic examination and flow cytometry figures, and especially in the results of heat treatment experiments. It is most likely that small size sporangiospores of *R. oligosporus* are heat resistant (data not shown). This is similar to the case where starvation also induced cross protection against heat challenge in *Escherichia coli* (Jenkins et al., 1988), and where a protein was synthesized at the onset of the starvation period, to enhance the heat resistance in starved cells of *Campylobacter jejuni* (Cappelier et al., 2000).

Heterogeneity of cells, originating from a genetically homogeneous population, has the obvious function to improve adaptation to unfavorable environmental conditions. Availability of sensitive detection systems provides new opportunities to study

heterogeneity of microbial populations. This may contribute to a better understanding of the mechanisms involved in selective survival of microorganisms under different stress conditions.

In conclusion, for practical purposes it has been shown that starter spores can be stored for more than 2 years, but that a considerable part of these spores are inactive because of dormancy and/or sublethal damage. It turns out that much improvement can be achieved by nutrient addition prior to germination. However, this work has also given rise to several new questions, such as:

(1) How are sporulation conditions, dormancy and germination ability of *R. oligosporus* sporangiospores related?

(2) What is the nature of bottlenecks in spores activated from sublethally damaged ones that prevent germination and mycelial growth?

(3) How are requirement and production (in fermentation) of essential nutrients such as L-alanine regulated?

(4) How do sporulation conditions influence the heterogeneity, in particular of sporangiospore size and their heat resistance?

(5) Genomic data of *Rhizopus oligosporus* should be produced that can support the study of the nature and functionality of specific proteins in the protein profiles observed in sporangiospores in transition between physiological states, as initiated by specific activating conditions.

Continued research will be needed to find full answers to these questions and understand the opportunities and limitations of the use of *R. oligosporus* sporangiospores. Nevertheless, the findings presented in this thesis already contribute significantly to our knowledge and make it possible to develop and validate efficient starters for tempe making which is not yet done in the context of this thesis. However, some recommendations for practical applications can be given:

-The use of 1 month-old starters is recommended with a MEB pretreatment resulting in maximum numbers of germinating spores. Crushing after mild drying is recommended instead of pulverization, to reduce losses of viability.

- For the tempe making process, soybean cooking water should be reused because it promotes more efficient germination of dormant spores of *R. oligosporus* in tempe starter cultures; this practice will also contribute to the nutritional value of tempe.

References

Bourret, J. A. (1986) Evidence that a glucose-mediated rise in cyclic AMP triggers germination of *Pilobolus longipes* spores. *Experimental Mycology* **10**, 60-66.

Brambl, R. (1975) Presence of polyribosomes in conidiospores of Botryodiplodia theobromae harvested with nonaqueous solvents. *Journal of Bacteriology* **122**, 1394-1395.

Breeuwer, P., and Abee, T. (2000) Assessment of viability of microorganisms employing fluorescence techniques. *International Journal of Food Microbiology* **55**, 193-200.

Breeuwer, P., De Reu, J. C., Drocourt, J.-L., Rombouts, F. M., and Abee, T. (1997) Nonanoic acid, a fungal self-inhibitor, prevents germination of Rhizopus oligosporus sporangiospores by dissipation of the pH gradient. *Applied and Environmental Microbiology* **63**, 178-185.

Brengues, M., Pintard, L., and Lapeyre, B. (2002) mRNA decay is rapidly Induced after spore germination of *Saccharomyces cerevisiae*. *The Journal of Biological Chemistry* **277**, 40505–40512.

Bunthof, C. J., Bloemen, K., Breeuwer, P., Rombouts, F. M., and Abee, T. (2001) Flow cytometric assessment of viability of lactic acid bacteria. *Applied Environmental Microbiology* **67**, 2326-2335.

Cappelier, J. M., Rossero, A., and Federighi, M. (2000) Demonstration of a protein synthesis in starved *Campylobacter jejuni* cells. *International Journal of Food Microbiology* **55**, 63-67.

Chitarra, G. S. (2003) Germination inhibitors of fungal spores: identification and mode of action. *Wageningen University, PhD-thesis, Wageningen, The Netherlands*.

Cotter, D. A., and Raper, K. B. (1966) Spore germination in *Dictyostelium discoideum*. *Proceedings of the National Academy of Science U.S* **56**, 880-887.

Davey, H. M., and Kell, D. B. (1996) Flow cytometry and cell sorting of heterogeneous microbial populations: the importance of single-cell analyses. *Microbiology Reviews* **60**, 641-696.

Dewerchin, M. A., and Van Laere, A. J. (1984) Trehalase activity and cyclic AMP content during early development of *Mucor rouxii* spores. *Journal of Bacteriology* **158**, 575-579.

Ekundayo, J. A., and Carlier, A. R. (1964) The germination of sporangiospores of *Rhizopus arrhizus*; spore swelling and germ-tube emergence. *Journal of General Microbiology* **35**, 261-269.

Freer, S. N., and Van Etten, J. L. (1978) Changes in messenger RNAs and protein synthesis during germination of *Rhizopus stolonifer* sporangiospores. *Experimental Mycology* **2**, 313-325.

Garraway, M. O., and Evans, R. C. (1984) Spore dormancy, activation, and germination. In *Fungal Nutrition and Physiology* ed. R. C. Evans, pp. 212-230. New York: John Wiley & Sons.

Griffin, D. H. (1994) *Spore dormancy and germination. In: Griffin, D. H.(Ed.), Fungal physiology*, New York: John Wiley & Sons, Inc., New York.

Halmann, M., and Keynan, A. (1962) Stages in germination of spores of *Bacillus licheniformis*. *Journal of Bacteriology* **84**, 1187-1193.

Hashimoto, C. D., Wu, C. D. R., and Blumenthal, H. J. (1972) Characterization of L-Leucine-induced germination of *Trichophyton mentagrophytes* microconidia. *Journal of Bacteriology* **112**, 967-976.

Hawker, L. E., and Madelin, M. F. (1976) The Dormant Spore. In *The Fungal Spore: Form and Function* ed. H. W.M, Vol. 1, pp. 1-72. John Willey and Sons.

Hobot, J. A., and Gull, K. (1977) The influence of glucose availability on events during germination of *Syncephalastrum racemosum* sporangiospores. *Journal of General Microbiology* **98**, 431-437.

Hunter, D. R., and Segel, I. H. (1971) Acidic and basic amino acid transport systems of *Penicillium chrysogenum*. *Archives of Biochemistry and Biophysics* **144**, 168-183.

Jenkins, D., Shultz, J., and Matin, A. (1988) Starvation induced cross protection against heat or H_2O_2 challenge in *Escherichia coli*. *Journal of Bacteriology* **170**, 3910-3914.

Kaprelyants, A. S., Gottschal, J. C., and Kell, D. B. (1993) Dormancy in nonsporulating bacteria. *FEMS Microbiology Reviews* **104**, 271-286.

Kaprelyants, A. S., and Kell, D. B. (1992) Rapid assessment of bacterial viability and vitality by Rhodamine 123 and flow cytometry. *Journal of Applied Bacteriology* **72**, 410-422.

Kaprelyants, A. S., and Kell, D. B. (1993) Dormancy in stationary-phase cultures of Micrococcus luteus: flow cytometric analysis of starvation and resuscitation. *Applied and Environmental Microbiology* **59**, 3187-3196.

Kaprelyants, A. S., Mukamolova, G. V., Davey, H. M., and Kell, D. B. (1996) Quantitative analysis of the physiological heterogeneity within starved cultures of *Micrococcus luteus* by flow cytometry and cell sorting. *Applied and Environmental Microbiology* **62**, 1311-1316.

Kell, D. B., Kaprelyants, A. S., Weichart, D. H., Harwood, C. R., and Barer, M. R. (1998) Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. *Antonie van Leeuwenhoek* **73**, 169-187.

Kell, D. B., Ryder, H. M., Kaprelyants, A. S., and Westerhoff, H. V. (1991) Quantifying heterogeneity: flow cytometry of bacterial cultures. *Antonie van Leeuwenhoek* **60**, 145-158.

Kiers, J. L. (2001) Effects of fermented soya bean on digestion, absorption and diarrhoea. *Wageningen University, PhD-thesis, Wageningen, The Netherlands*.

Kikoku, Y. (2003) Heat activation characteristics of *Talaromyces* ascospores. *Journal of Food Science* **68**, 2331-2335.

Levinson, H. S., and Hyatt, M. T. (1962) Nitrogenous compounds in germination and postgerminative development of *Bacillus megaterium* spores. *Journal of Bacteriology* **83**, 1224-1230.

Lloyd, D., and Hayes, A. J. (1995) Vigour, vitality and viability of microorganisms. *FEMS Microbiology Letters* **133**, 1-7.

Lovett, J. S. (1976) Regulation of protein metabolism during spore germination. In *The fungal spore* ed. W. M. Hess, pp. 189-242. Utah: John Wiley and Sons.

Mason, C. A., G., H., and Bryers, J. D. (1986) The death and lysis of microorganisms in environmental processes. *FEMS Microbiol. Rev.* **39**, 373-401.

Medwid, R. D. (1984a) Survival and germination of *Rhizopus oligosporus* sporangiospores. *Dissertation Abstracts International* **45**, 190pp.

Medwid, R. D. (1984b) Survival and germination of *Rhizopus oligosporus* sporangiospores. Ph.D. Thesis, Colorado State University.

Miller, L. P. (1962) L-proline and L-alanine as substitutes for higher temperatures in the germination of fungus spores. *Phytopathology* **52**, 743 (Abstr.).

Mirkes, P. E. (1974) Polysomes, rhibonucleic acid, and protein synthesis during germination of *Neurospora crassa* conidia. *Journal of Bacteriology* **117**, 196-202.

O'Connor, R., and Halvorson, H. O. (1961) L-alanine dehydrogenase: a mechanism controlling the specificity of amino acid-induced germination of *Bacillus cereus* spores. *Journal of Bacteriology* **82**, 706-713.

Pass, T., and Griffin, G. J. (1972) Exogenous carbon and nitrogen requirements for conidial germination by *Aspergillus flavus*. *Canadian Journal of Microbiology* **18**, 1453-1461.

Sarkar, P. K., Jones, L. J., Craven, G. S., Somerset, S. M., and Palmer, C. (1997) Amino acid profiles of kinema, a soybean-fermented food. *Food Chemistry* **59**, 69-75.

Shapiro, H. M. (1995) *Practical flow cytometry*, 3rd/Ed.: Wiley-Liss, Inc., New York, N.Y.

Stryer, L. (1995) Protein structure and function. In *Biochemistry* ed. L. Stryer, pp. 17-44. New York: W.H.Freeman and Company.

Sussman, A. S. (1966) *Dormancy and spore germination. In G.C. Ainsworth and A.S. Sussman (Eds.). The fungi*: New York: Academic.

Sussman, A. S. (1976) Activators of fungal germination. In *The fungal spores* ed. H. W.M., pp. 101-139. New York: John Wiley and Sons.

Sussman, A. S., and Douthit, H. A. (1973) Dormancy in microbial spores. *Annu. Rev. Plant Physiol.* **24**, 311-352.

Tanuwidjaja, L. (1985) Large scale tempe inoculum production. *Proceedings, Asian Symposium on Non-salted Soybean Fermentation. Tsukuba, Japan, July 1985. pp. 305-309. Tsukuba Science City: National Food Research Institute*.

Thevelein, J. M. (1984) Activation of trehalase by membrane-depolarizing agents in yeast vegetative cells and ascospores. *Journal of Bacteriology* **158**, 337-339.

Tripp, M. L., and Paznokas, J. L. (1981) Relationship between sporulation medium and germination ability of *Mucor racemosus* sporangiospores. *Journal of General Microbiology* **127**, 35-43.

Tripp, M. L., and Paznokas, J. L. (1982) Role of amino acids and endogenous protein in the germination of *Mucor racemosus* sporangiospores. *Journal of General Microbiology* **128**, 485-495.

Tunçel, G., Nout, M. J. R., and Rombouts, F. M. (1989) Effect of acidification on the microbiological composition and performance of tempe starter. *Food Microbiology* **6**, 37-43.

Van Etten, J. L., Bulla, L. A., J.R., and Julian, G. S. (1974) Physiological and Morphological correlation of *Rhizopus stolonifer* spore germination. *Journal of Bacteriology* **117**, 882-887.

Van Laere, A. J., Van Assche, J. A., and Carlier, A. R. (1980) Metabolism and chemical activation of *Phycomyces blakesleeanus* sporangiospores. *Experimental Mycology* **4**, 260-268.

Van Mulders, R. M., and Van Laere, A. J. (1984) Cyclic AMP, trehalase and germination of *Phycomyces blakesleeanus* spores. *Journal of General Microbiology* **130**, 541-547.

Wax, R., Freese, E., and Cashel, M. (1967) Separation of two functional roles of L-Alanine in the initiation of bacillus subtilis spore germination. *Journal of Bacteriology* **94**, 522-529.

West, T. P., Armstrong, R. L., and Magee, P. T. (1985) Changes in *Saccharomyces cerevisae* ascospore proteins during early germination. *FEMS Microbiology Letters* **28**, 255-258.

Yanagita, T. (1957) Biochemical aspects on the germination of conidiospores of *Aspergillus niger*. *Archiven der Mikrobiologie* **26**, 329-344.

Summary

Among the Asian fungal fermented foods, tempe is a soybean food of high nutritional value, fermented by the mould *Rhizopus oligosporus*. To initiate fermentation, starters containing sporangiospores of *R. oligosporus* are used. The objective of the present thesis is to better understand the factors that limit the productivity and shelf-life of tempe starters, with particular reference to the nature of dormancy and the transitions between dormant, activated, vital and viable *Rhizopus oligosporus* sporangiospores.

In Chapter 2, biomass and sporangiospore yield and viability of *Rhizopus oligosporus* as influenced by harvesting age and processing conditions, are described. By using in-vitro enzymatic digestion, fluorescence techniques with carboxyfluorescein diacetate (cFDA) and propidium iodide (PI), and culturing methods, it was observed that within 6 days of incubation on cooked rice substrate, about 8% w/w of fungal biomass was formed, containing 6.3 x 10^9 spores per gram. Of these spores, only 5-6% were viable. The remaining spores did not show damage to cytoplasmic membranes, and thus were not dead but probably dormant. Of the processing conditions, mild oven-drying had little negative effect on viability unlike pulverization which caused mechanical damage and loss of viability. The age of spores at harvest influences their storage stability. Spores harvested after 3 days suffering bigger losses during 2 and 3 months storage compared to spores harvested after 4 or 5 days.

Chapter 3 deals with dormant spores of *R. oligosporus* and their viability after activation. We aimed to assess the extent of dormancy and factors that could result in activation. Whereas heat treatments were unsuccessful, incubation for a few hours in malt extract broth (MEB) showed to be effective, with 80% of dormant spores being activated, compared with 11% in the control. Peptone and yeast extract, but not glucose played an important role in activating dormant spores. Metabolically active, swollen spores, followed by emergence of germ tubes were observed after activation in MEB for 25 min., 2 h and 4 h, respectively, at 37 °C. Simultaneously, some interesting transitions took place. Dormant spores represent 85-90% of the total spores at harvest after drying of newly made starter. Dormancy decreased to 21-32% after activation with MEB with a concomitant increase of metabolically active spores. As a result of storage, some dormancy was lost, yielding an increase of active spores from 11.2% at harvest to 28.8% after 3 months storage. Levels of active spores as measured by fluorescence microscopy correlated well with their viability as quantified by culturing. Activation of dormant spores increased their viability; levels of viable and active spores were maximum in 1 month old starter (61.7 and 75.9% of total spores, respectively) but decreased gradually, with concomitant increase of the number of dead spores (as indicated by the number of spores fluorescent with PI).

The activation effect of peptone on dormant spores of *Rhizopus oligosporus* has drawn our attention to the potential role of individual amino acids as activators (Chapter

4). L-alanine was shown to play an important role (of the same order as that of peptone) in the stimulation of germination of dormant spores (stored for 12 months). Alanine can serve as a single source of carbon as well as nitrogen for spore germination. L-proline, on the other hand, inhibits alanine uptake by blocking or congesting alanine transport into the spores, resulting in apparent low viability on agar media. L-leucine and L-isoleucine slightly favour spore germination, whereas neither L-arginine nor L-lysine have any stimulating effect. The stimulatory role of glucose was only evident in the presence of phosphate (in minimal medium). On the other hand, phosphate was found to play a facilitating role in spore germination. We observed that the ability and rate of germination of dormant and old sporangiospores of *R. oligosporus,* depend on their ability for the uptake of individual amino acids and/or glucose.

What is the relevance of all this for the functionality of tempe starters in soybean fermentation? Soybeans subjected to traditional preparation for tempe making are heavily leached because of prolonged soaking and cooking operations; germination of starter spores on such beans was found to be sub-optimal. This shows that there is scope for optimization of the bean pretreatment process in accordance with the physiological requirements of the starter.

In Chapter 3 it was shown that MEB is a good activation medium. During activation in this medium, transitions of physiological states were observed: the proportion of dormant spores decreased very rapidly, with a concomitant increase of metabolically active spores as measured by fluorescence microscopy. This study was expanded and carried out in a more sensitive manner using flow cytometry (FCM) with spores in tempe starter cultures that had been stored for 8, 10, 16, and 30 months (Chapter 5). It was observed that the shelf-life of tempe starter was not limited by the death of spores as was assumed previously, but by sublethal damage as well as dormancy of spores. Sublethally damaged and dormant spores can be resuscitated or activated respectively, into activated (live) spores using a suitable activation medium such as malt extract broth. During storage, the number of dormant and sublethally damaged spores increased while the number of metabolically active spores decreased. The longer the spores have been stored, the less dormant spores can still be activated; in contrast, the transition from sublethally damaged to activated spores did not decrease with longer storage. However, after a very long (30 months) period of storage, sublethally damaged spores could still be resuscitated but could not germinate anymore, whereas 30 months old dormant spores would not be activated anymore. Thus, besides live, dead, and dormant spores we distinguished a category of sublethally damaged spores. The shelf-life of spores in tempe starter is related to the physiological state of spores being sublethally damaged; a model of physiological state transitions of *R. oligosporus* sporangiospores was proposed.

In Chapter 6, protein profiles of dormant and activated sporangiospores of *R. oligosporus* obtained by two dimensional gel electrophoresis are studied. Results show that in dormant spores*,* there are a number of proteins or protein-like components that presumably have a function in maintaining dormancy. These proteins were degraded during the early stages of spore germination. A few newly synthesized proteins became detectable around 2 h of activation time; these could be germination-specific proteins but

may also participate in subsequent normal functions of mycelial cells. Many more new proteins were detected during the stage of germ tube emergence around 4h of activation time; these may be early stages in the development of mitochondria or cell wall components. In addition, it was observed that during the germination process, several protein spots increased; these may correspond to enzymes, ribosomes, and other proteins.

Finally, the general discussion reflects on dormancy, activation, and the transitions of physiological states. The aspect of heterogeneity of sporangiospore populations, as well as some remaining questions regarding the effect of sporulation conditions and regulation of alanine supply and demand, require further attention.

Samenvatting

Eén van de Aziatische schimmelgefermenteerde voedingsmiddelen is tempe, een door de schimmel *Rhizopus oligosporus* doorgroeid sojaproduct met hoge voedingswaarde. Voor het starten van de fermentatie wordt entmateriaal gebruikt dat de sporangiosporen van *R. oligosporus* bevat. Het doel van dit proefschrift is beter te begrijpen welke factoren de productiviteit en houdbaarheid van tempestarters beïnvloeden, met speciale aandacht voor de slaaptoestand (dormancy) en de overgangen van slaaptoestand naar geactiveerde, vitale en levende kiemkrachtige *R. oligosporus* sporangiosporen.

Hoofdstuk 2 beschrijft de invloed van het oogstmoment (leeftijd) en verwerkingsomstandigheden tijdens de productie van starter, op de schimmelmassa opbrengst en kiemkracht (viability) van *R. oligosporus*. Metingen met gebruik van in-vitro enzymatische verteringsmethoden, fluorescentiemetingen aan carboxyfluoresceinediacetaat (cFDA) en propidiumjodide (PI), en kweekmethoden, gaven aan dat na 6 dagen bebroeden op gekookte rijst ongeveer 8% m/m schimmelbiomassa en $6,3 \times 10^9$ sporangiosporen per gram waren gevormd. Van deze sporen waren echter slechts 5-6% kiemkrachtig. De overige sporen vertoonden geen schade aan het cytoplasmatisch membraan en waren dus niet dood, maar bevonden zich waarschijnlijk in slaaptoestand. Van de verwerkingsomstandigheden veroorzaakte drogen in de oven onder milde omstandigheden weinig schade, in tegenstelling tot vermalen hetgeen mechanische schade en verlies aan kiemkracht veroorzaakte. De leeftijd van de sporen bij de oogst beïnvloedt hun houdbaarheid. Jonge sporen (geoogst na 3 dagen gekweekt te zijn) leden sterker onder bewaring gedurende 2 – 3 maanden dan sporen die 4-5 dagen oud waren ten tijde van de oogst.

Hoofdstuk 3 betreft slapende sporen van *R. oligosporus* en hun kiemkracht na activering. Getracht werd de slapende sporen te kwantificeren, alsmede het effect van mogelijke activeringsbehandelingen. Terwijl hittebehandelingen niet bleken te helpen, bleek een bebroeding van enkele uren in mout extract bouillon (MEB) effectief te zijn waardoor 80% van de slapende sporen werden geactiveerd, vergeleken met 11% in de onbehandelde controleproef. Pepton en gistextract hadden een soortgelijk activerend effect, maar glucose als zodanig gaf geen activering. Na activering in MEB gedurende 25 minuten, 2 uur en 4 uur bij 37 °C werden respectievelijk metabolisch actieve sporen, gezwollen sporen en ontkiemende sporen waargenomen. Tegelijkertijd vonden interessante fysiologische veranderingen plaats. Direct na oogsten en drogen van de starter zijn 85-90% van de sporen in slaaptoestand. Activering in MEB veroorzaakt een vermindering tot 21-32% slapende sporen, gelijke tred houdend met een toename van metabolisch actieve sporen. Tijdens bewaring van tempestarter verminderde de slaaptoestand enigszins, resulterend in een toename van actieve sporen van 11,2% (oogst) tot 28,8% na 3 maanden bewaring. Hoeveelheden actieve sporen gemeten met behulp van fluorescentiemicroscopie

kwamen goed overeen met kiemkracht gemeten m.b.v. kweekmethoden. Activering van slapende sporen verhoogde hun kiemkracht; maximale kiemkracht (61,7% van de sporen) en actieve sporen (75,9%) werden gevonden in 1 maand bewaarde starter terwijl langere bewaring tot een vermindering leidde, met toename van het aantal dode sporen (op grond van meting van fluorescentie met PI).

Het activerend effect van pepton op slapende sporen van *R. oligosporus* leidde tot een verder onderzoek (hoofdstuk 4) naar de rol van afzonderlijke aminozuren. L-alanine bleek een duidelijk stimulerend effect te hebben – van dezelfde grootte orde als pepton - op de ontkieming van slapende sporen, die 12 maanden waren bewaard. L-alanine kan zowel de koolstof- als stikstofbehoefte voor ontkieming vervullen. L-proline echter, remt de opname van alanine door blokkering of verstopping van alaninetransport door de sporenwand, resulterend in lage kiemkracht in kweekmedia. L-leucine en L-isoleucine hebben een zwak stimulerend effect, terwijl L-arginine noch L-lysine enig meetbaar effect hebben. De stimulerende rol van glucose was uitsluitend aantoonbaar in de aanwezigheid van fosfaat (zoals gebruikt in minimaal medium). Daarentegen bleek fosfaat de ontkieming te bevorderen. De conclusie is dat het vermogen en de snelheid van ontkieming van slapende en langbewaarde *R. oligosporus* sporangiosporen afhangen van de opname van afzonderlijke aminozuren en/of glucose.

Men kan zich afvragen wat de praktische relevantie is voor de effectiviteit van starters in de tempefermentatie. Het bleek dat op traditionele wijze geweekte en gekookte sojabonen dermate veel nutriënten door uitloging in het water zijn kwijtgeraakt, dat de ontkieming van startersporen sub-optimaal is. Dit betekent dat de week- en kookstappen tijdens het tempebereidingsproces zouden kunnen worden verbeterd met het oog op de fysiologische behoeften van de starter.

In hoofdstuk 3 wordt getoond dat MEB een goed activeringsmiddel is. Tijdens bebroeding in MEB daalde het aantal slapende sporen snel en deze gingen over in metabolisch actieve sporen zoals gemeten m.b.v. fluorescentiemicroscopie. Dit onderzoek werd uitgebreid en nauwkeuriger uitgevoerd (hoofdstuk 5) met een flowcytometer (FCM) met sporen van uiteenlopende ouderdom (8, 10, 16 en 30 maanden bewaard). Het werd duidelijk dat de houdbaarheid van tempestarter niet zozeer wordt beperkt door het afsterven van sporen, zoals aanvankelijk verondersteld, maar door subletale beschadiging en slaaptoestand van de sporen. Subletaal beschadigde sporen kunnen worden geresusciteerd, en slapende sporen geactiveerd in geschikt activeringsmiddel zoals MEB, waarbij geactiveerde (levende) sporen ontstaan. Met toenemende bewaarduur nam het aantal slapende en subletaal beschadigde sporen toe, en het aantal metabolisch actieve sporen nam af. Des te langer de sporen zijn bewaard, des te minder slapende sporen kunnen worden geactiveerd; de mogelijkheid subletaal beschadigde sporen te resusciteren tot actieve sporen nam echter niet af in lang bewaarde sporen. Echter, na een zeer lange bewaarperiode (30 maanden) konden deze geactiveerde sporen niet meer ontkiemen, en 30 maanden bewaarde slapende sporen konden ook niet meer worden geactiveerd. Zo hebben wij in aanvulling op de categoriën dode, slapende en levende sporen, een categorie van subletaal beschadigde sporen kunnen toevoegen. De houdbaarheid van sporen in tempestarter wordt beïnvloed door de fysiologische toestand van sporen die subletaal

beschadigd raken; een model werd voorgesteld dat de overgangen tussen fysiologische stadia van *R. oligosporus* sporangiosporen aangeeft.

In hoofdstuk 6 wordt een vergelijking gemaakt van eiwitprofielen van slapende en geactiveerde sporen van *R. oligosporus*, verkregen m.b.v. 2-dimensionale gel-electroforese. In slapende sporen werden een aantal eiwitten of eiwitachtige stoffen onderscheiden die vermoedelijk een rol spelen bij het handhaven van de slaaptoestand. Deze eiwitten werden afgebroken tijdens de vroege stadia van de ontkieming. Reeds na 2 uur werd een aantal nieuwgevormde eiwitten zichtbaar; dit zouden onkiemingsgerelateerde eiwitten kunnen zijn, maar wellicht spelen ze ook in latere stadia een rol in het volgroeide mycelium. Tijdens het stadium van kiembuisontwikkeling na 4 uur activering, werden veel meer nieuwgevormde eiwitten aangetoond. Deze zouden vroege stadia kunnen vertegenwoordigen van de ontwikkeling van mitochondriën of celwandcomponenten. Tijdens het ontkiemingsproces werd ook waargenomen dat een aantal eiwitvlekken, reeds aanwezig in slapende sporen, in intensiteit toenam; deze zouden overeen kunnen komen met enzymen, ribosomale en andere eiwitten.

Tenslotte gaat de algemene discussie verder in op aspecten van de slaaptoestand, activering, en de overgangen tussen fysiologische toestanden. Resterende onderzoeksgebieden worden aangestipt, bijvoorbeeld de heterogeniteit van sporenpopulaties, de invloed van sporulatieomstandigheden en de regulering van alanine aanmaak en opname.

Tóm tắt

Trong số thực phẩm lên men ở Châu Á, tempeh là thức ăn làm từ đậu nành có giá trị dinh dưỡng cao, lên men từ nấm mốc *Rhizopus oligosporus* (R.o). Để khởi đầu sự lên men, mốc bột bào tử (MBT) của mốc này được sử dụng. Mục đích của luận án là để hiểu rõ hơn những nhân tố giới hạn đến hiệu suất và tuổi thọ của MBT làm tempeh, đặc biệt liên hệ đến tính tự nhiên của miên trạng (dormancy) và sự chuyển vị (transition) giữa các bào tử: miên trạng (dormant spores), hoạt hoá (activated spores), sống (vital spores), có khả năng sống (viable spores) của bào tử nấm mốc R.o.

Chương 2 mô tả sinh khối, sản lượng và khả năng sống của mấm mốc R.o bị ảnh hưởng bởi thời gian thu hoạch và điều kiện chế biến. Bởi sử dụng kỹ thuật phân hoá tố tiêu hoá (trong ống nghiệm) và kỹ thuật huỳnh quang với chất đánh dấu huỳnh quang xanh chỉ thị sống carboxyfluorescein diacetate (cFDA) và chất đánh dấu huỳnh quang đỏ chỉ thị chết propidium iodide (PI) và các phương pháp nuôi cấy, kết quả quan sát được trong vòng 6 ngày ủ trên cơ chất gạo cho thấy vào khoảng 8% (tính theo trọng lượng) sinh khối nấm được tạo thành chứa đựng 6.3×10^9 bào tử/g. Nhưng chỉ có 5-6% bào tử này có khả năng sống. Những bào tử còn lại không bị tổn thương màng tế bào chất, và như thế là chúng không chết mà có lẽ là miên trạng. Về điều kiện chế biến, sấy nhẹ (nhiệt độ không cao) có ít ảnh hưởng lên khả năng sống, nhưng công đoạn xay gây ra tổn thương cơ học và làm mất khả năng sống. Tuổi của bào tử lúc thu hoạch có ảnh hưởng đến sự ổn định trong bảo quản. Bào tử thu hoạch sau 3 ngày chịu sự mất khả năng sống lớn hơn trong suốt 2 và 3 tháng bảo quản so với bào tử thu hoạch sau 4 và 5 ngày.

Chương 3 đề cập về bào tử miên trạng của nấm mốc R.o và khả năng sống của chúng sau khi hoạt hoá. Mục đích là đánh giá tính miên trạng và những nhân tố mà có thể gây ra trong sự hoạt hoá. Trong khi sử lý nhiệt là không thành công, ủ vài giờ trong malt extract broth (MEB) đã cho thấy là rất hiệu quả với 80% bào tử miên trạng được hoạt hoá so với 11% ở đối chứng. Peptone và yeast extract nhưng không phải đường glu-cô (glucose) đóng vai trò quan trọng trong sự hoạt hoá bào tử miên trạng. Hoạt động trao đổi chất (metabolic active), bào tử phồng lên, tiếp theo bởi sự nổi lên của ống mầm theo tuần tự 25 phút, 2 giờ và 4 giờ được nhận thấy sau khi hoạt hoá trong MEB ở 37 độ C. Đồng thời một vài sự chuyển vị dã diễn ra. Ở MBT mới sản xuất, bào tử miên trạng đại diện 85-90% tổng số tại thời điểm thu hoạch và sau khi sấy. Chúng giảm xuống đến 21-32% sau khi hoạt hoá trong MEB với sự tăng lên đồng thời của bào tử hoạt động trao đổi chất. Như một kết quả của quá trình bảo quản, một ít bào tử miên trạng bị mất gây ra sự tăng lên của bào tử tự (tự nhiên không cần sự hoạt hoá) phản ứng trao đổi chất (active spores) từ 11.2% lúc thu hoạch đến 28.8% sau 3 tháng bảo quản. Mức độ của bào tử active spores định lượng bởi kính hiển vi huỳnh quang rất tương xứng với khả năng sống của chúng khi định lượng bởi sự nuôi cấy (culturing). Hoạt hoá của bào tử miên trạng đã làm tăng lên khả năng sống của chúng; mức độ của bào tử sống và bào tử active spores đạt tối đa ở MBT 1 tháng bảo quản (tương ứng là 61.7 và 75.9% của tổng số bào tử) nhưng giảm nhanh với sự tăng lên đồng thời của số bào tử chết (được chỉ thị bởi số lượng của bào tử phát huỳnh quang đỏ với PI).

Ảnh hưởng hoạt hoá của peptone lên bào tử miên trạng của R.o đã thu hút sự chú ý của chúng tôi đến vai trò tiềm năng của axít amin riêng lẻ như chất hoạt hoá. Trong Chương 4, axít-amin đã chứng tỏ đóng vai trò quan trọng trong sự hoạt hoá và nảy mầm của bào tử R.o trong MBT tempeh (12 tháng bảo quản) mà L-alanine đã đóng vai trò quan trọng (như peptone) trong việc kích thích sự nảy mầm của bào tử miên trạng. L-alanine được nấm này dùng như một nguồn cac-bon cũng như nguồn đạm cho sự nảy mầm. Trái lại, L-proline ức chế sự hấp thu alanine bởi làm nghẽn sự vận chuyển chất này vào bên trong bào tử mà kết quả thấy rõ khả năng sống thấp trên môi trường agar. L-leucine và L-isoleucine có hỗ trợ một ít cho sự nảy mầm, trong khi đó cả L-arginine lẫn L-lysine không có tác động kích thích nào. Vai trò kích thích của đường glu-cô chỉ thể hiện khi có sự hiện diện của phốt-phát (phosphate) (có trong môi trường tối thiểu). Mặt khác, phosphate đóng vai trò tạo điều kiện cho bào tử nảy mầm. Khả năng và tốc độ nảy mầm của bào tử miên trạng phụ thuộc vào khả năng hấp thu axít-amin và /hoặc đường glu-cô của chúng.

Sự liên hệ giữa kết quả tìm thấy và chức năng của MBT tempeh như thế nào trong lên men đậu nành? Đậu nành trải qua sự chuẩn bị để làm tempeh theo cách truyền thống bị giảm chất lượng bởi sự ngâm và nấu kéo dài; vì thế sự nảy mầm của MBT là dưới mức tối ưu. Điều này cho thấy cần có mục tiêu cho sự tối ưu hoá tiến trình sử lý đậu trong sự hài hoà với nhu cầu sinh lý của MBT.

Chương 3 chỉ ra rằng malt extract broth là một môi trường hoạt hoá tốt. Sự chuyển vị của bào tử được nhận thấy khi hoạt hoá trong môi trường này, sự chuyển vị của những trạng thái sinh lý đã được quan sát: số lượng bào tử miên trạng giảm xuống rất nhanh đồng thời với sự tăng lên số lượng của bào tử active spores khi được quan sát bởi kính hiển vi huỳnh quang. Nghiên cứu này được mở rộng và tiến hành theo một phương cách nhạy cảm hơn là sử dụng phương pháp phân tích tế bào (flow cytometry) cho những bào tử trong MBT mà đã bảo quản trong thời gian 8, 10, 16, và 30 tháng (Chương 5). Kết quả chỉ rằng tuổi thọ của MBT tempeh là không bị giới hạn bởi sự chết của bào tử như những giả thuyết trước đây mà do bởi tổn thương dưới mức gây chết (sublethal damage) và tính miên trạng của bào tử. Những bào tử sublethal damage và miên trạng có thể được làm tỉnh lại (resuscitated) hoặc được hoạt hoá (activated) (theo tuần tự) thành bào tử hoạt hoá (activated spores) bởi việc sử dụng môi trường phù hợp như là MEB. Trong suốt quá trình bảo quản số lượng bào tử miên trạng và bào tử tổn thương tăng lên trong khi số lượng bào tử active spores giảm. Bảo quản bào tử càng lâu, càng ít bào tử miên trạng có thể hãy còn hoạt hoá được; ngược lại sự chuyển vị từ bào tử tổn thương thành bào tử hoạt hoá là không giảm. Tuy nhiên, sau một giai đoạn dài bảo quản (30 tháng) bào tử tổn thương có thể hãy còn được làm tỉnh lại nhưng không thể nảy mầm được nữa. Vì thế ngoài bào tử: sống, chết và miên trạng chúng tôi đã phân biệt ra thêm một loại: bào tử tổn thương (sublethally damaged spores). Tuổi thọ của bào tử trong MBT có liên hệ đến trạng thái sinh lý của bào tử bị tổn thương; một mô hình của sự chuyển vị của các trạng thái sinh lý của bào tử R.o được đề xuất.

Chương 6, nghiên cứu và mô tả sơ lược protein của bào tử miên trạng (dormant spores) và bào tử hoạt hoá (activated spores) thu được bởi phương pháp điện di hai chiều. Kết quả chỉ rằng ở bào tử miên trạng có một số protein hoặc những thể giống protein mà có lẻ chúng có chức năng trong việc duy trì miên trạng. Những protein này được phân giải

trong suốt giai đoạn đầu của bào tử nảy mầm. Một vài protein mới được tổng hợp được phát hiện trong khoảng 2 giờ hoạt hoá; có thể là protein riêng biệt cho sự nảy mầm nhưng cũng có thể tham gia vào chức năng bình thường của tế bào sợi nấm. Protein mới được nhận thấy nhiều hơn trong suốt giai đoạn nối lên của ống mầm (germ-tube) khoảng 4 giờ hoạt hoá; những dự kiện này có thể là giai đoạn đầu trong sự phát triển những phần hợp thành của ty thể hoặc vách tế bào. Hơn nữa kết quả chỉ ra rằng trong suốt tiến trình nảy mầm, nhiều điểm protein tăng lên cường độ, chúng có thể là phân hoá tố (enzymes), thể tổng hợp protein (ribosomes) hoặc những protein khác.

Cuối cùng thảo luận chung phản ánh về tính miên trạng (dormancy) sự hoạt hoá (activation) và sự chuyển vị (transition) của các trạng thái sinh lý của bào tử. Phương diện không đồng nhất (heterogeneity) của bào tử cũng như vài câu hỏi còn lại đề cập đến ảnh hưởng của điều kiện môi trường hình thành bào tử, sự điều tiết việc cung cấp và nhu cầu về alanine của nấm mốc này yêu cầu nên có sự chú ý nhiều hơn.

Curriculum Vitae

Nguyen Van Thanh was born in Dong Thap province, Vietnam on April 15th, 1965. He is the fourth child of Mrs. Dang Thi To and Mr. Nguyen Van Chinh. After primary, secondary and high school education in Dong Thap, he entered the Department of Biology and Chemistry, Can Tho University (CTU) in 1982, and received his Bachelor degree in July 1986. From 1986 to 1997, he worked as a researcher in Biotechnology Research and Development Institute, CTU. In July 1998 he held a scholarship from NUFFIC (MHO-7 project) to pursue his studying in Wageningen University, The Netherlands, and obtained his Master degree in Biotechnology in January 2000. In 2001 he continued to obtain a scholarship from NUFFIC (MHO-7 project) to follow the sandwich PhD program at the Laboratory of Food Microbiology, Department of Agrotechnology and Food Sciences, Wageningen University. After completing his PhD degree he will continue working his task at the Biotechnology Research and Development Institute (BiRDI), Can Tho University (CTU), Vietnam.

List of Publications

Thanh, N. V., and Nout, M. J. R. (2002) *Rhizopus oligosporus* biomass, sporangiospore yield and viability as influenced by harvesting age and processing conditions. *Food Microbiology* **19**, 91-96.

Thanh, N. V., and Nout, M. J. R. (2004) Dormancy, activation and viability of *Rhizopus oligosporus* sporangiospores. *International Journal of Food Microbiology* **92**, 171-179.

Thanh, N.V., Rombouts, F.M., and Nout, M.J.R. Effect of individual amino acids and glucose on activation and germination of *Rhizopus oligosporus* sporangiospores in tempe starter. (*Submitted for publication*)

Thanh, N.V., Rombouts, F.M., and Nout, M.J.R. Characterization of viability and physiological state transitions of *Rhizopus oligosporus* sporangiospores in tempe starter culture by flow cytometry. (*Submitted for publication*)

Han, B. Z., Kuijpers, A.F.A., Thanh, N.V., and Nout, M.J.R. (2004) Mucoraceous moulds involved in the commercial fermentation of Sufu Pehtze. *Antonie van Leeuwenhoek* **85**: 253–257.

Training and supervision completed

Period	Place	Activity	Time, credit point
February, 2000	Wageningen University (WU)	Field work to Mushroom farms with Food Microbiology Group	1 day, 0.2cp
March, 2000	WU	Food Fermentation (attending the course)	3 weeks, 3cp
April, 2001	Cantho University (CTU), NUFFIC MHO project	Food Fermentation (taking part of teaching)	2 weeks, 2cp
June, 2001	WU	Field work to CBS with Food Microbiology Group	1 day, 0.2cp
March, 2002	CTU, NUFFIC MHO project	Workshop on Curriculum MSc Biotechnology	1 day, 0.2cp
April, 2002	CTU, NUFFIC MHO project	Food Fermentation (taking part of teaching)	2 weeks, 2cp
August 2002	CTU, NUFFIC MHO project	Workshop on financial management and auditing	1 week, 1cp
October, 2002	CTU, NUFFIC MHO project	Food Enzymology (attending the course)	2 weeks, 2cp
October, 2003	Centraalbureau voor Schimmelcultures, Utrecht (CBS)	Attended the course "Introduction to food-and airborne fungi"	1 week, 1cp
October, 2003	WU	VLAG course "Food perception and food preference"	1 week, 1cp
June, 2004	CTU, NUFFIC MHO project	MHO final Workshop at CTU and BiRDI	1 week, 1cp
Preparation of research proposal (PhD)			4cp
Preparation of research proposal (IFS)			2cp
Co-supervising of 1 MSc-biotechnology student doing his 6 months thesis project (in 2002)			1 month
2nd secretary of MHO7 project (2000-2004)			10 weeks

Acknowledgements

Cover: Several pictures representing major aspects of this thesis: *Rhizopus oligosporus* (sporangiospores in sporangium); fluorescing germ tubes and swollen spores after activation (4 h); Gel of protein profiles of activated (4 h) spores; FCM analysis: dot plots of green and red fluorescing spores of activated (1 h) suspension; and rice-based tempe starter (background).