SOLID-SUBSTRATE FERMENTATION OF SOYA BEANS TO TEMPE

Process innovations and product characteristics

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SOLID-SUBSTRATE FERMENTATION OF SOYA BEANS TO TEMPE
Process innovations and product characteristics

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Cover photograph: The Rotating Drum Reactor (Tekenlokatie de Dreyen)

STELLINGEN

1) Het door Raghava Rao *et al.* opgestelde model voor het ontwerpen van tray reactoren onderschat de invloed van de temperatuur tijdens SSF.

Raghava Rao, K.S.M.S.R.; Gowthaman, M.K.; Ghildyal, N.P.; Karanth, N.G. 1993 A Mathematical Model for Solid State Fermentation in Tray Bioreactors Bioprocess Engineering, 8, 255-262

2) De functie van alanine en proline, bij de ontkieming van *Rhizopus oligosporus* sporangiosporen, zijn door Medwid en Grant sterk overschat, ze dienen namelijk alleen als N-bron.

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

3) Het werkvolume dat door Smith en Aidoo wordt genoemd als belangrijkste nadeel van roterende trommel fermentoren weegt niet op tegen de voordelen van zo'n systeem.

Smith, J.E., Aidoo, K.E. 1988 Growth of fungi on solid substrates In: Physiology of industrial fungi. Ed:D.R. Berry. Blackwell Scientific Publications , Oxford 1988, 285 p.

4) De door Myliwadarso *et al.* gevonden concentratieverschillen in organische zuren in weekwater zijn gebaseerd op metingen rond de detectielimiet en derhalve onbetrouwbaar.

Mulyowidarso, R.K.; Fleet, G.H.; Buckle, K.A. 1991 Changes in the Concentration of Organic Acids During the Soaking of Soybeans for Tempe Production. International Journal of Food Science and Technology, 26, 607-614

5) De omschrijving van Klus *et al.* "tempe producerende bacteriën" geeft een verkeerde indruk van de belangrijkste flora tijdens de produktie van tempe.

Klus, K.; Borger-Papendorf, G.; Barz, W. 1993 Formation of 6,7,4'-trihydroxyisoflavone (factor 2) from soybean seed isoflavones by bacteria isolated from tempe. Phytochemistry 34, 979-981

6) Ikasari en Mitchell concluderen ten onrechte dat roterende trommel reactoren niet geschikt zijn voor protease productie.

L. Ikasari en D.A. Mitchell Protease production by Rhizopus oligosporus in solid state fermentation World Journal of Microbiology & Biotechnology 10, 320-324 1994

7) De door De Groote gekozen titel:"*Tempehproduktie in roterende* trommelreactor niet beter", geeft aan dat journalisten problemen hebben met het trekken van goed onderbouwde conclusies.

(Voedingsmiddelentechnologie 14 juli 1994 nr 14/15 p 29)

8) De veronderstelling van Ariffin *et al.* dat de breekkracht van tempe een goede maat is voor de hoeveelheid schimmelmycelium is onjuist.

R. Ariffin C. Apostolopoulos, A. Graffham, D. MacDougall, J.D. Owens (1994) Assessment of hyphal binding in tempe. Letters in Applied Microbiology, 18, 32-34

9) Microbiologen maken bij het berekenen van het kiemgetal misbruik van de wiskunde, door te veronderstellen dat het gemiddelde kiemgetal gelijk is aan de som van de log(kiemgetallen) gedeeld door het aantal log(kiemgetallen).

10) Door de intrede van software-pakketen met muisbesturing in het wetenschappelijk onderzoek is de functie van de muis als proefdier veranderd van lijden tot leiden.

11) De korting van AIO-salarissen ten bate van specifiek AIO-onderwijs is een vorm van oplichting.

13) Golf is een bijzonder competitieve sport, een speler met een handicap kan een professional verslaan.

Stellingen behorende bij het proefschrift:
Solid-Substrate Fermentation of Soya Beans to Tempe
Process innovations and product characteristics
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Wageningen
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ABSTRACT

De Reu J.C. (1995) *Solid-Substrate Fermentation of soya beans to tempe: process innovations and product characteristics.* Ph.D.-thesis, Wageningen Agricultural University, The Netherlands (154 p, English and Dutch summaries)

Solid-substrate fermentations (SSF) are restricted by heat- and mass transfer limitations, which might result in unfavourable growth conditions. One way to prevent such conditions is by agitation of the substrate. In this study a Rotating Drum Reactor (RDR) was designed for the fermentation of soya beans with *Rhizopus oligosporus*. The aim of the study was to develop a process for the controlled fermentation of soya beans into a microbiologically safe and protein rich product.

The reactor and the measurement and control system enable an automatic control of the process. The most important process parameters are: rotation speed, substrate temperature, rotation frequency and the relative humidity.

A major disadvantage of RDR that has been cited in literature is the sensitivity of micro-organisms towards agitation. In our study we have shown that the fungal activity in a discontinuous RDR remained high up to 70 hours while in the traditional non-agitated systems fungal activity decreases already after 36 hours of incubation. During fermentation several enzymes, viz. lipases, proteases, phytases and carbohydrases are formed by *R. oligosporus*. Due to the enzymatic activity, changes in the chemical composition of soya beans were observed. At increasing temperatures a decrease in the total fat content was observed. It was also observed that the level of free fatty acids was lower than expected based on the decrease in glyceride bound fatty acids. This might be explained by the fact that *R. oligosporus* used fatty acids as a source of carbon. It was also observed that the firmness of the product in the RDR was significantly less compared to the non-agitated samples. In the RDR we observed increased activities of exo-proteases and glycosidases compared with the traditional non-agitated systems. It was shown that lactic acid (> 0.05 % w/v, pH 4.2) delayed the germination of *Rhizopus oligosporus*.

There might be nutritional benefits from the the fermentation step in tempe manufacture through hydrolysis of soya bean cell walls, fats and proteins, making the product more easy to digest.

Key words: tempe, solid-substrate fermentation, *Rhizopus oligosporus*

VOORWOORD

Op deze plaats, wil ik iedereen bedanken die hebben bijgedragen aan het onderzoek en de totstandkoming van dit proefschrift. Zonder iemand te kort te doen wil ik op deze plaats enkele mensen noemen.

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CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

INTRODUCTION

A new challenge for an ancient technology? Tempe, a traditional Indonesian food obtained by fermenting soya beans with moulds is a cheap source of edible protein with interesting nutritional aspects. The small-scale traditional production process is labour intensive, uncontrolled and results in a variable final product quality.

Tempe might be used as an intermediate product for various food formulations, but to achieve that goal certain hurdles need to be cleared. Primarily, the design of a bioreactor which enables the production of a homogenous, nutritious and microbiologically safe product on an industrial scale.

AIM AND OUTLINE OF THIS THESIS

The aim of this study is to design, test and compare several types of laboratory-scale reactors and evaluate their suitability for solid-substrate fermentation. The major difficulties in solid-substrate fermentation are related to heat and mass transfer limitations. A better process control and scale-up can be accomplished by reducing or avoiding these problems. The widely practised fermentation of soya beans with *Rhizopus oligosporus* was used as the model process during this study.

In Chapter 2 an introduction into solid-substrate fermentation and the tempe process is given. In Chapter 3 a rotating drum reactor is described and the possibilities of using this type of reactor for better temperature control during SSF are discussed. An aerated packed-bed reactor and a model to predict the temperature pattern during the tempe process are described in Chapter 4. The model includes both microbiological and physical aspects of solid-substrate fermentations and could be used to optimize solid-substrate fermentations.

Based on the traditional static fermentation, several studies were carried out to characterize and optimize the traditional tempe process. In Chapter 5 the relation between the methods of soaking soya beans and the germination of *R. oligosporus* sporangiospores is described. Germination and growth of *R. oligosporus* as a function of the composition of the gas atmosphere is described in Chapter 6. In

Chapter 7 changes in the fatty acids during the tempe process are described for different temperatures and mould strains. In Chapter 8 changes in the protein fraction during the fermentation of soya beans with *R. oligosporus* are shown for both the traditional and rotating drum fermentations. In Chapter 9 the effect of polysaccharidases on the consistency and the non-starch polysaccharides content of soya beans during the fermentation is described. The general discussion is presented in Chapter 10.

CHAPTER 2

SOLID-SUBSTRATE FERMENTATION

DEFINITION AND PROPERTIES

Solid-substrate fermentations (SSF) are difficult to define precisely. Moo-Young and co-workers (1983) proposed the term 'solid-substrate fermentation' for all those processes which utilize water-insoluble materials for microbial growth in the absence of free water. In this definition water-soluble materials are excluded but they might be used in SSF as well. With increasing amounts of free water, solid-substrate fermentations progress from solid-state fermentation through slurry fermentations to fermentations of suspensions of solid particles. Examples of solid-substrate fermentations, their substrates and products are shown in Tables 1A and 1B.

Several advantages of solid-substrate fermentations (SSF) over submerged-liquid cultures (SLC) reported by Hesseltine (1977b), Cannel and Moo-Young (1980) and Mudgett (1986) include the following.

a) The medium is often quite simple, consisting of a cheap unrefined agricultural product which may contain all the nutrients necessary for microbial growth. This means that the substrate in general may require less enrichment (Cannel and Moo-Young, 1980).

b) The restricted availability of water may help to select against undesirable contaminants (Hesseltine, 1977b), especially bacteria and yeasts, although contamination by other fungi may be a problem. The less stringent need for aseptic procedures in SSF compared with SLC makes SSF more practical and suitable for low technology applications, where the workers are unskilled (Mitchell, 1992).

c) The concentrated nature of the substrate enables the use of smaller reactors in SSF compared to SLC to contain the same amount of substrate (Hesseltine, 1977b). Smaller reactor volumes result in lower capital and operating costs (Cannel and Moo-Young, 1980; Kargi *et al.*, 1985; Kumar and Lonsane, 1987b).

d) Forced aeration is reported to be easier in SSF than in SLC because the interparticle spaces allow transfer of fresh air to thin films of water at the substrate surfaces. These thin films can have a high surface area, allowing rapid oxygen transfer (Cannel and Moo-Young, 1980; Mudgett, 1980; Bajracharya and Mudgett, 1980).

e) Downstream processing requires less extraction solvent resulting in less waste (Hesseltine, 1977b).

Disadvantages of SSF compared to SLC include the following.

a) SSF is restricted to microorganisms which can grow at reduced moisture levels, namely fungi, some yeasts and streptomyces, and therefore the range of possible processes and products is more limited than with SLC (Hesseltine, 1977b; Cannel and Moo-Young, 1980).

b) Removal of metabolic heat generated during growth may be a problem, especially at large scale (Trevelyan, 1974; Hesseltine, 1977b; Aidoo *et al.*, 1982; Moo Young *et al.*, 1983; Aidoo *et al.*, 1984; Laukevics *et al.*, 1984).

c) The solid nature of the substrate causes problems in the monitoring of process parameters. Probes developed for SLC are often unsuitable for SSF. In addition, it is very difficult to ensure even distribution of any substances added during the process, so effective control of parameters such as pH, moisture content and substrate concentrations is a major problem. Biomass cannot be measured in-situ during SSF because fungi penetrate into and bind themselves tightly to the substrate particles (Mitchell, 1992).

d) Mass transfer in the solid phase is limited to diffusion (Cannel and Moo-Young, 1980).

e) Presently, important basic scientific and engineering parameters are still poorly characterized (Mitchell, 1992).

f) Cultivation times are often longer in SSF compared to SLC due to the lower specific growth rates of the microorganisms (Tengerdy *et al.*, 1983; Ghai *et al.*, 1983; Ramos-Valdivia *et al.*, 1983).

g) Extracts containing products obtained by leaching of fermented solids are often viscous in nature. This high viscosity restricts vacuum concentration of the crude extract (Mitchell, 1992).

DESIGN OF SSF BIOREACTORS

As with submerged liquid cultures, reactor design is an important factor determining the efficiency of SSF processes. Unfortunately, the design of solid-substrate bioreactors has, to date, been entirely empirical. Extensive mechanization and automation have been reported in Japan, although the exact situation is not clear as very little information on industrial SSF-bioreactors is available in the literature (Lonsane *et al.*, 1985).

Table 1A Non-agitated bioreactors, substrates and microorganisms used for solid-substrate fermentation and their products

Reactor type	Size	Substrate	Microorganism	Product	Reference
Heap		Cassava flour		Gari	Essers *et al.* (1992)
		Solid-Waste		Compost	Smith and Aidoo (1988)
Tray		Wheat bran	*Aspergillus niger*	Amyloglucosidase	Ghildyal *et al.* (1985)
		Soya beans	*Rhizopus* spp.	Tempe	Martinelli and Hesseltine (1964)
		Cassava waste	*Rhizopus oryzae*	Edible protein	Tanuwidjaja (1989)
		Cellulose	*Chaetomium cellulolyticum*	Edible protein	Hecht *et al.* (1985)
		Cassava	*Rhizopus oryzae*	Edible protein	Daubresse *et al.* (1987)
Packed Bed	0.08 l.	Cassava wet meal	*Aspergillus niger*		Raimbault and Alazard (1980)
	2 l.	Wheat bran	*Aspergillus oryzae*	Amylase	Sato *et al.* (1982)
	NP	Rice	*Aspergillus oryzae*	Enzymes	Narahara *et al.* (1982)
	0.08 l.	Sugar beet pulp	*Aspergillus phoenicis*	ß-glucosidase	Deschamps and Huet (1984a)
	6.3 l.	Starchy materials	*Saccharomyces cerevisae*	Ethanol	Sato *et al.* (1985)
			Aspergillus saitoi		
	0.5 l.	Buckwheat seeds	*Penicillium roqueforti*	Spores Inocula	Larroche and Gros (1986)
	4 l.	Fodder beets	*Saccharomyces cerevisae*	Ethanol	Gibbons and Westby (1986)
	50 l.	Corn grits	*Saccharomyces cerevisae*	Ethanol	Sato *et al.* (1988)
	126 l.	Sugar beet pulp	*Trichoderma viride*	Edible protein	Durand *et al.* (1988)
	1 l.	Cassava wet meal	*Aspergillus niger*		Saucedo-Castaneda *et al.* (1990)
		Mustard seed meal	*Aspergillus clavatus*	Detoxification	Smits *et al.* (1992)
	11 l.	Basal liquid medium	*Schwanniomyces castelli*	Ethanol	Saucedo-Castaneda *et al.* (1992)

NP, not presented

Table 1B. Agitated bioreactors, substrates and microorganisms used for solid-substrate fermentation and their products

Reactor type	Size	Substrate	Microorganism	Product	Reference
Rotating Drum	ϕ 37cm	Wheat	*Aspergillus ochraceus*	Ochratoxin A	Lindenfelser and Ciegler (1975)
	2 l.	Wheat bran	*Aspergillus niger*	Enzymes	Nishio *et al.* (1979)
	4.8 l.	Wheat bran	*Aspergillus awamori*	Enzymes	Silman (1980)
	60 l.	Sugar cane	*Saccharomyces oviformis*	Ethanol	Er-El *et al.* (1981)
	30 l.	Buckwheat seeds	*Penicillium roqueforti*	Spores inoculum	Larroche and Gros (1986)
	5.7 l.	Sorghum	*Saccharomyces cerevisae*	Ethanol	Weiland and Scholz (1990)
	4.7 l.	Soya beans	*Rhizopus oligosporus*	Tempe	De Reu *et al.* (1993)
Fluidized bed		3 kg yeast	*Saccharomyces cerevisae*	Ethanol	Moebus and Teuber (1982)
		Wheat bran	*Saccharomyces cerevisae*		Tanaka (1986)
	1.8 l.	Potatoes + medium	*Saccharomyces cerevisae*	Proteins	Hong *et al.* (1989)
Rocking	1.3 l.	Corn grit	*Rhizopus oligosporus*		Sargantanis *et al.* (1993)
	1.3 l.	Corn grit	*Rhizopus oligosporus*		Ryoo *et al.* (1991)
Agitated tank	60 l.	Sugar beet pulp	*Aspergillus phoenicis*	ß-glucosidase	Deschamps and Huet (1984b)
	3.7 m^3	Sugar beet pulp	*Trichoderma viride*	Edible protein	Durand and Chereau (1988)
	40 l.	Apple pomace	*Saccharomyces cerevisae*	Ethanol	Ngadi and Correira (1992)
	126 m^3	Sugar beet pulp	*Aspergillus tamarii*	Protein enrichment	Xue *et al.* (1992)
Continuous	83 l.	Fodder beets	*Saccharomyces cerevisae*	Ethanol	Gibbons *et al.* (1984)
	12 l.	Animal waste and corn	Lactobacilli and Yeasts	Feed	Hrubant *et al.* (1989)

Considerations important in the design of SSF bioreactors include:

a) Whether mixing is required, and how it should be achieved.
b) The optimum degree of aeration.
c) The rate at which heat needs to be removed.
d) The measurement and control of process parameters.
e) Solids handling.
f) Sterilization and the prevention of recontamination.
g) The mode of processing.
h) The scale-up criteria.

ad a) Mixing

SSF processes can be divided into three groups based on the mixing regime that is used. Viz. static, periodically agitated and continuously agitated. Agitation facilitates the maintenance of homogeneous conditions within the bioreactor, especially with respect to the temperature and the gaseous environment (Hesseltine, 1977a). The lack of agitation determines the depth of the substrate that can be used. In static SSF, temperature gradients of up to 3°C per cm have been observed (Rathbun and Shuler, 1983). In addition, if forced aeration is not used for static SSF, the oxygen in the interparticle spaces decreases to limiting levels (Rao *et al.*, 1993).

However, the mixing of solid-substrate might also have deleterious effects, including adverse effects on substrate porosity, disruption of the attachment of microorganisms to the substrates, and damage of the fungal mycelia due to shear forces caused by abrasion between particles. Shear forces in SSF are complex and are therefore difficult to characterize. For example, aerial hyphae are crushed onto the surface of the substrate, reportedly resulting in an inhibition of sporulation (Bajracharya and Mudgett, 1979; Silman, 1980).

The damage of the mycelia depends strongly on the rotation frequency and speed, e.g. 3 revolutions per day resulted in scarcely injured mycelium and good sporulation occurred, while a frequency of 3 revolutions per 5 hours damaged the mycelium and reduced the spore content (Larroche and Gros, 1986b). In other cases, such as the protein enrichment of solid substrates, inhibition of sporulation is actually advantageous since sporulation makes the product less attractive.

ad b) Aeration

Since most SSF processes involve aerobic microorganisms, the transfer of oxygen to the biomass at the surfaces of solid particles is of prime importance. Aeration

can also play a role in the removal of metabolic heat and of gaseous and other volatile metabolites from the interparticle spaces and the bioreactor headspace. There is little information available concerning the mechanism and efficiency of oxygen transfer from the gas phase to the microorganism in SSF. However, five steps are potentially important in the transfer process:

1) Transfer of oxygen into interparticle spaces.
2) Diffusion of oxygen across stagnant gas films at the substrate surface.
3) Transfer across the interface into the liquid film at the substrate surface.
4) Uptake of oxygen by the microorganism, either directly from the stagnant gas film or from the liquid film at the substrate surface.
5) Diffusion of oxygen into substrate particle itself (intra-particle diffusion)

Of these, only the first two can be influenced by the reactor design and operation.

ad c) Heat removal

SSF processes are characterized by the generation of large quantities of heat (Lonsane, 1985). The rate at which heat needs to be removed depends on the metabolic activity of the microorganisms and the quantity of substrate in the bioreactor. Unfortunately the solid nature of the substrate and its low moisture content lead to very low heat transfer rates in SSF (Mitchell *et al.*, 1992).

The various strategies for heat removal can be summarized as follows:

a) Forced aeration with moist air to remove heat by conduction.
b) Forced aeration with dry air to remove heat by evaporation.
c) Cooling the external surface of the bioreactor.
d) Situation of the bioreactor in a temperature controlled room or water bath.

ad d) Measurement and control of process parameters

In non-agitated systems, process parameters will almost certainly vary at different locations within the bioreactor, making it virtually impossible to obtain a representative sample.

On-line pH measurement in SSF is difficult to achieve. In most cases, adequate contact cannot be assured between standard glass-bulb electrodes and the substrate. Even with flat-ended electrodes, constant contact with the substrate cannot be ensured especially if the substrate is agitated. Good buffering capacity of some of the substrates used in SSF helps in eliminating the need for pH control during fermentation (Chahal, 1983).

Water is used in only limited amounts in SSF systems (Lonsane *et al.*, 1992), and when available in lower or higher quantities than the optimum values,

the process productivity is significantly affected (Lonsane *et al.*, 1985). The water activity (a_w) in the medium, can be measured by determining the equilibrium relative humidity (RH). In the a_w range where SSF is operated i.e., 0.80-0.99, the use of traditional RH-probes with capacitive elements is limited due to the danger of condensation and consequently saturation. Gervais (1989) improved such probes by ventilation of the capacitive elements. Möller *et al.* (1987) reported humidity measurement in gas/solid fluidized beds with the near infrared technique.

In laboratory-scale fermenters, oxygen uptake and carbon dioxide evolution are measured directly with gas analyzers or indirectly by gas chromatography for head space analysis. Temperatures are easy to measure with a variety of probes such as thermocouples, thermistors, Pt elements or infrared techniques.

ad e) Solids handling

As shown in Tables 1A and 1B there is a wide range of raw materials that are used in SSF. Solids in SSF vary widely in shape, size, abrasiveness, shear-sensitivity and fluidity. Each of these factors should be considered when selecting appropriate machinery for handling and transport of solids in industrial scale SSF processes.

ad f) Sterilization and the prevention of contamination

Although SSF is often carried out non-aseptically, in particular cases it may be necessary to sterilize the substrate, the bioreactor, or the air used for forced aeration. Sterilization of substrates by steam within the bioreactor has been attempted, but even cooking of the substrate is difficult to achieve at large scales (Daubresse *et al.*, 1987) unless provision is made for adequate mixing. Substrates are often cooked separately from the bioreactor. It is often not practical to steam sterilize the bioreactor and associated equipment, and chemical sterilization or disinfection is often applied.

Contamination can be prevented quite easily during the cultivation period itself in the case of closed bioreactors. In open bioreactors such as tray bioreactors (Table 1A) one must rely on dense and vigorous inocula (Lonsane *et al.*, 1982) and relative conditions such as an acidic pH (Nout and Rombouts, 1990) and a suitable low water activity.

ad g) The mode of processing

Most SSF applications involve batch cultures. Effective implementation of fed batch or continuous techniques at a large scale will require the application of automated solids-handling techniques. The problem of prevention of contamination

also becomes important in extended processes.

ad h) Scale-up strategies

Information available on scale-up strategies for SSF systems is scarce and empirical approaches have been used. Scale-up is further complicated by the involvement of various types of bioreactors, intense heat generation and non-homogeneity in the system. The fermentation plants are, consequently, labour and energy intensive and the development of well-founded scale-up criteria, such as for SLC processes, is vital for extensive commercialization of SSF systems (Lonsane *et al.*, 1992). Scale up criteria have to be concentrated on the problems mentioned under a,b,c,d, and f.

Bioreactors

Bioreactors for SSF can be divided into agitated and non-agitated ones. In Figures 1 a-e the major types are shown. The non-agitated systems are the simplest bioreactors.

Heap fermentations are used in composting (Cannel and Moo-Young, 1980) and for the production of some fermented African cassava products (Essers *et al.*,1992).

Figure 1a. Heap Fermentation

Tray bioreactors have been used successfully at laboratory, pilot, semi-commercial and commercial stages. A major disadvantage of tray reactors is that at a large scale, they are not easily automated and therefore tend to be labour intensive. In addition, a large area is required since the thickness of substrate in trays is limited to only a 5 - 10 cm. by heat transfer problems (Rathbun and Shuler, 1983).

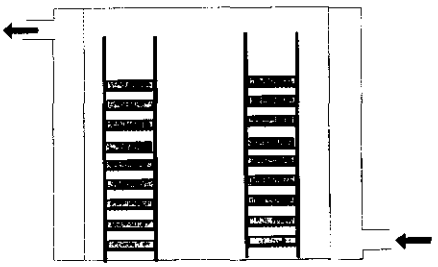

Figure 1b. Tray reactor

A commonly used type of non-agitated bioreactor is the packed-bed bioreactor. Packed-bed bioreactors contain a static substrate supported on a perforated base plate, through which forced aeration is applied. Many variations of this basic design are possible. The typical design is cylindrical-shaped. Most commonly the forced aeration is applied at the bottom. The humidity of the incoming air can be kept high to minimize water loss from the substrate. The advantage of packed-bed reactors is that they remain relatively simple, while allowing better process control (especially temperature and humidity) than is possible with trays. Disadvantages associated with packed-bed bioreactors include non-uniform growth, poor heat removal and problems with scale-up (Mitchell *et al.*, 1992).

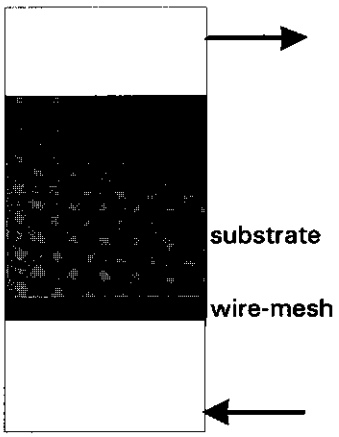

Figure 1c. Packed-Bed Reactor

The agitated bioreactors might be divided into rotating drum reactors, air-fluidized bed reactors and stirred tank bioreactors. Rotating drum reactors are characterized as horizontal or inclined cylinders. Aeration, if applied, is with low pressure air fed into the reactor headspace. The mixing provided by the tumbling action in rotating drum reactors is relatively gentle, and of all methods of automated mixing should cause the least damage to microorganisms or to the substrate structure (Mitchell *et al.*, 1992). Stirred bioreactors are of two main types depending on whether the axis of the

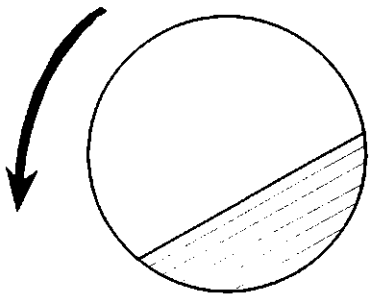

Figure 1d. Rotating Drum Reactor

bioreactor is horizontal or vertical. Horizontal stirred bioreactors are quite similar to rotating drum reactors except that the mixing is provided by an internal scraper or paddles, rather than by rotation of the body of the bioreactor. Vertical stirred bioreactors are often subjected to forced aeration. Vertical stirred bioreactors differ from packed-bed bioreactors by the fact that they are agitated, which may either be continuous or intermittent. If gas is passed at a flow rate beyond the fluidization velocity, the solids become suspended in the gas stream. In this state the bed is said to be fluidized. Special features and advantages of air-solid fluidized reactors include: the provision of very good aeration, removal of metabolic heat by the airstream, quick elimination of metabolic products, highly effective mixing is achieved and higher productivities compared with traditional SSF processes (Mitchell *et al.*, 1992).

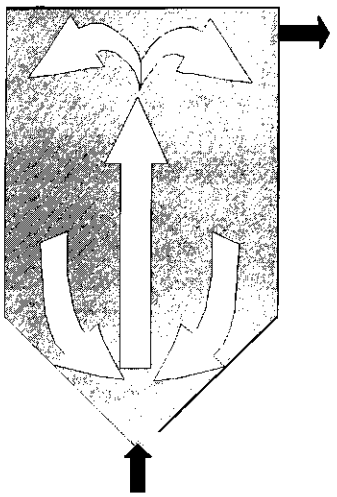

Figure 1e. Gas-Fluidized-Bed Reactor

TEMPE

As shown in Table 1A, tempe production is an example of solid-substrate fermentation in a tray reactor. Tempe is a traditional Indonesian fermented food in which fungi, particularly *Rhizopus* spp. play an essential role. Yellow seeded soya beans are the common and most popular raw material; the resulting 'tempe kedele' is usually referred to as 'tempe'. Other possible raw materials are described by Nout and Rombouts (1990). Generally speaking, fresh tempe of good quality 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. The major desirable aspects of tempe are its attractive flavour and texture and certain nutritional properties (Shurtleff and Aoyagi, 1979). With its high protein content (40-50 % dry matter), tempe serves as a tasty protein to complement

starchy staples, and can substitute for meat and fish. In 1988, the annual production of tempe in Indonesia was estimated at 765,000 tonnes. An annual increase of 5% per year is expected for the next decade (Karta, 1990). The principle of the tempe process is presented in Figure 2.

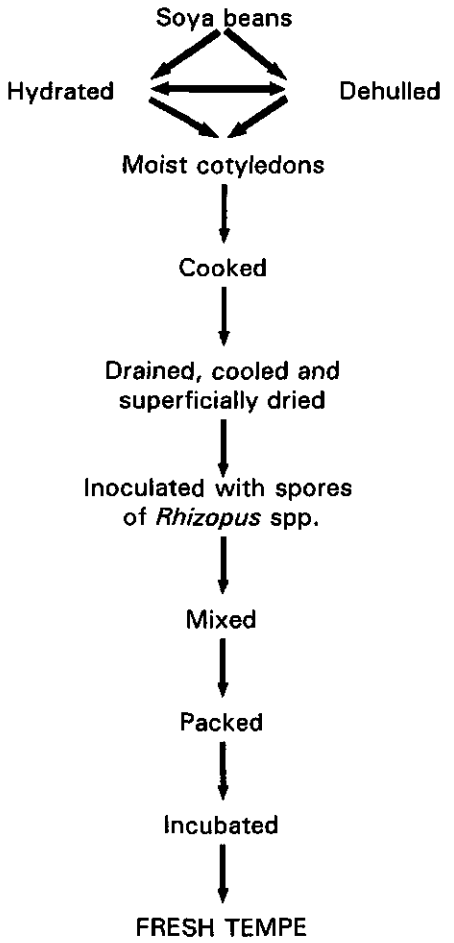

Figure 2. Principle of the tempe process (adapted from Ko and Hesseltine, 1979).

Several genera of fungi have been isolated from traditional tempe (Ko and Hesseltine, 1979; Winarno and Reddy, 1986) but *Rhizopus* spp. are considered to be essential for tempe making. Other genera are considered to be chance contaminants which may not even have grown but are present as spores. The most important genera of the class of the Zygomycetes which are commonly found in

foods, are confined to a single order, the Mucorales. The genera *Mucor, Rhizopus, Rhizomucor* and *Absidia* are classified in the family of the Mucoraceae. According to the revised nomenclature of Schipper (1984) and Schipper and Stalpers (1984) the genus *Rhizopus* consists of the species *R. stolonifer*, *R. oryzae* and the *R. microsporus* group. The *R. microsporus* group includes the species *R. homothallicus* and *R. microsporus*. The latter is subdivided into four varieties: *microsporus*, *rhizopodiformis*, *oligosporus* and *chinensis*. *R. arrhizus* has been placed in the *R. oryzae* group since it is considered to be an extreme form of *R. oryzae*.

Rhizopus microsporus var. *oligosporus* (in short *R. oligosporus*) is considered most suitable for tempe making (Sharma and Sarbhoy, 1984; Hesseltine, 1985b; Ko, 1985). Hesseltine *et al.* (1985a) reported that several *Rhizopus* species were able to grow both aerobically and anaerobically, however, *R. oligosporus* Saito NRRL 2710 failed to grow anaerobically. *Rhizopus* spp. produce a variety of carbohydrases, lipases, proteases, phytases and other enzymes, and some of the changes in the substrate macromolecules during tempe fermentation have been studied.

The effect of tempe fermentation on the total nitrogen content is negligible, but increases of free amino acids occur. However, the amino acid pattern of raw beans and tempe are similar, implying that there is no de-novo synthesis of amino acids, but only a degradation and consumption of soya protein by the growing fungus (Baumann *et al.*, 1990).

Changes in the lipids fraction are described by Wagenknecht *et al.* (1962), Sudarmadji and Markakis (1978), Paradez-Lopez *et al.* (1987), and Hering *et al.* (1991). Lipolysis yields predominantly linoleic acid, as well as oleic, palmitic, linolenic and stearic acid. Hering *et al.* (1991) observed that the fungus metabolized the soya bean lipids and synthesized, respectively changed, the fat composition only a minimal amount, depending on the strain and the environmental conditions used.

Changes in vitamin contents in soya bean tempe, are described by Shurtleff and Aoyagi (1979), Okado *et al.* (1983), Murata (1985), Keuth and Bisping (1993), and Tunçel (1993). Increased levels were reported for riboflavin (vitamin B_2), nicotinic acid, pantothenic acid, pyridoxin (vitamin B_6), folacin, cyanocobalamin (vitamin B_{12}) and biotin.

During the processing of soya beans to tempe antinutritional factors are removed or inactivated. Protein-type trypsin inhibitors are largely inactivated during cooking or steaming (Roozen and De Groot, 1985). Flatulence producing factors such as stachyose and raffinose are removed by soaking and boiling (Winarno and

Reddy 1986). Phytic acid of raw substrate increased during soaking due to pre-germination enzyme activities, but it is degraded during steaming and fermentation into lesser inositol phosphates and inorganic phosphate (Winarno and Reddy, 1986; Sutardi and Buckle 1985 a and b). During soaking and heating haemagglutonins and tannin are also leached out, inactivated and decomposed, respectively.

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CHAPTER 3

TEMPERATURE CONTROL IN SOLID-SUBSTRATE FERMENTATION THROUGH DISCONTINUOUS ROTATION

ABSTRACT

A laboratory-scale system for controlled dynamic solid-substrate fermentation was developed and tested. The fermentation takes place in a stainless steel discontinuously rotating drum reactor, under controlled conditions of temperature, gas composition, relative humidity and direction and rate of rotation. The system was tested on a model fermentation of soya beans with *Rhizopus oligosporus*. In contrast with the traditional tempe fermentation, a granular product is obtained and build-up of heat and mass gradients is restricted. Despite the discontinuous rotation, the fungal growth continues as evidenced by the production of heat. The rate of cooling depends on the temperature of the gas flushed through the reactor, the gas flow rate and the length of the rotation period. As a consequence of the homogeneous temperature control, the fungal heat development continued up to 70 hours of fermentation. This is in clear contrast with the traditional tempe fermentation which is already limited after 36 hours by its own heat accumulation.

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J.C. de Reu, M.H. Zwietering, F.M. Rombouts, M.J.R. Nout (1993)
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INTRODUCTION

Tempe is a traditional Indonesian food in which fungi, particularly *Rhizopus* spp. play an essential role. Yellow-seeded soya beans are the most common and popular raw material (Nout and Rombouts, 1990).

The equipment required for daily production capacities of 7 - 1800 kg has been described by Shurtleff and Aoyagi (1980). When scaling up the production by increasing the fermentor size, problems can be expected due to accumulation of metabolic heat (14960 Btu/kg dry solids (1 BTU = 1054 J) (Mudgett, 1986); 2514 kJ/kg of fermented solids (Aidoo *et al.*, 1982)). Consequently, the temperature within a bed or package of tempe may rise 10 - 16 °C above that of the environment. A steep temperature gradient of 3°C/cm bed thickness during active growth has been reported in a fermentor employing a bed height of 6.5 cm (Rathbun and Shuler, 1983). The availability of oxygen is determined by diffusion, which in turn is determined by porosity, particle size and consistency of the substrate (Alvarez-Martinez, 1987; Mitchell *et al.*, 1988). Due to these limitations to mass transfer, oxygen levels decreased to approximate 2 % v/v and carbon dioxide increased to 22 % v/v. However at 1 - 6.5 % v/v oxygen rapid fungal growth still occurred (Rathbun and Shuler, 1983), implying that the oxygen levels measured in tempe would not be growth-limiting.

A change in the reactor configuration can improve the process. Instead of a static packed-bed, we developed a rotating drum reactor (RDR). The use of rotating drum reactors has been recently reported for the production of, e.g. ethanol (Kargi and Curme, 1985; Weiland and Scholz, 1990), ochratoxin A (Lindenfelser and Ciegler, 1975), enzymes (Silman, 1980), spores (Larroche and Gros, 1986), koji (Mudgett, 1986) and for the cultivation of plant cells (Shibasaki *et al.*, 1992).

Some authors consider that the rotation of a fermentation medium would have a negative effect on growth and sporulation (Cannel and Moo-Young, 1980; Silman, 1980). According to Smith and Aidoo (1988) the main disadvantage of RDR is that the useful space for fermentation is only approximately 30 % of the total drum volume.

In contrast to research on submerged fermentations, there are no commercial laboratory-scale systems available for solid-substrate fermentations. Consequently, we developed a device that can be used for studies on solid-substrate fermentations. This paper describes a RDR system with advanced

measurement and control features, and illustrates the temperature control in a solid-substrate fermentation based on the tempe model.

MATERIALS AND METHODS

Organism

Rhizopus oligosporus LU 575 NRRL 5905, classified as *Rhizopus microsporus* var. *oligosporus* was grown 7 days at 30°C and maintained on malt extract agar (Oxoid, CM 59). Sporangiospore suspensions were obtained by scraping the sporangia off the agar and suspending them into sterile distilled water with 0.1 %(v/v) Tween 80. The viable count varied between $5x10^5$ and 10^6 cfu/ml.

Reactor and sensors

Experiments were carried out in a RDR as depicted in Figure 1. The RDR had an inner diameter of 20 cm and a length of 15 cm resulting in a 4.7 liter volume.

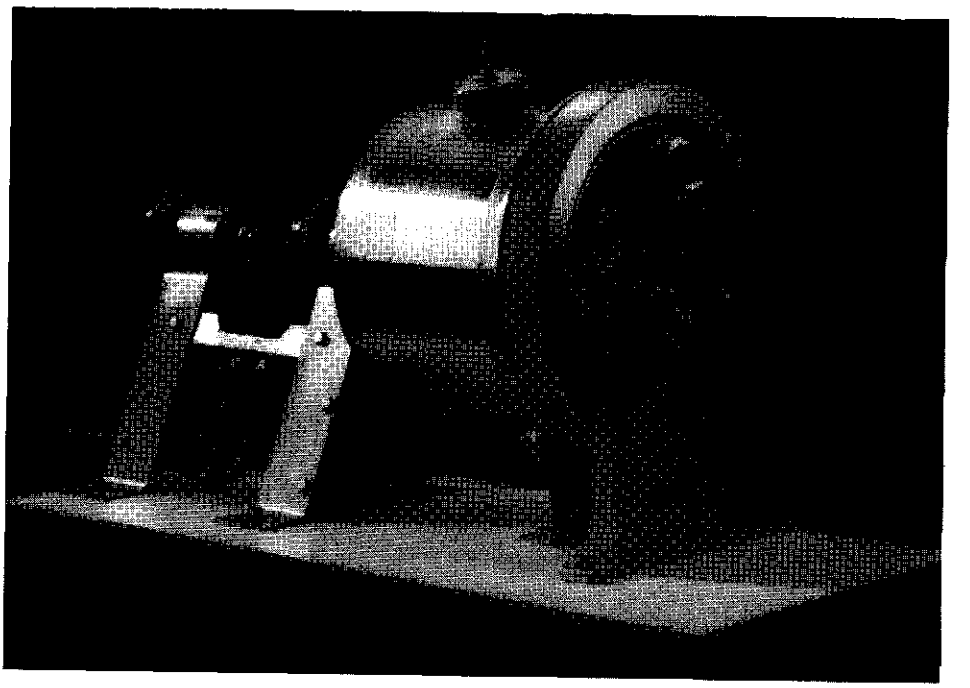

Figure 1. The rotating drum reactor

The reactor consisted of two stainless steel main parts, namely the rotating drum itself and the static wall which includes weld-in sockets for the sensors.

Two Pt 100 Ω-sensors with a diameter of 3 mm, (Tempcontrol, Voorburg, The Netherlands) were used to measure the temperature, one situated in the substrate and the other in the headspace of the reactor. An autoclavable oxygen probe (ϕ 25 mm, length 70 mm, Ingold, Urdorf, Switzerland) was placed in the headspace, and was calibrated under process conditions prior to the start of the fermentation. A non-autoclavable probe for relative humidity (I400, Rotronic, Bassersdorf, Switzerland) with a built-in Pt 100 Ω-sensor was placed in the headspace after autoclaving.

The RDR was used in a temperature controlled incubator. In order to prevent beans from sticking to the wall, the rotation was supplemented by a sweeping action. This was achieved by a wiper consisting of spring loaded horizontal and vertical parts. During fermentation, homogeneous samples could be obtained, after rotation through the sample port.

Gas flow system

Humidified air was provided through the gas inlet tube. The outlet was connected to an oxygen and carbon dioxide analyzer (Uras 3, Hartmann & Braun, Frankfurt am Main, Germany).

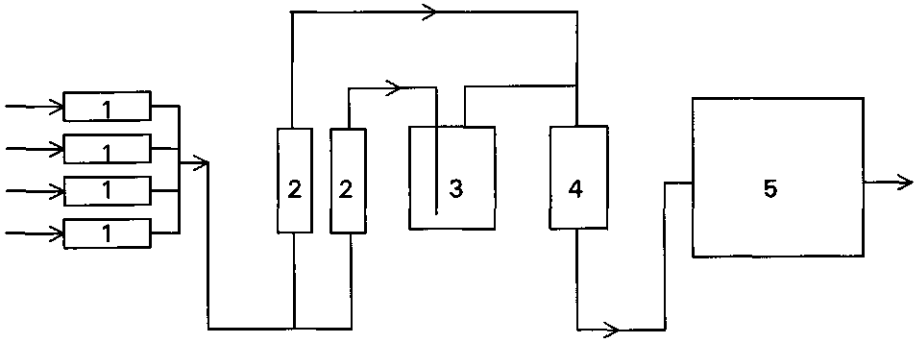

Figure 2. Schematic view of the gas flow system: 1 mass flow controller valves, 2 rotameters, 3 humidifier, 4 in-line relative humidity measuring unit, 5 rotating drum reactor

The relative humidity of the ingoing gas was controlled by mixing a water-saturated flow and a dry gas flow. The relative humidity was measured in the gas stream before the gas inlet. The gas composition could be controlled using four mass flow controllers (10, 100, 1000 and 2000 sccm, Type 1259B, MKS Instruments, München, Germany) and one multi-gas controller (147, MKS

Instruments, München, Germany) (Figure 2). Gas of desired composition could be produced by mixing oxygen, carbon dioxide, nitrogen and air, with flow rates varying from 0 to 3 l/min and relative humidity levels ranging from 50 to 100 %.

Measurement and control system

All sensors were linked to a computer-controlled measurement and control system consisting of hardware formed by a distributed controller (μDCS 6000, Analog Devices, Oosterhout, The Netherlands), controller software (FIXDMACS 1.3, Intellution, Norwood, MA, USA) and a personal computer (Vectra QS/16S with 80387 coprocessor, Hewlett Packard, Grenoble, France). The rotation speed (0 - 30 rpm), direction (forward/reverse) and duration could be programmed and controlled by FIXDMACS programming blocks, but manual intervention was also possible. The process data were stored on harddisk prior to analysis (plotting, ASCII transfer to Lotus etc.).

Fermentation process

Yellow seeded soya beans (*Glycine max*) were dehulled by dry abrasion and soaked overnight with accelerated acidification (Nout *et al.*, 1987). Subsequently the beans (pH soak water < 4.2) were washed with tap water and boiled for 20 minutes, cooled and superficially dried (15 - 30 minutes, at room temperature). The RDR was autoclaved for 20 minutes at 121 °C, before filling it with inoculated soya beans (1 kg/run). After inoculation, beans were transferred to the previously autoclaved and cooled RDR.

RESULTS AND DISCUSSION

Effect of aeration

One way to prevent high substrate temperatures is to use forced evaporation to absorb the metabolic heat generated. The degree of evaporation may be controlled by the flow rate and the relative humidity of the gas (Barstow *et al.*, 1988). According to Ryoo *et al.* (1991) mixing dry and wet gas achieves better control of evaporation than increasing the gas flow rate. In addition to heat removal, gas flow may serve to control the composition of the atmosphere in the reactor. Initially we used compressed air as aeration gas since it is cheapest. To simulate the situation in a traditional static fermentation we tested the effect of air flow on the substrate temperature in the RDR at stand-still. Different flow

rates (0, 0.25, 0.5, 0.75, 1, 1.5 and 2 l/min) with moisturized air (RH 95 %) were used.

The substrate temperature showed a similar pattern as the growth curve for fungal biomass including a lag phase, an exponential phase and a stationary or autolytic phase with concomitant temperature decrease. In Figure 3 the substrate temperature during a static fermentation and during two discontinuously rotated fermentations with an air flow rate of 1 l/minute is presented. Table 1 summarizes results with air flow rates ranging from 0.25 - 2.00 l/min. This table shows that flow rates < 0.75 l/min were limiting to the metabolic activity. The highest temperatures were reached with air flow rates between 0.75 and 1.5 l/min. Due to heat removal the temperature of the gas increased 3 - 4 °C. Flow rates exceeding 1.5 l/min resulted in lower temperatures due to increased heat removal.

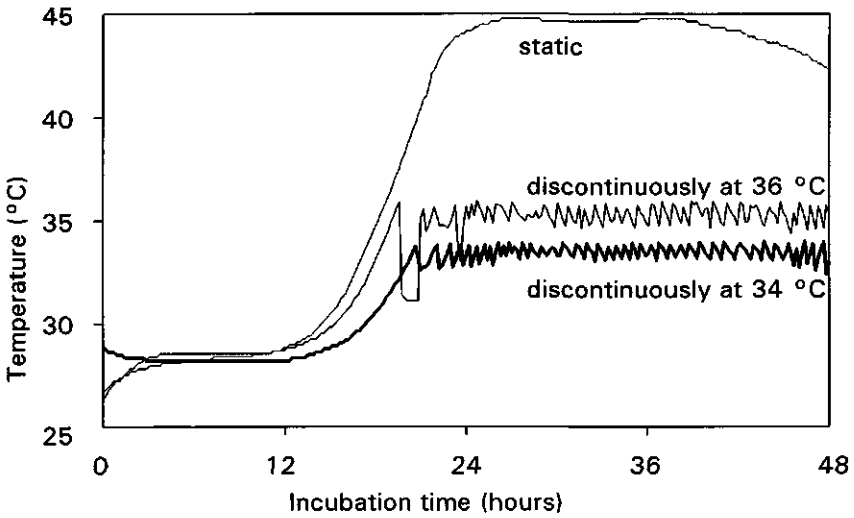

Figure 3. Development of substrate temperature in the rotating drum reactor during static and discontinuously rotated fermentations. Gas flow: air of 29 °C, 95 % RH at 1 l/min. Surrounding temperature 30 °C. Discontinuously rotated: rotation temperature (T_R) 34 °C and rotation period (t_r) 1 min, rotation temperature (T_R) 36 °C and rotation period (t_r) 1 min.

Table 1. Influence of air flow on the temperature of substrate and gas in the reactor during a static fermentation in the rotating drum reactor

Flow rate (l/min)[2)]	T_{max} substrate (°C)	T_{max} gas (°C)	Temperature slope[1)] (°C/h)
0.25	29.41	28.98	0.22
0.50	39.26	32.41	1.06
0.75	45.56	34.91	2.21
1.00	44.61	34.34	1.81
1.50	45.41	33.74	1.76
2.00	40.75	33.69	1.53

1) Maximum rate of temperature increase during active growth phase

2) Relative humidity of air flow = 95 %
Surrounding temperature = 30 °C
Temperature of gas at inlet = 29 °C

Effect of rotation temperature and rotation period

The aim of rotating was to minimize heat and mass transfer limitations and to create a homogeneous product by breaking the tempe mass into smaller particles. Several authors reported that rotation of fermentation media would have a negative effect on growth and sporulation (Cannel and Moo-Young, 1980; Silman, 1980). One of our aims was to investigate whether growth inhibition would occur as result of discontinuous rotations. An advantage of discontinuous over continuous rotation would be that less energy is required for rotation and that the fungal mycelium would be exposed to a minimum of shear. In principle, a compromise between maximum growth (minimum rotation) and minimum temperature gradients (maximum rotation) would be required.

One may programme the discontinuous rotation according to a predefined timescale. However, due to variations in the lag phase, the actual start of the active growth is difficult to predict. Consequently, we choose to couple the rotation programme directly to the actual fungal metabolic activity as indicated by the substrate temperature.

Due to the mixing with gas of a lower temperature, the substrate temperature is reduced to T_{RS} (Figure 4) at the end of rotation. During subsequent

stand-still the substrate temperature increases again to T_R. The rate of temperature rise could be expressed as slope α (Figure 4).

To achieve adequate size reduction of the block formed, rotation into both reverse and forward directions was required. A rotation scheme in which the rotation temperature (T_R), rotation rate (rpm), rotation direction and rotation period (t_r) were defined in a programme block of the FIXDMACS programme is shown in Table 2. There was no rotation during the lag phase. As a result of spore germination and growth, the substrate temperature was allowed to increase until the defined rotation substrate temperature (T_R) was reached (Figure 4).

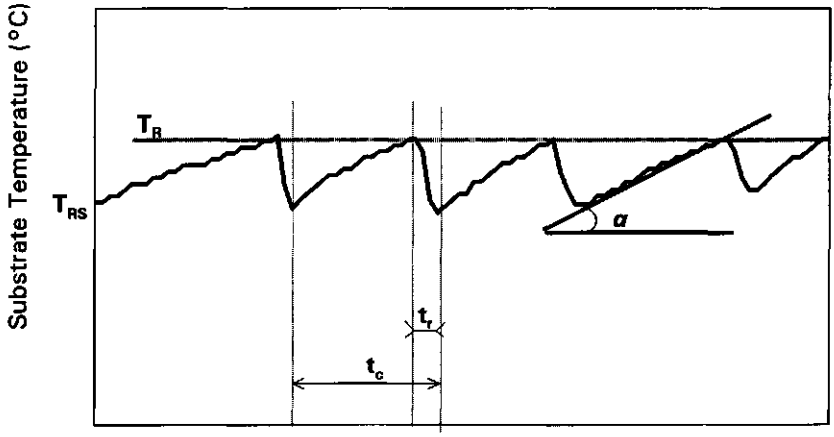

Figure 4. Detailed view of the temperature pattern during discontinuously rotated fermentation: T_R = rotation temperature (°C); T_{RS} = temperature at end of rotation period (°C); t_c = cycle period (h); t_r = rotation period (h).

$$\alpha: \quad slope \ \frac{T_R - T_{RS}}{t_c - t_r}$$

Table 3 summarizes the temperature fluctuations obtained at rotation temperatures ranging from 32 °C to 41 °C and rotation periods of 1 and 5 minutes. Air of 95 % relative humidity and 30 °C was put through at 1 l/min. With increasing rotation temperature and rotation periods, the temperature difference $T_R - T_{RS}$ increased. This was due to the similarly increasing difference

between T_R and T_{gas}, and the increased exposure to the gas, respectively. An increase of t_c from 1 to 5 minutes resulted in almost all cases in a decrease of α. This effect may have been caused by increasing damage to mycelium by longer rotation. In addition, the stronger cooling effect of prolonged rotation could also influence α. We did not investigate the pure effect of prolonged rotation on mycelium disruption. However this could be done by ensuring constant difference between the temperature of the in-going gas and the substrate temperature in order to avoid the effect of cooling at increased t_c.

Table 2. Rotation scheme as defined in a programme block of FIXDMACS for an experiment with a rotation temperature (T_R) of 34 °C and rotation period (t_r) of 1 minute

Step	Event	Rotation rate (rpm)		Rotation time (sec)
1	If Tsub[1] > 34 °C GOTO step 3	0		0
2	GOTO step 1	0		0
3	Rotate forwards	6		7
4	Rotate backwards	4		7
5	Rotate forwards	6		7
6	Rotate backwards	4		7
7	Rotate forwards	6		32
8	GOTO step 1	0		0
			total:	60 sec

[1]Tsub = *substrate temperature*

In Figure 3 the temperature curves at T_R 34 °C and T_R 36 °C both with t_r 1 min are illustrated together with the temperature curve of a static fermentation in the same reactor at the same air flow (1 l/min, 30 °C, 95 % RH). In the traditional static tempe process, the substrate temperature regulates the growth of the mould. Figure 3 shows that at a maximum substrate temperature of 44 °C, the growth was strongly inhibited and the accumulated heat was dissipated slowly resulting in a decrease of the substrate temperature beyond 36 hours of incubation. In the RDR the slope (α) increased gradually to a constant level and could be maintained until the chemical composition of the substrate became

inhibitory, probably by substrate limitation. The slope α increased with higher rotation temperatures (T_R); whereas the maximum slope during the static process was 1.8 °C/h (Table 1, 1 l/min), the slope α in the dynamic process was maximum (5.8 °C/h) at T_R = 38.5 °C. The increase of the temperature slope (α) resulted from two effects, namely firstly the production of metabolic heat which we assume to be maximum at the optimum growth temperature (approx. 37 °C), and secondly the levelling of a temperature gradient within the beans which was created by the cooling effect during rotation. As a consequence of the homogeneous temperature control, the fungal heat development continued for up to 70 hours of fermentation. This is in clear contrast with the traditional tempe fermentation which was limited already after 36 hours by its own heat accumulation.

Table 3. Temperature control during discontinuous rotated fermentation in a rotating drum reactor flushed with air of 29 °C, 95 % relative humidity, at 1 l/min for different rotation temperatures and rotation periods. Surrounding temperature = 30 °C

Rotation temperature T_R (°C)	Rotation period t_r (min)	Temperature drop T_R-T_{RS} (°C)	Cycle time t_c (min)	Average Slope α $(T_R-T_{RS})/(t_c-t_r)$ (°C/h)
32	1	0.36 ± 0.12	9 ± 7	2.8
32	5	0.30 ± 0.11	16 ± 9	1.4
34	1	0.92 ± 0.22	39 ± 11	1.5
34	5	2.09 ± 0.80	83 ± 65	1.2
36	1	1.17 ± 0.41	40 ± 13	1.8
36	5	2.31 ± 0.42	73 ± 36	1.9
38.5	1	2.91 ± 0.46	39 ± 11	5.8
38.5	5	3.69 ± 0.58	142 ± 81	2.3
41	1	3.03 ± 0.43	36 ± 15	5.7

The avoidance of uncontrolled and stormy heat accumulation with consequent excessive and limiting temperatures opens interesting prospects for increasing the efficiency of substrate modification in fungal solid-substrate

fermentations.

Compared with rotating drum systems described elsewhere (Silman 1980; Kargi and Curme 1985; Lindenfelser and Ciegler 1975; Shibasaki 1992) which operate under fixed conditions for temperature, rotation speed and air flow rate which do not have options for data logging or computer control, the present system has a number of advantages. In addition to measurement and control of multiple parameters, the set values and actions can be activated by the process itself, e.g. temperature activated rotation as in Table 2. The optimization of tempe processes requires investigation of kinitics of substrate modification under defined and controlled conditions. This is presently being studied.

ACKNOWLEDGEMENTS

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CHAPTER 4

A MODEL FOR SOLID-SUBSTRATE FERMENTATION OF *RHIZOPUS OLIGOSPORUS* IN A PACKED-BED REACTOR

ABSTRACT

In this study an aerated packed-bed reactor with off-gas circulation was developed and tested for the fermentation of soya beans with *Rhizopus oligosporus*. The design of the system enabled us to study the effect of cooling by forced evaporation in a reactor without axial and radial temperature or gas concentration gradients.

Based on the carbon dioxide formation a relation between temperature and specific growth rate of *Rhizopus oligosporus* was determined on soya beans. The optimum temperature for growth was 40 °C and the maximum temperature was estimated at 46.7 °C.

The use of packed-bed reactors to study heat removal by means of forced aeration is limited due to the formation of temperature gradients (max 0.5 °C/cm) and shrinkage of the substrate.

A mathematical model was developed to predict the substrate temperature as function of incubation time, air flow rate and the initial biomass content. The model predictions were fairly accurate, if initial amount of biomass and the substrate composition were estimated by trial and error. The reaction kinetics used in the model are still too simple, the effects of substrate, water and space limitations on growth should be included into the model, in order to describe the decline in activity after approx. 30 hours.

This chapter has been submitted as:

A model for solid-substrate cultivation of *Rhizopus oligosporus* in a packed-bed reactor

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INTRODUCTION

Solid-substrate fermentation (SSF) systems, involving the growth of microorganisms on moist substrate in the absence of free water, have been used extensively from ancient times in the Oriental, Asian and African countries for the production of fermented foods, starter inocula, mushrooms, dough fermentations, etc. (Lonsane *et al.*, 1992). Despite their long history, however, the design of these systems is still more an art than a technology.

Mitchell (1992) reviewed models, growth patterns and growth kinetics in SSF and concluded that there exists only a limited understanding of the principles underlying the patterns and kinetics of microbial growth in SSF. Recently, several models for the behaviour of solid-state fermentors have been published, which combine simple black-box kinetic models with physical transport phenomena (Saucedo-Castañeda *et al.*, 1990; Raghava Rao *et al.*, 1993; Sargantanis *et al.*, 1993; Rajagopalan and Modak, 1994). The usefulness of these reactor design models is hampered by their poor validation or erroneous assumptions, as will be briefly explained below.

Saucedo-Castañeda *et al.* (1990) described a model for cultivation of *Aspergillus niger* on cassava meal in an aerated packed-bed fermentor. They used the logistic growth law with temperature dependent specific growth rate and maximum biomass level. The description of the physical behaviour of the fermentor contains some fundamental errors: water evaporation is neglected (even though it is generally accepted that it has a large influence on the fermentor temperature), the heat capacity of the moist air is assumed to equal that of the moist substrate-mycelium mixture (although they differ by almost a factor 1000). The authors are unable to predict experimental results with this model starting from independently determined kinetic and estimated physical parameters. They revert to a fitting procedure using no less than 10 parameters.

Raghava Rao *et al.* (1993) have modeled SSF in a tray-fermentor. Without experimental evidence, they neglect temperature gradients and assume that oxygen depletion in the layer of solid substrate is the key factor determining the rate of the fermentation process. Their model is based on coupled transport by diffusion through the interparticle gas phase and zero-order conversion kinetics, but it lacks a proper description of (the effect of environmental conditions on) growth and conversion kinetics as well as experimental validation. Results presented by the same group in other papers (Ghildyal *et al.*, 1993, 1994) show that oxygen is not depleted even in extremely deep substrate layers, while the temperature can easily

reach inhibitory levels in an 80 mm deep layer.

Rajagopalan and Modak (1994) also modeled SSF in a tray-fermentor. Their model is based on the logistic growth model extended with Monod-kinetics to allow for oxygen limitation. The authors claim to use the temperature dependence of the specific growth rate determined by Saucedo-Castañeda *et al.* (1990) for *A. niger* growing on cassava meal, but their growth rate function does not agree with the source. Model predictions based on the kinetics of *A. niger* growing on cassava meal are compared to measurements with *Rhizopus oligosporus* growing on soya beans published by Rathbun and Shuler (1983); differences between the growth kinetics of both fungi are neglected. Diffusion of oxygen through interparticle voids and oxygen transfer from the interparticle voids to the 'biofilm' covering the solid particles are taken into account as the rate limiting steps in the oxygen supply to the fungus; oxygen transport in the 'biofilm' is neglected. The values for the diffusion coefficients of oxygen and carbon dioxide in the interparticle voids are only approx. 1 % of the values in air, which in our opinion is irrealistic. Oxygen transfer limitation plays a very important role in the simulations, which must be attributed to the very low value of the mass transfer coefficient used in the model ($k_g a = 1.8\ 10^{-3}\ s^{-1}$). This value is irrealistic; assuming $Sh = 2$, $ID = 10^{-5}\ m^2/s$ and $dp = 10^{-3}$ m, we estimate that $k_g a = 160\ s^{-1}$. Other mechanisms must cause the growth rate decrease observed in the experiments. Finally, the enthalpy balance used in the model contains several serious errors: the heat capacity of the water in the solids and the effects of water evaporation are neglected.

Sargantanis *et al.* (1993) have modeled an intermittently rotating drum reactor with *Rhizopus oligosporus* growing on corn grits. They used the logistic growth law with specific growth rate and maximum biomass level depending on temperature and moisture concentration. Without experimental evidence they assume homogeneous reactor contents, even though the description of their experimental set-up shows that no measures have been taken to prevent temperature gradients. The model's predictions of temperature development in uncontrolled fermentation are very inaccurate.

In this study a model was developed and tested that predicts the temperature increase during growth of *Rhizopus oligosporus* on soya beans in a packed-bed reactor with forced aeration through the bed. Off-gas circulation was applied to avoid radial and axial gradients in the bed. Adiabatic operation was approached by maintaining the incubator temperature at 0.1 °C below the reactor temperature. This system is therefore suitable to study heat removal by means of forced aeration within a reactor with homogeneous growth.

MATERIALS AND METHODS

Culture

Rhizopus oligosporus NRRL 5905 was grown and maintained on Malt Extract Agar (MEA, Oxoid CM59) slants. Sporangiospore suspensions were prepared as described previously (De Reu *et al.*, 1993).

Reactor and sensors

Experiments were carried out in a Packed-Bed Reactor (PBR) as depicted in Figure 1.

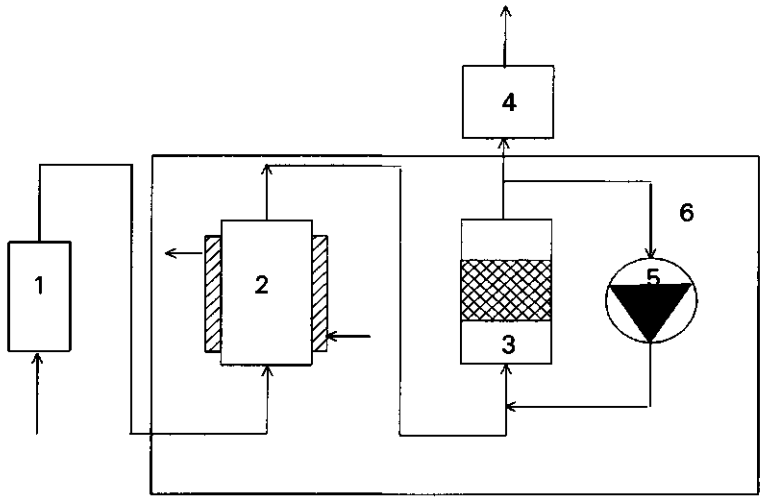

Figure 1. The experimental set-up: 1 Mass flow controller 2 Temperature controlled humidifier 3 Packed-bed reactor 4 Oxygen and carbondioxide analyzers 5 Recirculation pump 6 Incubator.

The PBR had an inner diameter of 6 cm and a length of 26 cm. The reactor consisted of glass with a removable bottom and top. Three Pt 100 Ω-sensors with a diameter of 3 mm, (Tempcontrol, Voorburg, The Netherlands) were used to measure the temperature, two situated in the substrate and the third in the headspace of the reactor. A non-autoclavable probe for relative humidity (I400, Rotronic, Bassersdorf, Switzerland) with a built-in Pt 100 Ω-sensor was placed in the headspace after autoclaving the reactor. The PBR was used in a temperature

controlled incubator. The incubator temperature was initially kept on 30 °C. When the substrate temperature increased due to metabolic heat beyond 30 °C, the setpoint was kept on the temperature of the substrate - 0.1 °C, to avoid external heating of the reactor and radial temperatures gradients.

Gas flow system

The gas flow was controlled by a mass-flow controller (2000 sccm/min, type 1259B, MKS Instruments, Munich, Germany). Air was saturated to 100 % relative humidity at 30 °C in a temperature controlled humidifier. The off-gas was split, one was connected to an oxygen (Servomex, Zoetermeer, the Netherlands) and carbon dioxide analyzer (Uras 3, Hartmann & Braun, Frankfurt am Main, Germany). To avoid axial temperature gradients in the packed-bed the second air flow was linked to a compressor (2 l/min, type V-147, KNF, Germany) for recirculation. The recirculated air was mixed with the fresh humidified air before the gas-inlet of the reactor.

Measurement and control system

All sensors were linked to a computer-controlled measurement and control system consisting of hardware formed by a distributed controller (μDCS 6000, Analog Devices, Oosterhout, The Netherlands), controller software (FIXDMACS 1.3, Intellution, Norwood, MA, USA) and a personal computer (Vectra QS/16S with 80387 coprocessor, Hewlett Packard, Grenoble, France). The process data were stored on a hard disk prior to analysis (plotting, ASCII transfer to Lotus etc.)

Fermentation process

Yellow seeded soya beans (*Glycine max*) were dehulled by dry abrasion and soaked overnight (30 °C) with accelerated acidification (Nout *et al.*, 1987). Subsequently the beans (pH soak water < 4.2) were washed with tap water and boiled for 20 minutes, cooled and superficially dried (15 - 30 minutes, at room temperature). The PBR was autoclaved for 20 minutes at 121 °C, before filling it with inoculated soya beans (100 g/run). After inoculation with sporangiospore suspension 1 % v/w beans, the beans were transferred to the previously autoclaved and cooled PBR.

Determination of specific growth rate and lag phase

To determine the temperature dependence of the specific growth rate, the growth of *Rhizopus oligosporus* was monitored by measuring carbon dioxide in the headspace of a closed flask. Narahara (1982), Nishio (1979) and Okazaki (1979,

1980) have shown that the carbon dioxide production can be used to determine the specific growth rate during SSF.

Autoclaved flasks (1 dm^3) with 15 grams of inoculated beans were incubated at temperatures varying between 5 and 50 °C. At regular time intervals samples of the headspace were collected and analyzed on a Chrompack CP 9001 (TCT/PTI 4001) GLC. Specific growth rates were calculated by fitting the logistic growth law modified to allow for a lag phase, on the measured amount of CO_2.

The logistic growth law with lag phase give the following expression for the amount of product.

$$t \leq \lambda \qquad P(t) = P_0$$

$$t > \lambda: \quad P(t) = P_0 + Y_{P/X} X_0 \left[\frac{X_{max}}{(X_{max} - X_0) e^{-\mu(t-\lambda)} + X_0} - 1 \right]$$

or

$$t > \lambda: \qquad P(t) = (P_0 - Y_{P/X} X_0) + \frac{Y_{P/X} X_0 X_{max}}{(X_{max} - X_0) e^{-\mu(t-\lambda)} + X_0}$$

This equation contains six parameters. It turned out that the fitting program frequently gave invalid results when this equation was used. Therefore, the equation was simplified by assuming that $M_p(t) = Y_{P/X} X(t)$ during the entire batch fermentation. This gives:

$$t \leq \lambda: \qquad P(t) = P_0$$

$$t > \lambda: \qquad P(t) = \frac{P_0 P_{max}}{(P_{max} - P_0) e^{-\mu(t-\lambda)} + P_0}$$

Evidently, the assumption above frequently will not hold in the beginning of an experiment, while it will be reasonable at the end. However, the parameter of main interest for our study, viz. μ, is not affected by the assumption, while the value for λ will be overestimated as shown in Appendix 1.

We used the simplex method provided by Simulation Tool for Easy Modelling (Resource Analysis Delft, The Netherlands) to determine the values for μ, λ, $M_{P,0}$

and $M_{P,\,max}$.

Model

The major assumptions underlying the mathematical model are:

- The fungus grows exponentially; its specific growth rate is solely affected by temperature, not by any other limitation, i.e. the model is not based on the logistic growth law.
- The PBR is fully homogeneous and adiabatic.
- The off-gas from the fermentor is at equilibrium with the solids.
- Biomass, carbon dioxide and water are the only fermentation products; Sulphur and Phosphorus are neglected in the stoichiometry; the nitrogen source is ammonium liberated by proteolytic enzymes.
- The maintenance requirements of the fungus, and the heat capacities and densities of all compounds in the reactor are independent of temperature in the range of interest.

Stoichiometry

The model is based on the following reactions for growth and maintenance respectively:

$$CH_{nh}O_{no} + Y_O\ O_2 + Y_N\ NH_3 \rightarrow Y_X\ CH_{1.8}O_{0.5}N_{0.15} + Y_C\ CO_2 + Y_W\ H_2O$$

$$CH_{nh}O_{no} + Y_{Om}O_2 \rightarrow Y_{Cm}CO_2 + Y_{Wm}H_2O$$

The nature of the substrate is not known beforehand. These equations as well as the following, allow for a definition at a later stage.

From the elemental balances, the stoichiometric coefficients can be calculated as follows:

$$Y_C = 1 - Y_X$$

$$Y_N = 0.15\ Y_X$$

$$Y_O = 1 - 1.0875 Y_X + 0.25nh - 0.5no$$

$$Y_W = -0.675 Y_X + 0.5no$$

$$Y_{Cm} = 1$$

$$Y_{Om} = 1 + 0.25nh - 0.5no$$

$$Y_{Wm} = 0.5nh$$

Rate equations

The production rates of biomass, substrate, oxygen, carbon dioxide and water are:

$$r_X = \mu M_x$$

$$r_S = -\frac{1}{Y_x} rx - m_s M_x$$

$$r_O = -\frac{Y_O}{Y_x} r_X - Y_{om} m_s M_X$$

$$r_C = \frac{Y_C}{Y_X} r_X + Y_{cm} m_s M_X$$

$$r_W = \frac{Y_W}{Y_x} r_x + Y_{wm} m_s M_x$$

The heat production associated with the metabolism is assumed to be proportional to the oxygen consumption (Roels, 1983):

$$r_q = -H_O r_O$$

Mass and enthalpy equations

For biomass, substrate and carbon dioxide the mass balance equations read:

$$\frac{dM_i}{dt} = r_i \text{ with } i = x,s,c$$

Note that we do not calculate the carbon dioxide concentration in the off-gas, but solely the cumulative amount of carbon dioxide.

The mass balance equation for intra- plus extracellular water reads:

$$\frac{dM_w}{dt} = r_w + F_a(c_{wi} - c_w)$$

Note that incorporation of water in the cytoplasm will cause a decrease of the amount of extracellular water available to the biomass, which may limit growth (Larroche *et al.*, 1992). We have not yet included this in our model, as we have as yet no reliable means of measuring the water activity.

The enthalpy balance equation reads:

$$\frac{d}{dt} \sum_{i=s,x,w,r} M_i c_{pi} T =$$

$$= r_q + r_{com} + U_w A_w (T_s - T) + F_a(c_{pa} T_i + c_{wi}(c_{pwv} T + \Delta H_w) - c_{pa} T - c_w(c_{pwv} T + \Delta H_w))$$

In this balance, the accumulation terms relating to the gas phase have been neglected, as they are orders of magnitude smaller than the accumulation terms relating to the solid matrix including water. Saturated water vapour concentrations were calculated with the equations given by Beukema (1980).

RESULTS AND DISCUSSION

Figure 2 shows the measured amounts of carbon dioxide and the predictions of the modified logistic equation at 18.9 and 29.5 °C. Figure 3 shows the specific growth rates determined at incubation temperatures from 10 to 46.7 °C. The square root model proposed by Ratkowski *et al.* (1983) was fitted to the experimental data. Although a good fit was obtained up to 45.5 °C, the model had to be modified to allow for the sharp decrease in specific growth rate above this point.

$$T_{min} < T < 45.5\ °C: \quad \mu = [b(T - T_{min})(1 - e^{c(T - T_{max})})]^2$$

$$45.5 < T < 46.7\ °C: \quad \mu = 0.536\left[1 - \frac{(T - 45.5)}{1.2}\right]$$

This implies that the value of T_{max} has no physical meaning here, a phenomenon also reported by McMeekin *et al.* (1993). Parameter values are given in Table 1.

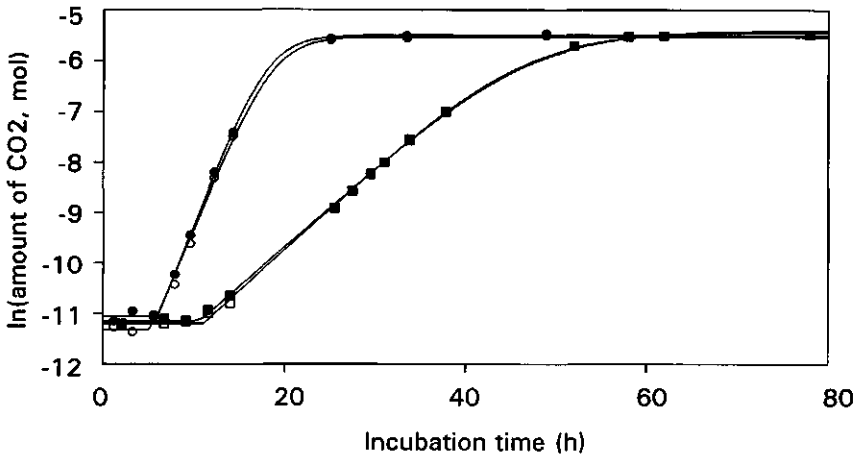

Figure 2. The natural logarithm of the amount of carbon dioxide and the predictions of the modified logistic equation for growth of *R. oligosporus* on soya beans incubated at 18.9 (□, ■) and 29.5 (○, ●) °C.

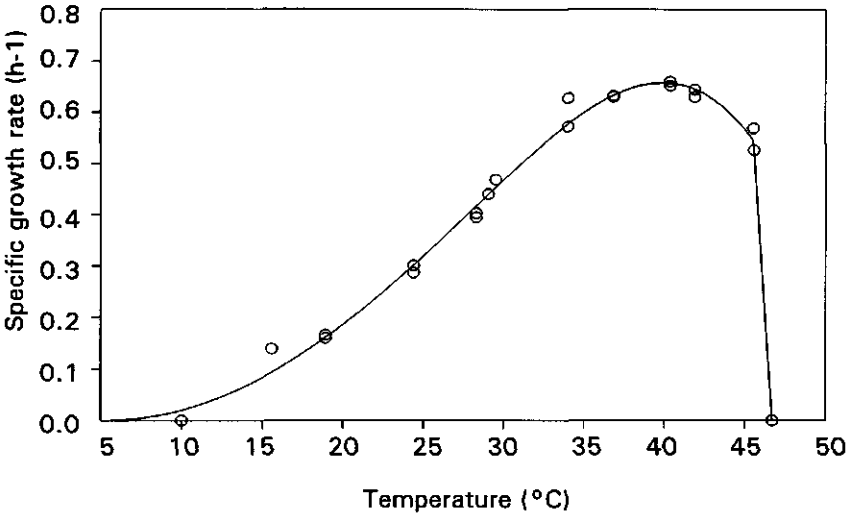

Figure 3.. The effect of the temperature on the specific growth rate of *Rhizopus oligosporus* on soya beans. ○ exp. data — model

Table 1. Parameters for the Square root model obtained by curve fitting of the specific growth rates of *R.oligosporus* on soya beans

Parameter	Value
Tmin	5.32 °C
Tmax	54.95 °C
b	0.030 $h^{-1/2}°C^{-1}$
c	0.099 $°C^{-1}$

The initial amount of carbon dioxide showed a large variance ($1.7\ 10^{-5} \pm 1.4\ 10^{-5}$ moles, N = 20). All experiments gave virtually the same values for the maximum amount of carbon dioxide (average $4.06\ 10^{-3}$ moles, SD $2.03\ 10^{-4}$, N = 19). This value corresponded with exhaustion of oxygen. The decline in growth rate observed in these batch experiments was not included in the PBR model, as oxygen limitation is not likely to occur in the aerated PBR.

Packed-bed reactor

Figure 4 shows the experimental substrate temperature (Fig. 4a) and cumulative carbon dioxide production (Fig. 4b) for four experiments with a fresh-air flow rate of 0.42 dm^3/min. Initially the substrate and reactor are heated to 30 °C. During the first 8-10 hours the heat and carbon dioxide production rates remain very low. Then both rates increase rapidly, causing a rapid temperature increase to 45-47 °C. The temperature remains at this level for 6-12 hours and then decreases slowly.

The reproducibility of the solid-state cultivation of *R. oligosporus* on soya beans is acceptable, except for the onset of the temperature increase. This phenomenon has been observed in the packed-bed reactor, as well as in a rotating-drum reactor (De Reu, 1993) and in tray systems. A similar problem has been reported by Mitchell *et al.* (1991). Probable causes are a lack of reproducibility in the spore cultivation process, the bean pretreatment - especially the lactic acid fermentation - or the inoculum distribution. The consequences for modelling are discussed below.

Temperature differences between the substrate temperature measured at 4 cm (T_{centre}) and 2 cm (T_{bottom}) from the bottom of the bed and in the headspace of the incubator are shown in Figure 5 for experiment 1.

The assumption that the bed is homogeneous is not completely true, which

may cause deviations between model predictions and experiments near the upper temperature limit of the fungus.

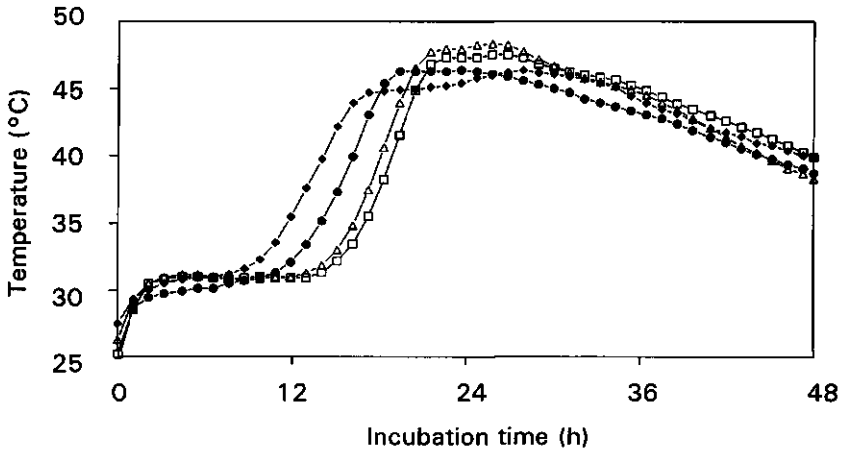

Figure 4a

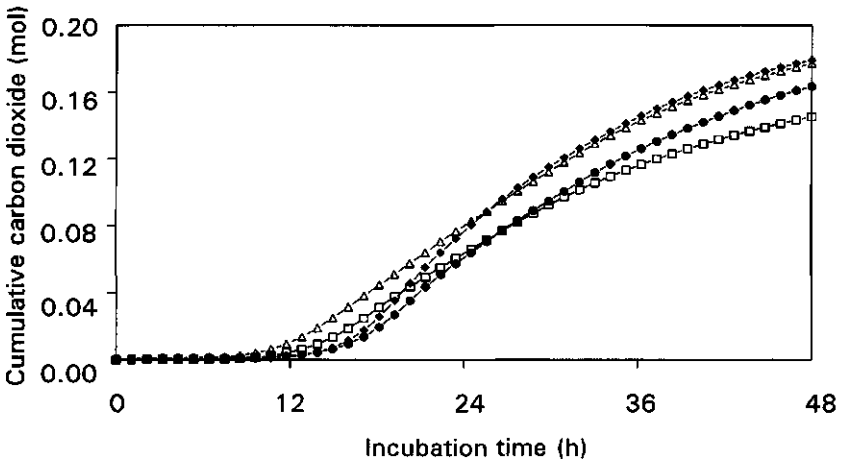

Figure 4b

Figure 4. The substrate temperature (Fig. 4a) and the cumulative carbon dioxide production (Fig. 4b) measured in a packed-bed reactor during fermentation of soya beans with *Rhizopus oligosporus*, air flow rate 0.42 dm^3/min, □ expt. 1, ▵ expt. 2, ● expt. 3, ◆ expt. 4.

The same holds for the assumption that equilibrium between gas and solids is reached. The difference between off-gas temperature and bed temperature after approx. 24 hours is a consequence of (1) the high heat production rate and (2) the (observed) shrinkage of the bed which causes channelling.

Temperature measurements in the off-gas recycle lines using dry air, revealed that the enthalpy transferred to the gas by the compressor was at most 4×10^{-3} W. Most of this enthalpy was transferred to the air surrounding the gas recycle lines, resulting in an enthalpy flux from the compressor to the reactor (r_{com}) of at most 4×10^{-4} W. This contribution can be neglected in the enthalpy balance.

When the beans were inoculated and packed in the reactor their initial temperature was approx. 26 °C. As shown in Figure 4a the soya beans equilibrated within 3 hours with the incubator temperature (30 °C). Based on this temperature increase we were able to estimate U_wA_w 0.5 ± 0.1 W/°C (n = 5).

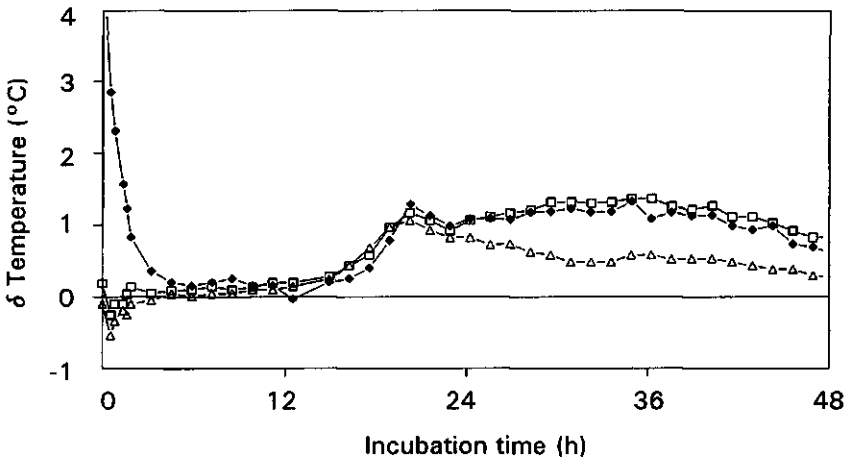

Figure 5. Temperature differences between the substrate temperature measured at 4 cm (T_{centre}) and 2 cm (T_{bottom}) from the bottom of the bed in the headspace of the reactor (T_{gas}) for experiment 1 in a packed-bed reactor during fermentation of soya beans with *Rhizopus oligosporus*, air flow rate 0.42 dm³/min. △ T_{centre}- T_{bottom}, □ T_{centre}-T_{gas}, ◆ $T_{incubator}$ -T_{gas}

Model predictions

Figure 6 shows the temperature and cumulative carbon dioxide production predicted by the model, together with experimental results. The parameter values

used in the calculation are shown in Table 2.

Table 2. Parameter estimations used in the model to calculate the temperature pattern and cumulative carbon dioxide production in a packed-bed reactor.

Parameter	Value	Units	Comments or source
b	0.030	$h^{-1/2}$ °C^{-1}	Table 1
c	0.099	°C^{-1}	Table 1
c_{pa}	28.8	J mol^{-1} °C^{-1}	
c_{pr}	840	J kg^{-1} °C^{-1}	
c_{ps}	30.0	J $Cmol^{-1}$ °C^{-1}	assumed to be valid for all organic solids
c_{pw}	75.2	J mol^{-1} °C^{-1}	
c_{pwv}	36.0	J mol^{-1} °C^{-1}	
c_{px}	33.0	J $Cmol^{-1}$ °C^{-1}	
ΔH_0	4.6 10^5	J mol^{-1}	
ΔH_w	4.35 10^4	J mol^{-1}	
F_a	1.12	mol h^{-1}	
$M_c(t=0)$	0.0	Cmol	
M_r	1.341 kg	kg	measured
$M_s(t=0)$	2.5	Cmol	initially the soya beans contained 40 % (w/w) dry matter
$M_w(t=0)$	3.33	mol	and 60 % (w/w) water
$M_x(t=0)$	2.0 10^{-6}	Cmol	estimated by trial and error, see text
m_s	0.0144	Cmol $Cmol^{-1}$ h^{-1}	Roels, 1983
nh	2		estimated by trial and error, see text
no	0.1		estimated by trial and error, see text
r_{com}	0.0	W	measured, see text
T(t=0)	26	°C	measured
T_i	30	°C	measured
T_{max}	54.95	°C	Table 1
T_{min}	5.32	°C	Table 1
T_s	varying	°C	measurements
U_wA_w	0.5	W °C^{-1}	estimated from warming-up
Y_x	0.65	Cmol $Cmol^{-1}$	Roels, 1983

Two parameters were estimated by trial and error in order to obtain maximum agreement between predictions and experiments, viz. the initial amount of biomass and the substrate composition. The initial amount of biomass was estimated at 2 10^{-6} Cmol for the experiments shown in Figure 6. A value of (4.6 ± 0.5) 10^{-6} Cmol was expected from 4 dry weight measurements on one spore suspension. Lipids were chosen as substrate in the simulations, because this gave the best simultaneous fit of temperature and carbon dioxide profiles. Compared to glucose, lipids will result in a higher ratio between heat and carbon dioxide production. *R. oligosporus* uses fat as carbon source during growth on soya beans (De Reu *et al.*, 1994). Incubation in petri dishes at 37 °C resulted in a loss of *ca* 30% of the lipids after 69 hours. These observations support the choice of oleic acid as substrate.

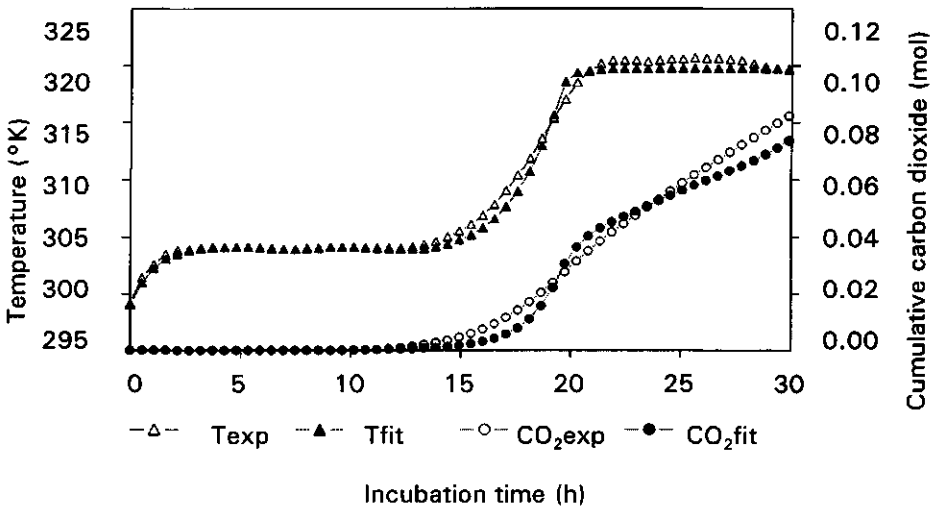

Figure 6. The temperature and cumulative carbon dioxide production predicted by the model together with experimental results (expt. 1) obtained in a packed-bed reactor during fermentation of soya beans with *Rhizopus oligosporus*, air flow rate 0.42 dm^3/min.

The model describes the experimental temperature and carbon dioxide profiles well during the first 30-40 hours of the fermentation, despite the fact that the packed bed is not completely homogeneous and the gas is not fully in equilibrium with the solids. The quantity of carbon dioxide produced in the batch experiments for determination of the specific growth rate (0.027 moles per 0.1 kg of wet beans) is reached after 17-23 hours in the packed-bed experiments. Although the growth

kinetics have not been experimentally validated from this point on, they appear to remain valid during the first 30-40 hours of the fermentation. The subsequent decline in observed carbon dioxide production rate and temperature is not predicted by the model. This must probably be attributed to the very simple reaction kinetics used in the model. Substrate, water or space may become limiting; these effects have not been included in the model.

Frequently, the logistic growth law is assumed for modelling growth of fungi (e.g. Saucedo-Castañeda *et al.*, 1990). The temperature decrease (after 30 h of incubation) shown in Figure 4a cannot be predicted by using the logistic growth law in our model: the predicted temperature decline is always too sharp. Therefore, a more complex model will be necessary to describe solid-state cultivation of *R. oligosporus* on soya beans. We are currently studying growth kinetics in more detail.

CONCLUSIONS

1) The use of packed-bed reactors to study heat removal by means of forced aeration is limited due to the formation of temperature gradients (max 0.5 °C/cm) and shrinkage of the substrate, therefore the assumption that growth is homogeneous in the reactor will not hold for longer fermentation times.

2) The model predictions were fairly accurate, if initial amount of biomass and the substrate composition were estimated by trial and error.

3) The reaction kinetics used in the model are still too simple, the effects of substrate, water and space limitations on growth should be included into the model, in order to describe the decline in activity after approx. 30 hours.

NOMENCLATURE

Parameter	Units	Meaning
b	$h^{-1/2}$ $°C^{-1}$	constant in specific growth rate equation
c	$°C^{-1}$	constant in specific growth rate equation
c_{pa}	J mol^{-1} $°C^{-1}$	heat capacity dry air
c_{pr}	J $Cmol^{-1}$ $°C^{-1}$	heat capacity of reactor
c_{ps}	J kg^{-1} $°C^{-1}$	heat capacity of substrate (all solids)
c_{pw}	J mol^{-1} $°C^{-1}$	heat capacity of water

c_{pwv}	J mol^{-1} $°C^{-1}$	heat capacity of water vapour
c_{px}	J $Cmol^{-1}$ $°C^{-1}$	heat capacity of biomass
c_{wi}	mol mol^{-1}	ration between water vapour and dry air concentration in fresh air
c_w	mol mol^{-1}	ration between water vapour and dry air concentration in off-gas
d_p	m	particle size
ID	m^2/s	diffusion coefficient gas
ΔH_o	J mol^{-1}	oxidation enthalpy
ΔH_w	J mol^{-1}	oxidation enthalpy of water at 0 °C
c_{wo}	[mol/m^3]	water vapour concentration off gas
F_a	mol $^{h-1}$	flow rate
$k_g a$	s^{-1}	mass transfer coefficient
λ	[h]	lag phase
mu	h^{-1}	specific growth rate
M_c	Cmol	cumulative amount of carbon dioxide
M_r	kg	weight of reactor
M_s	Cmol	amount of substrate
M_w	mol	amount of intra-plus extra- cellular water
M_x	Cmol	amount of biomass
m_s	Cmol $Cmol^{-1}$ h^{-1}	maintenance coefficient
nh		number of hydrogen atoms per Cmol substrate
no		number of oxygen atoms per Cmol substrate
P_o	mol	initial amount of CO_2
P_{max}	mol	maximal amount of CO_2
P(t)	mol	amount of CO_2
r_{com}	W	heat flux from compressor to reactor
r_C	Cmol h^{-1}	carbon dioxide production rate
r_O	mol h^{-1}	oxygen production rate
r_q	J h^{-1}	heat production rate
r_S	Cmol h^{-1}	substrate production rate
r_W	mol h^{-1}	water production rate
r_X	Cmol h^{-1}	biomass production rate
Sh	[-]	Sherwood number
t	h	time
T	°C	reactor temperature
T_i	°C	fresh air temperature
T_{max}	°C	constant in specific growth rate equation
T_{min}	°C	constant in specific growth rate equation
T_s	°C	incubator temperature

U_wA_w	W °C^{-1}	product of reactor surface area and overall heat transfer coefficient
X_0	Cmol	initial biomass concentration
X_{max}	Cmol	maximum biomass concentration
X(t)	Cmol	amount of biomass
Y_C	Cmol Cmol^{-1}	ratio between carbon dioxide production and substrate consumption (growth- related)
Y_{Cm}	Cmol Cmol^{-1}	ratio between carbon dioxide production and substrate consumption (maintenance- related)
Y_N	mol Cmol^{-1}	ratio between nitrogen source consumption and substrate consumption (growth-related)
Y_O	mol Cmol^{-1}	ratio between oxygen consumption and substrate consumption (growth-related)
Y_{Om}	mol Cmol^{-1}	ratio between oxygen consumption production and substrate consumption (maintenance-related)
$Y_{P/X}$	Cmol Cmol^{-1}	ratio between carbon dioxide production and biomass production
Y_W	mol Cmol^{-1}	ratio between water production and substrate consumption (growth-related)
Y_{Wm}	mol Cmol^{-1}	ratio between water production and substrate consumption (maintenance-related)
Y_x	Cmol Cmol^{-1}	ratio between biomass production and substrate consumption

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APPENDIX 1. CONSEQUENCES OF SIMPLIFYING THE LOGISTIC GROWTH MODEL

The original equation for the amount of product is based on the logistic law for the amount of biomass and the assumption that the ratio between product and biomass formation is constant:

Logistic law for biomass $t < \lambda \qquad X(t) = X_0$

$$t \geq \lambda \qquad X(t) = \frac{X_0 \cdot X_{max}}{(X_{max} - X_0) \cdot e^{-\mu \cdot (t-\lambda)} + X_0} \tag{A2.1}$$

Assumption $$P(t) - P_0 = Y_{PX} \cdot (X(t) - X_0) \tag{A2.2}$$

Logistic law for product $t < \lambda \qquad P(t) = P_0$

$$t \geq \lambda \qquad P(t) = (P_0 - Y_{PX} \cdot X_0) + \frac{Y_{PX} \cdot X_0 \cdot X_{max}}{(X_{max} - X_0) \cdot e^{-\mu \cdot (t-\lambda)} + X_0} \tag{A2.3}$$

In order to improve the reliability of the fitting program used to determine the specific growth rate μ and the length of the lag phase λ from batch experiments, the equation for the amount of product derived from the logistic growth law was simplified. The assumption underlying the simplification and the simplified equation for the amount of product are given below:

Simplifying assumption $P'(t) = Y_{PX} \cdot X(t)$ for all values of time. (A2.4)

Simplified equation $t < \lambda \qquad P(t) = P_0$

$$t \geq \lambda \qquad P'(t) = \frac{P_0 \cdot P_{max}}{(P_{max} - P_0) \cdot e^{-\mu \cdot (t-\lambda)} + P_0} \tag{A2.5}$$

In order to simplify an evaluation of the consequences of the simplification in situations where the assumption is not valid, the original and simplified equations have been made dimensionless, using the following dimensionless groups:

$$\psi_0 = \frac{P_0}{Y_{PX} \cdot X_0} \qquad \chi_0 = \frac{X_0}{X_{max}} \qquad \pi_0 = \frac{P_0}{P_{max}} \qquad \pi_t = \frac{P(t)}{P_0} \qquad \pi'_t = \frac{P'(t)}{P_0}$$

$$\theta = \mu \cdot t \qquad \kappa = \mu \cdot \lambda \tag{A2.6}$$

The two dimensionless equations are (in Mathcad notation):

Original equation (A2.7)

$$\pi_t(\theta, \psi_0, \chi_0, \kappa) := \text{if}\left[\theta < \kappa, 1, \left(1 - \frac{1}{\psi_0}\right) + \frac{1}{\psi_0 \cdot (1 - \chi_0) \cdot \exp(-\theta + \kappa) + \psi_0 \cdot \chi_0}\right]$$

Simplified equation $\pi'_t(\theta, \pi_0, \kappa) := \text{if}\left[\theta < \kappa, 1, \frac{1}{(1-\pi_0)\cdot\exp(-\theta+\kappa)+\pi_0}\right]$ (A2.8)

From our experiments we know the values of P_0 and P_{max}, and hence that of π_0:

Initial amount of product $P_0 := 1.7\cdot10^{-5}$ Cmol

Maximal amount of product $P_{max} := 4.06\cdot10^{-3}$ Cmol

Hence $\pi_0 := \frac{P_0}{P_{max}}$ $\pi_0 = 4.2\cdot10^{-3}$ $\frac{1}{\pi_0} = 239$

The value of ψ_0 is difficult to measure exactly, because this requires a measurement of the initial amount of biomass (X_0) as well as the ratio between product and biomass formation (Y_{PX}). We have estimated ψ_0 based on dry weight measurements in larger quantities (20 cm^3) of spore suspension and an assumption regarding Y_{PX}:

Measured initial amount of biomass $X_0 := 6.9\cdot10^{-7}$ Cmol per 15 initial wet bean weight

Assumed yield $Y_{PX} := 0.5$ Cmol product per Cmol biomass (Roels, 1983)

Estimated value of ψ_0 $\psi_0 := \frac{P_0}{Y_{PX}\cdot X_0}$ or $\psi_0 = 49$

Note that χ_0 and π_0 are related. Eqn (A2.2) gives:

$\chi_0 := \left[\psi_0\cdot\left(\frac{1}{\pi_0}-1\right)+1\right]^{-1}$ or $\chi_0 = 8.5\cdot10^{-5}$

The predicted values of π_t and π'_t for four values of ψ_0 are shown in Figure A2.1. Note that the measured value of π_0 has been used in all calculations.

For values of ψ_0 ranging from 10 to 100, i.e. in the order of magnitude of the estimated value in our experiments, the main effect of the simplification is a shift of the curve to the left. The slope of the straight part of the curve is relatively unaffected. In other words, if we fit π'_t to data that obey π_t, the lag phase will be overestimated, but the specific growth rate will still be reasonably accurate. Values of ψ_0 below 1 may give more serious problems in fitting, as the shape of the straight part of the curve changes significantly.

In order to verify this qualitative conclusion, the consequences of the simplification were determined by fitting π'_t to 'data' generated with π_t, using our program Logifit. The measured value for π_0 (4.2 10^{-3}), the estimated value for ψ_0 (50) and a neglegible lag phase ($\kappa = 0$) were used to calculate π_t. In order to

allow for a deviation between the specific growth rate used in π_t and the specific growth rate giving the best fit of $\pi`_t$, a dummy parameter $\alpha$ was introduced in the equation for $\pi`_t$, which represents the ratio of the fitted and original specific growth rates.

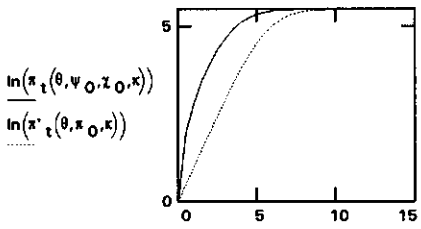
The equation used for fitting is:

$$\pi`_t(\theta, \pi_0, \kappa) = \text{if}\left[\theta < \kappa, 1, \frac{1}{(1-\pi_0)\cdot\exp(\alpha\cdot(-\theta+\kappa)) + \pi_0}\right] \qquad \text{(A2.9)}$$

with $$\alpha = \frac{\mu_{fit}}{\mu} \qquad \text{(A2.10)}$$

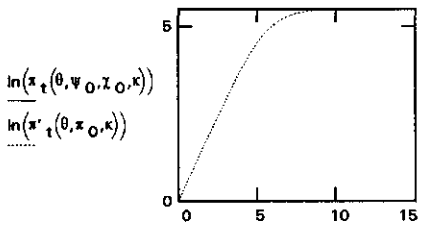
Figure A2.2 shows a graphical comparison of π_t and the fitted $\pi`_t$. It is clear that a reasonably accurate fit can be obtained. Table A2.1 shows the parameter values obtained from Logifit. If the estimated value of ψ_0 is correct, the simplified model still gives a reasonably accurate value for the specific growth rate (deviation -12%), but it gives a gross overestimation of the lag phase (dimensionless lag phase $\kappa=\mu\lambda=3.5$). The simplified model $\pi`_t$ does not give information about the initial amount of biomass.
Conclusion: useful values for μ can be expected, but the value for λ will be an artefact.

$\theta := 0, 0.5 .. 15 \quad \pi_0 = 4.2 \cdot 10^{-3} \quad \psi_0 := 0.1 \quad \chi_0 := \left[\psi_0 \cdot \left(\frac{1}{\pi_0} - 1 \right) + 1 \right]^{-1} \quad \chi_0 = 4 \cdot 10^{-2} \quad \kappa := 0$



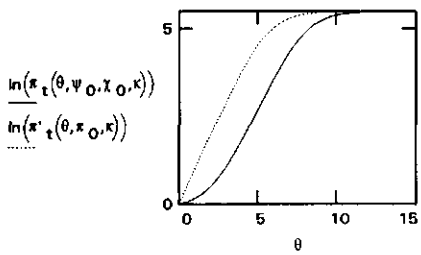
Large inoculum or small initial amount of product.

$\theta := 0, 0.5 .. 15 \quad \pi_0 = 4.2 \cdot 10^{-3} \quad \psi_0 := 1 \quad \chi_0 := \left[\psi_0 \cdot \left(\frac{1}{\pi_0} - 1 \right) + 1 \right]^{-1} \quad \chi_0 = 4.2 \cdot 10^{-3} \quad \kappa := 0$



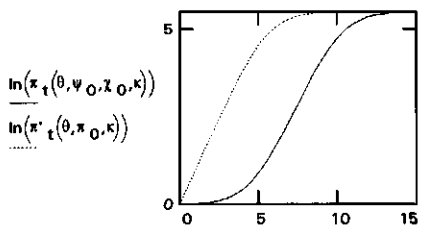
Both lines coincide, because eqn (A2.4) is fulfilled.

$\theta := 0, 0.5 .. 15 \quad \pi_0 = 4.2 \cdot 10^{-3} \quad \psi_0 := 10 \quad \chi_0 := \left[\psi_0 \cdot \left(\frac{1}{\pi_0} - 1 \right) + 1 \right]^{-1} \quad \chi_0 = 4.2 \cdot 10^{-4} \quad \kappa := 0$



Small inoculum or large initial amount of product.

$\theta := 0, 0.5 .. 15 \quad \pi_0 = 4.2 \cdot 10^{-3} \quad \psi_0 := 100 \quad \chi_0 := \left[\psi_0 \cdot \left(\frac{1}{\pi_0} - 1 \right) + 1 \right]^{-1} \quad \chi_0 = 4.2 \cdot 10^{-5} \quad \kappa := 0$



Even smaller inoculum or larger initial amount of product.

Figure A2.1 Predicted values of π_t and π'_t for ψ_0 = 0.1, 1, 10 and 100, and π_0 = 4.2 10^{-3} and $\kappa = 0$.

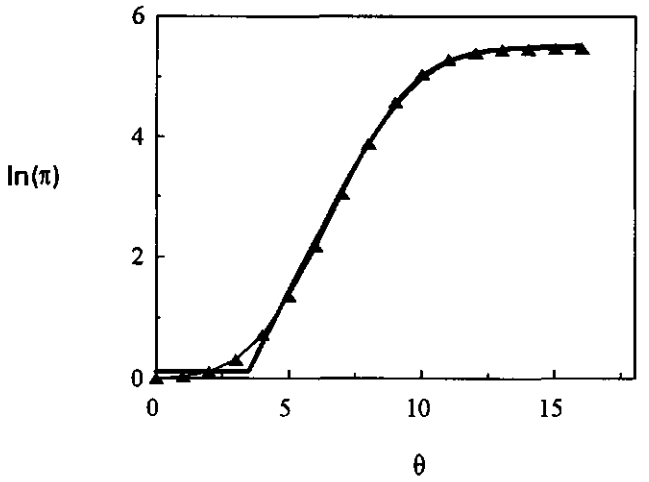


Figure A2.2 Natural logarithm of the dimensionless product concentration π versus the dimensionless time θ. Symbols and thin line show the values calculated with π_t, using the measured value for π_0 ($4.2\ 10^{-3}$), the estimated value for ψ_0 (50) and $\kappa = 0$. The fat line shows the values calculated with $\pi`_t$. Parameter values for $\pi`_t$ were determined by fitting the 'data' (symbols) using Logifit.

Table A2.1 Parameter values obtained by fitting $\pi`_t$ on the 'data' (symbols in Figure A2.2) generated with π_t, using Logifit.

	value found with $\pi`_t$	original value used in π_t	deviation (%)
π_0	$4.5 \cdot 10^{-5}$	$4.2 \cdot 10^{-5}$	7
α	0.88	1	-12
κ	3.5	0	nd
ψ_0	nd	50	nd

CHAPTER 5

INFLUENCE OF ACIDITY AND INITIAL SUBSTRATE TEMPERATURE ON GERMINATION OF *RHIZOPUS OLIGOSPORUS* SPORANGIOSPORES DURING TEMPE MANUFACTURE

ABSTRACT

During the soaking of soya beans according to an accelerated acidification method organic acids were formed, resulting in a pH decrease from 6.0 to 3.9. After 24 h of fermentation at 30 °C lactic acid was the major organic acid (2.1 % w/v soak water), while acetic acid (0.3% w/v soak water) and citric acid (0.5% w/v soak water) were also found. During cooking with fresh water (ratio raw beans: water, 1:6.5) the concentrations of lactate/lactic acid and acetate/acetic acid in the beans were reduced by 45 % and 51 %, respectively.

The effect of organic acids on the germination of *Rhizopus oligosporus* sporangiospores was studied in liquid media and on soya beans. Germination in aqueous suspensions was delayed by acetic acid: within six hours no germination occurred at concentrations higher than 0.05 % (w/v incubation medium), at pH 4.0. When soya beans were soaked in the presence of acetic acid, the inhibitory concentration depended on the pH after soaking. Lactic acid and citric acid enhanced germination in liquid medium, but not in tempe.

Inoculation of soya beans with *R. oligosporus* at various temperatures followed by incubation at 30 °C resulted in both increased and decreased periods for the lag phase of fungal growth. A maximum difference of 3 hours lag phase was found between initial bean temperatures of 25 and 37 °C.

When pure cultures of homofermentative lactic acid bacteria were used in the initial soaking process, less lactic acid and acetic acid were formed during soaking than when the accelerated acidification method was used. This resulted in a reduction of the lag phase before growth of *R. oligosporus* by up to 4.7 h.

This chapter has been accepted as:
Influence of acidity and initial substrate temperature on germination of *Rhizopus oligosporus* sporangiospores in tempe fermentation.
J.C de Reu, F.M. Rombouts and M.J.R. Nout
Journal of Applied Bacteriology

INTRODUCTION

Tempe is an Indonesian food product prepared by fermenting dehulled cooked soya beans with the fungus *Rhizopus oligosporus*. The manufacturing process (Figure 1) was reviewed by Ko and Hesseltine (1979), Winarno and Reddy (1986) and Nout and Rombouts (1990).

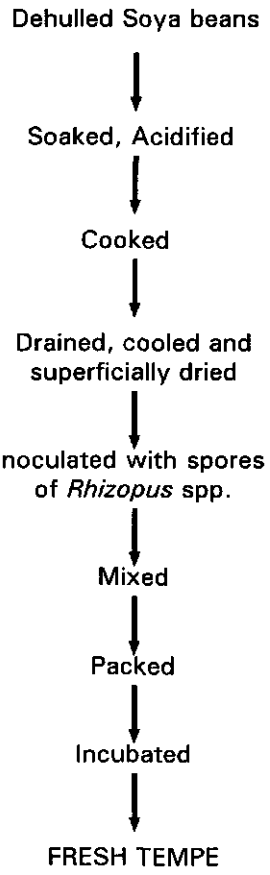


Figure 1. Principle of the tempe process (adapted from Nout and Rombouts, 1990)

During the traditional process in Indonesia the soya beans undergo a natural microbial fermentation during the hydration (soaking) process (Winarno and Reddy,

1986). During this period of 12 - 48 h at approx. 28°C, the microflora residing in the beans and the soaking vessels acts as a starter for mixed lactic/acetic acid fermentations that lower the pH from 6.5-7.0 (initial) to 4.5-5.5. The predominant species occurring during such soaking operations include the lactic acid bacteria *Lactobacillus casei, Enterococcus faecium* and *Streptococcus dysgalactiae* as well as other bacteria e.g. *Staphylococcus epidermidis, Klebsiella pneumoniae, Kl. ozaenae, Enterobacter cloacae, E. agglomerans, Citrobacter diversus* and *Bacillus brevis*, and the yeasts *Pichia burtonii, Candida diddensiae* and *Rhodotorula rubra* (Mulyowidarso *et al.*, 1989). In temperate climates with a different natural microflora as well as a more stringent disinfection of equipment, the natural acidification of soya beans does not always occur, or is very slow. Poor acidification and centrifugal washing/cooling results in cotyledons of near neutral pH and high moisture content providing excellent conditions for unwanted acid-sensitive bacteria, e.g. *Bacillus* spp. and Enterobacteriaceae (Nout and Rombouts, 1990).

The importance of raw material pH is illustrated by the investigation of Tanaka *et al.* (1985) who carried out challenge tests with *Clostridium botulinum, Staphylococcus aureus, Salmonella typhimurium* and *Yersinia enterocolitica* in non-acidified soya beans inoculated with *R. oligosporus* NRRL 2710. Under the conditions used, these harmful organisms were able to grow and some produced their toxins.

Steinkraus *et al.* (1965) advocated the addition of lactic acid or acetic acid during soaking in order to reduce chances of microbial spoilage. Soaking of beans (1 kg dry beans: 3 l of water) with 0.125 % (v/v) acetic acid was insufficient to prevent bacterial spoilage. In an attempt to control bacterial contamination of tempe, soaking in 0.25 % (v/v) acetic acid gave promising results, while soaking in a 0.5 % (v/v) acetic acid solution required twice the normal time for fermentation (Steinkraus *et al.*, 1965). Soaking in 1 % lactic acid solution suppressed microbial growth (final total counts $< 10^2$ cfu/ml soak water) and sliminess of the soak water (Nout *et al.*, 1985). Chemical acidification (soaking in 1 % lactic acid) gave a poor tempe quality with mixed culture usar-style starter (Nout *et al.*, 1992) and acceptable quality with pure culture of *R. oligosporus* NRRL 5905.

Nout *et al.* (1987) found that inoculation with soak water of a previous batch provided a method of obtaining a vigorous lactic acid fermentation, under laboratory conditions. *Lactobacillus plantarum* was the major species in the soak water of soya beans treated with this method of accelerated acidification.

Mulyowidarso *et al.* (1991) investigated the concentrations of organic acids and carbohydrates in soak water and soya beans during the traditional soaking method involving a natural fermentation. They found that lactic acid (0.65 % w/v) and malic acid (0.35 % w/v) were the major acids, after 24 h of soaking with natural fermentation.

The physiological requirements of *R. oligosporus* sporangiospores for germination were studied by Medwid and Grant (1984). They found that the optimum conditions for germination were 42°C and pH 4.0. Initial swelling occurred only in the presence of a suitable carbohydrate. Subsequent production of germ tubes required sources of both carbon and nitrogen.

One of the major factors determining the process capacity of tempe production is the lag time before the fungal fermentation stage (varying from 10 to 24 h, at 30°C). Removal of the skin of beans, the availability of oxygen, the relative humidity, the pH of the soaked beans and temperature were major factors influencing mould growth (Steinkraus *et al.*, 1960).

The aim of this study was to determine the concentrations of organic acids present in soya beans after soaking with bacterial fermentation or acidification and after cooking, and to investigate the effect of these organic acids at representative levels, and of temperature on germination of sporangiospores of *R. oligosporus* in liquid suspensions and on soya beans. The knowledge obtained should provide the means to reduce or standardize the lag phase which would result in a better process control.

MATERIALS AND METHODS

Cultures

Rhizopus oligosporus NRRL 5905 was grown (7 days at 30 °C) and maintained on Malt extract agar (MEA, Oxoid CM59) slants. Sporangiospore suspensions were prepared as described previously (De Reu *et al.*, 1993). The viable count varied between 5 x 10^5 and 1 x 10^6 colony forming units/ml. For each series a fresh sporangiospore suspension was prepared.

Pure cultures of *Lactobacillus plantarum, Pediococcus pentosaceus* or *Lactobacillus acidophilus* were isolated from soya bean soak water and identified as described by Hounhouigan *et al.* (1993). The pure cultures were harvested from MRS-broth (Oxoid, CM 359) which was incubated for 2 days at 30 °C.

Preparation of tempe

1. Soaking conditions

Tempe was prepared from dehulled yellow soya beans (*Glycine max*). Raw beans, 300 g, were soaked with 900 ml of water for 24 h under the following sets of conditions: (I) at 5°C, to minimize microbial action; (II) at 5°C with the addition of acetic acid (0.125, 0.25 or 0.5 % w/v) to the soak water (III) at 5°C with the addition of lactic acid (1.5 %) and acetic acid (0, 0.125, 0.25 or 0.5 % w/v) to the soak water (IV) at 30 °C with the addition of lactic acid (2.0 % w/v) to the soak water (V) at 30 °C with the addition of acetic acid (0.5 % w/v) to the soak water (VI) at 30 °C with the addition of soaking water from an accelerated acidification (Nout *et al.*, 1987) (10 % v/v) to the soak water (VII) at 30 °C with the addition of 90 ml pure cultures of *L. plantarum*, *P. pentosaceus* or *L. acidophilus*, to 810 ml of soak water.

2. Cooking

After soaking the beans were rinsed gently with approximately 0.5 l of tap water, drained, and cooked in 2 l of tap water in an uncovered pan (5l; diam. 25 cm) for 20 min boiling time. The water was then drained away and the beans were spread on a wire mesh at room temperature to cool.

3. Fermentation of cooked beans

The cooked beans were inoculated with *R. oligosporus* by mixing a sporangiospore susension (1 % v/w) with 450 g of cooked beans which were then packed into a perforated box (lxhxw: 20x3x10 cm) and incubated at 30 °C in an incubator with forced ventilation (IKS, Leerdam, The Netherlands).

In order to determine the effect of the initial bean temperature of the cooked beans on germination of sporangiospores of *R. oligosporus*, beans that had been soaked by method (VI), cooked and dried were transferred to polythene bags and incubated at 25°, 30°, 37° or 42° for 1 h were inoculated, packed into a perforated box, re-incubated at the same temperature for 1 h then transferred to 30°C for further incubation.

Determination of pH and organic acids

The pH of duplicate samples of soak water and of soaked soya beans was measured with a pH meter (WTW, type 522, Weilheim, Germany) and a pH electrode (Schott-Geräte, type N61, Hofheim a.Ts., Germany).

To determine organic acids, soak water samples were deproteinated by the addition of 5 % trichloroacetic acid to give a final concentration of 0.1% (v/v).

After centrifugation (10 min, 1350 g) the supernatants were stored at -20°C until analysis. Just prior to HPLC analysis, the thawed supernatant was filtered through 8 μm filters (Millipore, SCWP04700, Ireland) and 0.45 μm filters (Schleicher & Schuell, FP030/2, Dassel, Germany) filters. Organic acids were determined by HPLC using an Aminex HPX-87H stainless steel column (Biorad) and a refractive index detector (Shodex, SE-61) at 35 °C. The acids were eluted with H_2SO_4, 0.005 mol l^{-1} as the mobile phase and a flow rate of 0.6 ml min^{-1}.

Determination of titratable acidity

To determine titratable acidity, 10 g of soya beans were homogenized with 90 ml distilled water in a Waring blender (30 sec at high speed). This mixture was titrated to pH 8.5 using 0.1 mol l^{-1} NaOH and maintained at that level for 5 min. Subsequently the total volume of NaOH used was measured, and the titratable acidity was expressed as ml 0.1 mol l^{-1} NaOH per g of sample. Duplicate determinations were made.

Determination of the effect of pH, organic acids and temperature on germination of sporangiospores of *R. oligosporus*

Sporangiospore suspensions (1 ml) were centrifuged for 2 min at 10,000 g in an Eppendorf centrifuge (Microcen 13, Herolab, Wiesloch, Germany). The pellets were resuspended in the required media.

The basal medium for spore germination consisted of a solution of 0.37 % w/v glucose, 0.09 % w/v $(NH_4)_2SO_4$ and 0.1 % w/v $MgSO_4.7H_2O$ sterilized by autoclaving 20 min at 121°C, to which 0.1 % v/v of filter-sterilized stock solution of minerals (0.2 mg $FeSO_4.7H_2O$, 0.2 mg $ZnSO_4.7H_2O$ and 0.2 mg $MnSO_4.4H_2O$ per ml) was added. To the basal medium, lactic acid (90 % (DL), Cat no. 366 Merck, Germany) or acetic acid (100%, Cat no. 63, Merck, Germany) or citric acid (Cat no. 244, Merck, Germany) were added in concentrations ranging from 0 - 1.5 % w/v incubation medium. Subsequently, the pH of the medium was adjusted to pH 4.0 $\pm$ 0.05 using 0.5 mol l^{-1} HCl or 0.5 mol l^{-1} NaOH, the resulting media were filter-sterilized (0.45 μm) and 1 ml was added to the pellet of a 1 ml sporangiospore suspension. The spores were resuspended and the suspensions were incubated in a water bath at 25°, 30°, 37° or 42 °C. At hourly intervals each sample was mixed, 20 μl was removed to measure germination, and the incubation was continued. Germination was defined as the extension of a germ tube to a length equal to one-half the diameter of the spore (Medwid and Grant, 1984). The percentage of germinated spores was determined microscopically (Carl

Zeiss, Standard 20, 400 x, Germany). For each sample 8 microscopic fields with at least 10 spores were counted. The average percentage of germination and the standard deviation were calculated. The Student's t-test was used to evaluate statistically significant differences between the results.

Measurement of germination of, and growth from, sporangiospores of *R. oligosporus* during production of tempe

Because direct measurement of the germination of the fungal spores on soya beans is impractical, increase in temperature of tempe was used as an indicator of germination of spores and growth of the fungus. The temperatures were monitored with thermocouples (Tempcontrol, Voorburg, The Netherlands) in the centre (h 1.5 cm, 5 cm from the longest side, 8 cm from the shortest side) of the box. Thermocouples were connected with a data logger (Squirrel 1205 Grant, U.K.). The data were processed using Lotus 1-2-3 (Lotus Development Corporation, Cambridge, MA, USA). The lag phase was defined as the time required to reach the stage of exponential temperature increase.

RESULTS

Bacterial fermentation, pH and organic acids in soak water

Figure 2 shows changes of pH and the concentrations of organic acids in soak water when soaking method (VI) was used. During the first 15 h of soaking, the pH decreased rapidly towards pH 3.9. After 15 h of soaking a further increase in organic acid concentrations did not affect the pH value very much.

Table 1. The formation of acetic and lactic acids in soak water during the soaking of soya beans with pure cultures and with accelerated acidification after 24 h at 30 °C in a non-agitated system

Strain	pH after soaking	acetic acid (% w/v)	lactic acid (% w/v)
Lactobacillus plantarum	4.15	0.14	1.78
Lactobacillus acidophilus	4.20	0.14	1.61
Pediococcus pentosaceus	4.24	0.16	1.56
Accelerated acidification	4.12	0.29	2.14

Lactic acid was the major organic acid in soak water: after 24 h of soaking 2.1 % (w/v soak water) was found. Acetic acid increased from 0 to 0.3 % (w/v soak water) after 24 h of soaking. During soaking the amount of citric acid varied between 0.35 and 0.5 % (w/v soak water). Other organic acids were not detected.

Table 1 shows the acidification of soak water using pure cultures of homofermentative lactic acid bacteria compared to the accelerated acidification method. The data represent averages of duplicate results. Compared with the accelerated acidification method, soaking of soya beans with pure cultures of lactic acid bacteria resulted in less total acidity with relatively less acetic acid.

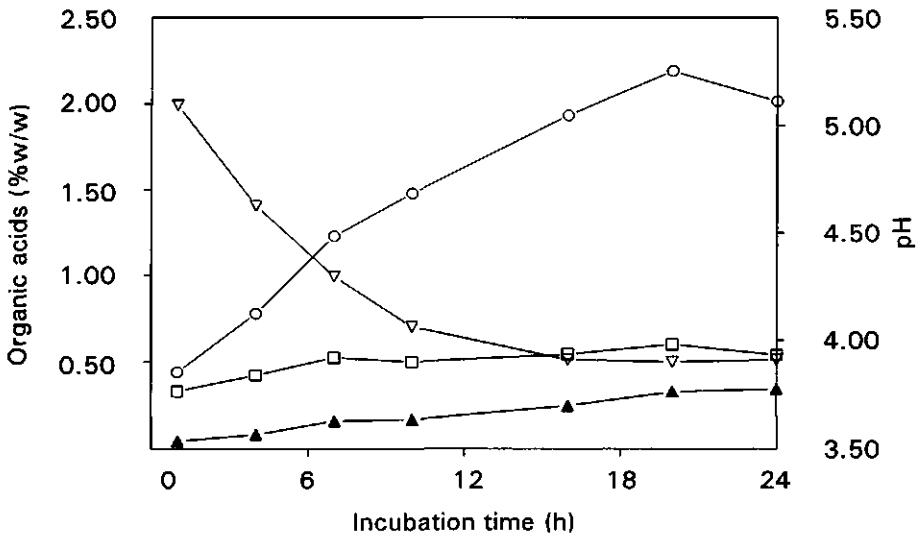


Figure 2. Evolution of pH and organic acids (% w/w soak water) during soaking with accelerated acidification of soya beans at 30 °C. -▲- acetic acid; -○- lactic acid ; -□- citric acid ; -▽- pH

Reduction in concentration of organic acids during cooking

After soaking in 2.0 % (w/v soak water) lactic acid at 5 °C or in 0.5 % (w/v soak water) acetic acid, the beans were rinsed with tap water and cooked in fresh tap water. The decrease of titratable acidity in beans is shown in Table 2. The results were based on duplicate trials. A reduction of 45 % of titratable acidity in soya beans soaked in 2 % (w/v soak water) lactic acid was observed after 20 minutes of boiling. For acetic acid, a reduction of 51 % was found. After the major

reduction between 0 and 20 minutes cooking a slight apparent increase of titratable acidity was found. This might be caused by a different buffering capacity resulting from permeability alterations due to prolonged cooking.

Table 2. Reduction of titratable acidity (ml 0.1 mol/l NaOH (g sample)$^{-1}$) by cooking and cooling of non-acidified soya beans and soya beans acidified by soaking in lactic acid and acetic acid*

	Titratable acidity				
	Control	Soaked in 2.0 % (w/v) lactic acid		Soaked in 0.5 % (w/v) acetic acid	
Pretreatment: Cooking Time (min)	(I) (ml/g)	(IV) (ml/g)	(%)	(V) (ml/g)	(%)
0	0.42	1.70	100	1.00	100
20	0.16	0.93	55	0.49	49
40	0.17	0.98	58	0.53	53
60	0.17	0.99	58	0.53	53

* The figures are the averages of duplicate results.

The effect of organic acids and temperature on germination of sporangiospores of *R. oligosporus* in liquid media

Based on the observed concentrations of organic acids in soaked and cooked beans, the effects of lactic acid, acetic acid and citric acid on spore germination were investigated in liquid media.

In Table 3 the effects of lactic, acetic and citric acids, the spore density of the suspension, and the incubation temperature on the germination of *R. oligosporus* sporangiospores are shown. After 2 hours of incubation the various stages of fungal growth became visible, viz. spore swelling, germ tube formation, hyphal extension and branching. The percentage of germinated spores increased with time of incubation up to 6 hours, after which it became very difficult to distinguish between germinated and non-germinated spores, due to the dense network formed by the growing hyphae. After 6 hours of incubation the pH decreased from 4.0 to 3.6.

Table 3. The effect of lactic acid, citric acid and acetic acid on germination (%) of spores of *Rhizopus oligosporus* incubated at 30 °C for six hours in a liquid medium* adjusted to pH 4.0, and the effect of temperature on germination

Expt	Sample	Acid concentration %(w/v medium)	Spores/field	Germination percentage (%)
1	control	0.00	13.0 ± 3.3 †	45.1 ± 14.1†
	lactic acid	0.50	12.5 ± 3.9	59.2 ± 7.6
	lactic acid	1.00	12.8 ± 2.3	63.4 ± 11.7
	lactic acid	1.50	12.4 ± 2.8	56.5 ± 15.1
2	control	0.00	29.5 ± 6.5	19.1 ± 3.3
	citric acid	0.25	24.1 ± 6.8	28.3 ± 7.9
	citric acid	0.50	19.4 ± 3.4	37.7 ± 7.4
	citric acid	1.00	20.8 ± 3.9	17.5 ± 7.6
3	control	0.00	18.1 ± 3.2	42.7 ± 5.1
	acetic acid	0.05	15.5 ± 6.3	10.6 ± 5.6
	acetic acid	0.10	23.6 ± 10.2	0.5 ± 1.4
	acetic acid	0.15	22.4 ± 10.8	0.0 ± 0.0
4	control	0.00	17.9 ± 4.8	43.4 ± 10.3
	lactic acid	1.00	13.3 ± 3.3	50.2 ± 5.8
	lactic acid	1.50	11.5 ± 2.3	64.0 ± 12.4
	citric acid	0.25	16.4 ± 4.4	46.0 ± 8.7
	citric acid	0.50	20.4 ± 9.8	59.4 ± 13.7
	acetic acid	0.05	11.5 ± 1.6	22.6 ± 11.2
	acetic acid	0.10	12.7 ± 2.3	0.0 ± 0.0
5	10 * diluted		5.8 ± 2.8	70.1 ± 27.8
	control		59.3 ± 12.1	11.1 ± 4.6
	4 * concentrated		196.3 ± 20.0	2.4 ± 1.1
6	25 °C		20.6 ± 12.2	4.3 ± 3.0
	30 °C		39.5 ± 6.3	12.7 ± 6.5
	37 °C		31.2 ± 10.5	20.6 ± 6.6
	42 °C		44.7 ± 12.6	11.4 ± 5.3

* medium composition: 0.37 % w/v glucose, 0.09 % w/v $(NH_4)_2SO_4$, 0.1 % w/v $MgSO_4.7H_2O$ and (0.2 μg/ml of $FeSO_4.7H_2O$, $ZnSO_4.7H_2O$ and $MnSO_4.7H_2O$)

† mean (n = 8) ± S.D.

The addition of lactic acid (1.0 % w/v, Table 3, expt 1) to a liquid medium resulted in an significant ($p<0.05$) increase from 45.1 ± 14.1 to 63.5 ± 11.7 % germination. Citric acid at concentrations of 0.25 and 0.50 % (w/v incubation medium) (expts 2 and 4) increased ($p<0.05$) the germination percentage of *R. oligosporus* sporangiospores, to a similar extent as lactic acid.

In contrast, acetic acid (Table 3, expts 3 and 4) delayed the germination. No germination at all was found within 6 hours at concentrations of 0.10 and 0.15 % (w/v incubation medium), while only a low percentage of spores germinated at 0.05 % (w/v incubation medium) acetic acid.

In experiment 4 (Table 3) the effects of the three organic acids on germination of a single spore suspension were tested. After 6 hours of incubation a slight increase in the germination percentage was found for lactic (1.5 % w/v, $p<0.01$) and citric acid (0.5 % w/v, $p<0.05$), while with acetic acid germination remained significantly ($p<0.01$) lower.

The influence of the spore density on germination was also investigated. To that purpose, 10-fold diluted and 4-fold concentrated spore suspensions were compared with the usual spore suspension (Table 3, expt 5). The percentage of spores that germinated increased with increasing dilution. To investigate whether this phenomenon was due to substrate limitation, germination in Malt Extract Broth was measured; this medium is richer in organic carbon and nitrogen. Germination percentages increased significantly ($p<0.05$) from 43.9 ± 22.2 in our experimental medium to 74.0 ± 22.0 in the Malt Extract Broth (pH 4.0).

Germination percentages at 25°C were low, but increased with temperature to a maximum at 37° C (Table 3).

Changes in temperature during fermentation of cooked beans

After soaking and cooking, evaporative cooling reduced the temperature of the soya beans to approximately room temperature. Variations of initial bean temperatures influenced the growth rate and the length of the lag phase of fungal growth as measured by increase in temperature (Figure 3). A maximum difference of 3 hours was found between 37° and 25°C for the periods of most active heat production. However, if the incubation temperature was maintained at 25°C or 37°C a longer and a shorter lag phase, respectively were observed (data not shown).

The effects of various soaking conditions on the length of the period before excessive heat production (lag phase) during tempe fermentation are shown in Table 4. The addition of acetic acid to the soak water failed to cause a significant

increase in the lag phase (Table 4, expt A). A combination of 0.5 % (w/v) acetic acid and 1.5 % (w/v) lactic acid in the soak water resulted in an increase in the lag phase from 14.0 to 19.5 hours (Table 4, expt B).

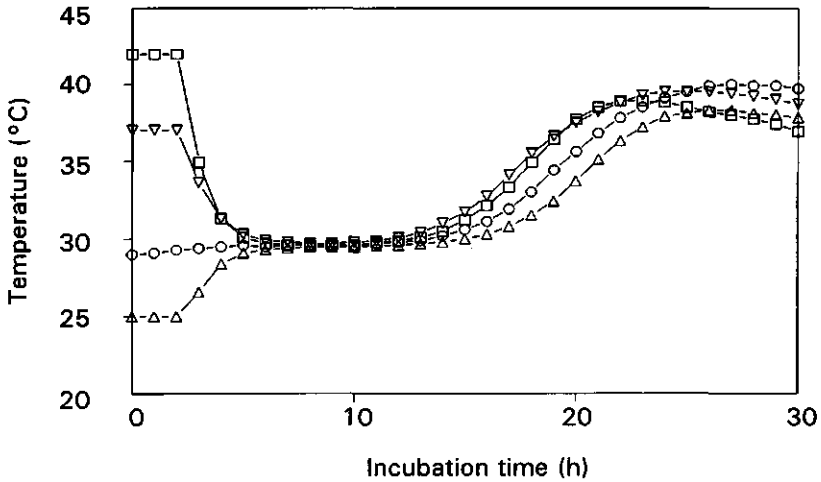


Figure 3 Evolution of temperature of soya beans undergoing fungal fermentation with *Rhizopus oligosporus*, as a function of the initial bean temperature:
△ 25 °C; ○ 30 °C; -▽- 37 °C; □ 42 °C

Based on expt B (Table 4) and expt 3 (Table 3) it is clear that acetic acid might inhibit the tempe fungal growth stage. To avoid such inhibition, two alternative approaches to soaking were tested. The first one was to use pure cultures of homofermentative lactic acid bacteria as a means of reducing the accumulation of acetic acid. Under anaerobic conditions homofermentative strains produce only lactic acid from hexoses, but as shown in Table 1 acetic acid was also found, implying oxidase activity and/or that certain substrates (e.g. pentoses) were available in the soak water enabling acetic acid formation. Compared with the method of accelerated acidification a reduction from 19.5 to 14.8 h in lag phase was seen when the beans were soaked with an inoculum of *P. pentocaseus* (Table 4, expt C). Compared with acidification by *P. pentosaceus* an increase of the fungal lag phase was found for beans soaked with *L. plantarum* and *L. acidophilus* from 14.8 to 16.3 and 16.5 h, respectively. A further reduction of the fungal lag phase was obtained with soaking in 2.0 % (w/v soak water) lactic acid. Soaking in 0.5 % (w/v soak water) acetic acid at 30 °C, and subsequent cooking in 0.5 % (w/w) acetic acid resulted in a lag phase beyond 48 hours.

Table 4. The effect of the soaking treatments on the pH of the soak water and of soya beans, the lag phase of *Rhizopus oligosporus* and the temperature increase during the tempe process. Incubation temperatures: expts A and B at 5 °C, expts C and D 30 °C.

Expt	Soak condition	Treatment	pH* after soaking	pH† after cooking	lag phase (h)	temperature increase (°C/h)
A	non acidified	I	6.57	6.85	11.4	1.7
	0.125 % (w/v) acetic acid	II	6.00	6.55	10.8	1.6
	0.25 % (w/v) acetic acid	II	5.55	6.15	10.5	1.6
	0.50 % (w/v) acetic acid	II	5.10	5.69	11.4	1.6
B	1.5 % (w/v) lactic acid	III	4.23	4.81	13.3	1.3
	1.5 % (w/v) lactic acid 0.125 % (w/v) acetic acid	III	4.24	4.77	14.0	1.2
	1.5 % (w/v) lactic acid 0.25 % (w/v) acetic acid	III	4.24	4.68	14.1	1.2
	1.5 % (w/v) lactic acid 0.50 % (w/v) acetic acid	III	4.24	4.60	19.5	1.2
C	*Lactobacillus plantarum*	VII	4.15	4.35	16.3	1.0
	Lactobacillus acidophilus	VII	4.20	4.45	16.5	1.0
	Pediococcus pentosaceus	VII	4.24	4.52	14.8	1.0
	Accelerated acidification (24 h)	VI	4.12	4.36	19.5	1.1
	Lactic acid (2.0 % w/v soak water)	IV	4.01	4.40	12	1.0
	Acetic acid (0.5 % w/v soak water)	V	4.73	4.57‡	> 48	0
D	Accelerated acidification (4 h)	VI	4.86	6.54	11.3	2.1
	Accelerated acidification (12 h)	VI	4.37	5.34	11.8	1.8
	Accelerated acidification (16 h)	VI	4.21	4.75	11.9	1.5
	Accelerated acidification (24 h)	VI	4.11	4.37	13.5	1.3

*) = soakwater; †) = soya beans; ‡) = cooked in 0.5 % (w/w water) acetic acid

The second alternative to prevent inhibitory concentrations of acetic acid is to reduce the soaking period during accelerated acidification, for example from 24 to 12 h. By doing this, spoilage of tempe by acid-sensitive bacteria might cause problems if the soya beans are not adequately acidified. A small reduction in the lag phase was found from 15.3 to 11.3 (h) if the soaking period was reduced from 24 to 4 h.

In Table 4 it is shown that the temperature increased faster when the soya beans were less acidified. A temperature increase varying between 1.0 to 1.5 °C/h was observed for beans with a pH between 4.35 and 4.8, while a maximum increase of 2.1 °C/h was found for beans (pH of beans 6.54) soaked during 4 hours according the method of accelerated acidification.

DISCUSSION

The formation of both lactic acid (2.1 % w/v soak water) and acetic acid (0.3 % w/v soak water) during soaking according to the method of accelerated acidification for 24 h at 30 °C, was expected since the major species in the soak water, *L. plantarum*, is a facultative heterofermentative lactic acid bacterium. Compared with the method of accelerated acidification, lower concentrations of acetic acid (0.14 % versus 0.29 % w/v soak water) were obtained with pure cultures of *L. plantarum*. This might be due to a higher initial count, or to adaptation of the microflora to the substrate method, or to accompanying heterofermentative lactic acid bacteria in the method of accelerated acidification. The increase of citric acid observed during the first six hours of soaking is possibly due to endogenous metabolism that would accompany the initial stages of bean germination (Mulyowidarso *et al.*, 1991). Compared with the results of Mulyowidarso *et al.* (1991) less variety in organic acids but higher concentrations of lactic acid (2.0 versus 0.65 % w/v soak water) and acetic acid (0.3 versus 0.08 % w/v soak water) were obtained using the accelerated method. This is due to the acidification method used. In the traditional soaking method with natural acidification a variety of microorganisms were active resulting in the production of 10 different organic acids. In a method with accelerated acidification, the higher initial count of lactic acid bacteria and the lower initial pH favour the production of lactic acid and acetic acid.

As expected, a reduction in organic acids during cooking was caused by leaching of free acid from the beans into the fresh water. Increasing the boiling time from 20 to 60 minutes did not result in further significant changes of titratable

acidity.

In liquid media acetic acid at concentrations of ≥ 0.05 % (w/v, at pH 4.0) delayed the germination of *R. oligosporus* sporangiospores. No comparable literature data are available. Slight positive effects on the germination percentages were found for 1.0-1.5 % (w/v) lactic acid and 0.25-0.50 % (w/v) citric acid.

Germination percentages of spores in liquid media varied between 45 % and 19 % in the control (expts. 1 and 2, Table 3), this was influenced by the spore density. This may have been due to either substrate limitation or self-inhibition. The formation of self-inhibitors affecting germination was reported earlier for *Aspergillus niger* by Barrios-González *et al.* (1989). At increased concentrations of the media components, no increases in the germination percentage were obtained compared with the control expertiments. Malt Extract Broth, a medium richer in organic carbon and nitrogen, yielded higher levels of germination, but 100 % germination was not achieved. Possibly, not all spores are able to germinate.

We found that the optimum temperature for germination was 37 °C, whereas 42 °C was reported by Medwid and Grant (1984) for *R. oligosporus* NRRL 2710. In Table 3 (expt 6) germination percentages increased when the temperature was increased from 25 ° to 37 °C. The same effect was seen in the experiments with the soya beans (Fig. 3). The lag phase before the excessive heat production on beans with an initial temperature of 37 °C was shorter compared with those equilibrated at 30 ° or 25 °C.

In soya beans, higher concentrations of acetic acid were necessary to achieve the same inhibitory effect as in liquid media. This can be explained by differences in the quantity of undissociated acetic acid. Eklund (1989) reported that levels of 0.1 % of undissociated acetic acid had an inhibitory action on growth of moulds. If the pH of the soya beans is above the pK_a of acetic acid (4.73), acetic acid became less inhibitory as shown in Table 4 (expt A). If combinations of acetic acid and lactic acid were used (expt B, Table 4) in the soak water of soya beans, the delaying effect of acetic acid (6.2 h) became less important than if beans had also been cooked in acetic acid (> 36h) (expt C, Table 4). This can be explained by the fact that in the latter case the concentration of free, undissociated acid in the beans will be higher.

A typical example of the complexity of lag phase prediction is the variation in time 13.5 versus 19.5 h, (Table 4, expts. C and D), obtained after soaking the beans according the same soaking treatment VI. As there were no significant differences in the pH of the soak water and the beans after cooking, factors other than the concentration of organic acids also influenced the lag phase. The

difference in lag phase was probably caused by the inoculum, as for each experiment a separate, fresh sporangiospore suspensions was prepared.

The use of pure cultures of homofermentative lactic acid bacteria is one alternative soaking method to obtain lower concentrations of acetic acid compared with the method of accelerated acidification. Based on the amounts of acetic acid (Table 1) no differences were expected between the different strains, but due to the higher concentrations of lactic acid the pH of the soya beans after cooking increased less for the beans soaked with lactobacilli than for those soaked with *P. pentosaceus*. Therefore the amounts of undissociated acetic acid were higher for the lactobacilli which resulted in an increased fungal lag phase.

In this study it was found that the lag phase of *R. oligosporus* during the tempe process depended on spore viability, temperature, concentration of undissociated organic acid and pH. By increasing the initial bean temperature and decreasing the quantity of acetic acid, a shorter lag phase can be obtained. To prevent spoilage by acid-sensitive bacteria, acidification is necessary. Addition of lactic acid to the beans results in a shorter lag phase (12 versus 19.5h, Table 4 expt C), but according to Nout *et al.* (1985) and Tünçel *et al.* (1989) soaking with fermentation is preferred to obtain tempe of superior quality. Thus pure cultures inocula of homofermentative lactic acid bacteria are a better way to ensure minimum processing time with adequate product quality compared with chemical acidification.

Furthermore, selection of conditions for germination of spores at maximum rate and extent may enable tempe manufacturers to minimize total processing time, and to reduce development of contaminating microorganisms during the critical initial period of fungal fermentation.

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CHAPTER 6

EFFECT OF OXYGEN AND CARBON DIOXIDE ON GERMINATION AND GROWTH OF *RHIZOPUS OLIGOSPORUS* ON MODEL MEDIA AND SOYA BEANS

ABSTRACT

The micro-colony technique enables studying the effects of several atmospheric conditions on fungal growth by measuring the radius of the colony, while excluding effects of those conditions on germination of the sporangiospores.

Various concentrations of oxygen and carbon dioxide in the gas environment were found to influence growth of *Rhizopus oligosporus* on Malt Extract Soya Peptone Agar. Maximum radial growth rate was 1.48 mm/h and the maximum specific growth rate was 0.109 (h^{-1}) at 30 °C. Oxygen became limiting below 1 % (v/v), but growth remained possible at levels of 0.001 % oxygen. Carbon dioxide stimulated growth at limiting oxygen levels. The specific growth rate increased from 0.043 (h^{-1}) at 0.5 % (v/v) oxygen and 0 % (v/v) carbon dioxide to 0.096 (h^{-1}) at 0.5 % (v/v) oxygen and 5 % (v/v) carbon dioxide. A mixture of 0.5 % (v/v) oxygen and 35 % (v/v) carbon dioxide inhibited growth. Delay of sporangiospore germination due to low (<0.001 %) amounts of oxygen was not observed with the techniques used.

Fungal activity in a Rotating Drum Reactor was stronger affected by low levels of oxygen than was biomass formation on model media. High concentrations of carbon dioxide inhibited growth in the Rotating Drum Reactor at non-limiting levels of oxygen. It is concluded that aeration and heat removal are both essential aspects of optimization of large-scale solid-substrate bioreactors with *Rhizopus oligosporus*.

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Effect of oxygen and carbon dioxide on germination and growth of *Rhizopus oligosporus* on model media and soya beans
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Applied Microbiology and Biotechnology

INTRODUCTION

Solid-substrate fermentations (SSF) are generally characterized by the growth of micro-organisms on water-insoluble substrates in the presence of varying amounts of free water (Mitchell and Lonsane, 1992). Solid-substrate fermentation deals with three different phases, a solid phase, a liquid phase bound to the solid matrix and a gaseous phase.

Tempe is an example of a static solid substrate fermentation, in which the inoculated soya beans are packed in 4-6 cm thick beds and covered with banana leaves or polyethylene sheets. Incubation takes 50-20 h at 25-37 °C. *Rhizopus* spp. are considered to be essential for tempe making (Nout and Rombouts, 1990). Rathbun and Shuler (1983) reported that in tempe, oxygen levels decreased to approx. 2 % (v/v) and carbon dioxide increased to 22 % (v/v). The availability of oxygen and the removal of carbon dioxide are determined by diffusion, which in turn is determined by porosity, particle size and consistency of the substrate (Alvarez-Martinez, 1987; Mitchell *et al.*, 1988). The effects of low concentrations of oxygen and high levels of carbon dioxide are not well described, but Hesseltine *et al.*, (1985) and Soccol *et al.*, (1994) reported that 16, respectively 19 different strains of *Rhizopus spp.* were able to grow under anaerobic conditions.

Steinkraus *et al.*, (1965) reported that when the layer of fermenting beans was thicker than approximately 5 cm, mould growth became less rapid and less heavy in the centre of the bean mass than it was in thin layers. According to Steinkraus this phenomenon was due to the fact that oxygen was essential to the growth of the mould. However, reaching the maximum growth temperature could be another reason for poor growth.

Mitchell (1992) reviewed models on growth patterns, growth kinetics and growth in solid-substrate cultivation and concluded that presently only a limited understanding exists of the principles underlying the patterns and kinetics of microbial growth in SSF. However, the modeling of SSF is currently at a stage which should see rapid advances over the next few years (Mitchell, 1992). Raghava Rao *et al.* (1993) described a model for SSF in tray reactors which is based on the effects of oxygen diffusion and its effect on the growth of micro-organisms. However, by assuming isothermality in the bioreactor the major problem of solid-substrate fermentation, viz. heat removal was neglected.

The kinetics of microbial expansion were first analyzed in depth by Pirt (1967). Trinci (1971) refined the model for colony growth of filamentous fungi. Initially growth is exponential and, this exponential expansion occurs until the

colony diameter reaches about 0.2 mm (Trinci, 1969). Exhaustion of nutrients beneath the centre of the colony causes growth inhibition in this region, and subsequently only the peripheral zone contributes to the radial expansion.

Micro-colonies are single colonies obtained from spread plates of sporangiospore suspensions. If micro-colonies are used, as in the present study, the radial expansion of the fungal colony is:

$$r_t = \mu_{rad}\, t + r_0 \qquad (1)$$

r_t	= colony diameter at time t	(mm)
μ_{rad}	= radial growth rate	(mm/h)
r_0	= colony radius after inoculation	(mm)

This simple model (Eq. 1) describes the radial expansion of fungal colonies but it cannot describe the kinetics of biomass production since it ignores the variations of mycelial density occuring in the zone behind the peripheral growth zone.

The biomass increase can initially be described knowing that:

$$\mu_x = \frac{dX}{Xdt} \qquad (2)$$

μ_x	= specific growth rate
X	= biomass at time t

At a macroscopic level, the value of μ_x has been estimated using empirical equations based on phenomenological parameters, i.e., the maximum specific rate of growth, μ_{max}, and the maximum biomass density, X_m, in the 'logistic equation' (Eq. 3). This approach has been used to describe the fungal growth curves in liquid or solid substrates (Okazaki *et al.*, 1980; Oriol *et al.*, 1987).

$$\mu_x = \mu_{max}[1 - \frac{X}{X_m}] \qquad (3)$$

The aim of this study is to determine and to model the effects of oxygen and carbon dioxide on germination and growth of *Rhizopus oligosporus* NRRL 5905

during incubation on Malt Extract Soya Peptone Agar plates in order to evaluate the comparative importance of control of heat removal and gaseous atmosphere composition.

MATERIALS AND METHODS

Culture

Rhizopus oligosporus NRRL 5905 was grown and maintained on Malt Extract Agar (Oxoid CM59) slants. Sporangiospore suspensions were prepared as described previously (De Reu *et al.*, 1993). The viable count varied between $5x10^5$ and 10^6 colony forming units/ml.

Incubator

An aerated air tight incubator (70 x 40 x 40 cm, l x d x h) was connected to a multi-gas controller (MKS, Münich, Germany) which controlled 4 mass flow controllers (10, 100, 1000 and 2000 sccm/min, MKS, Münich, Germany). Gas mixtures consisting of various proportions of oxygen, carbon dioxide and nitrogen were humidified till 100 % before entering the incubator. The flow rate was set at 1.0 l/min. Non-inoculated petri-dishes were placed in the incubator to equilibrate with the gas atmosphere for 4 hours, before the inoculation took place. During the experiments the petri-dishes were removed without disturbing the gas atmosphere of the incubator, by using a small lock (Figure 1). The incubation temperature was set and controlled at 30 ± 0.5 °C

Inoculation

-Micro-colony-

1 ml of a 1000-fold diluted sporangiospore suspension was used in pour plates with Malt Extract (100 g/l, Oxoid), Soya Peptone (50 g/l, Oxoid) and technical Agar (1 % w/v technical agar, Oxoid) (MESPA1). After 20 h of incubation at 30 °C, the colonies were cut out using a sterile cork borer with an internal diameter of 8 mm and moved to the centre of a ready-poured MESPA1.5-plate (18 ml; containing 1.5 % w/v technical agar) for the growth experiments.

-Spore suspensions-

To evaluate the effects of oxygen and carbon dioxide on the germination of *Rhizopus oligosporus* the MESPA1.5 plates were inoculated in the centre with 20 µl of a fresh spore suspension.

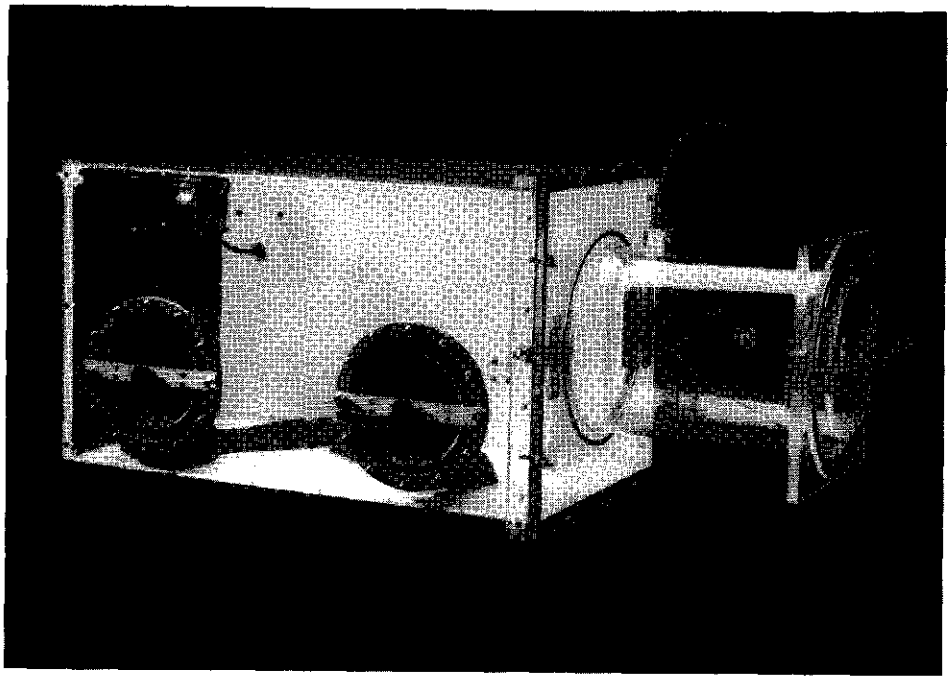

Figure 1. The airtight incubator

Biomass monitoring

At regular time intervals petri-dishes were removed in triplicate. After measuring the radius of the colony, the agar + mycelium and 300 ml of demineralized water were heated in a microwave oven until the suspension was boiling. The mixture was filtered through a pre-weighed filter. Subsequently the filters were dried for 2 days at 80 °C, before determining the mycelium dry weight gravimetrically.

Growth curves

The growth curves based on the natural logarithm of mycelium dry weight and colony diameters were fitted using linear regression to determine the specific growth rate and radial growth rate. Parameters and the 95 % confidence limits were calculated.

Rotating Drum Reactor

Growth experiments on soaked and cooked soya beans were carried out in a 4.7

l Rotating Drum Reactor (RDR) (De Reu *et al.*, 1993). The soya beans were prepared as described previously (De Reu *et al.*, 1993). During the experiments a discontinuous rotating scheme was used to avoid a temperature increase beyond 36 °C. After reaching the rotation temperature (36°C) the system was rotated clockwise and counter clockwise during 1 minute at 8 r.p.m. The slope of the temperature increase after the rotation period was used as an indirect method for measuring fungal activity.

pH and formol titration

A 20 g sample of fermented material was homogenized with 40 ml water in a Waring blender during 1 minute at maximum speed. The pH was measured in this suspension in duplicate. To this suspension 0.4 ml 8 mol/l HCl was added. 10 ml of demineralized water was added to 2 grams of the suspension and this diluted suspension was titrated with 0.1 mol/l NaOH to a constant pH of 8.5. Subsequently, 5 ml of formaldehyde (37 %, pH 8.5) was added, and after 2 min the suspension was titrated with 0.1 mol/l NaOH to pH 8.5. The volume of NaOH that was required for this second titration was expressed as ml (0.1 mol/l NaOH)/g sample.

RESULTS

Effect of Oxygen on growth on MESPA1.5

Figures 2 and 3 show the biomass dry weight and the colony diameter of micro-colonies at controlled concentrations of oxygen. The data represent averages of triplicate results. Due to variations in the initial colony diameters (3-8 mm) the data for the first 4 hours are not shown; after this period the colony diameters increased linearly. The differences in biomass dry weight and colony diameters between 1, 5 and 20.9 % oxygen are relatively small. At lower oxygen concentrations (0.5 and 0.0001 % oxygen v/v) the biomass dry weight hardly increased. When the natural logarithm of biomass dry weight was plotted against time, a linear increase was observed; this implies that the colonies did not reach the stationary phase before reaching the edge of the petri-dish and therefore the logistic curve fitting was not used. Only the experiment at 20.9 % oxygen was in a phase between exponential growth and the stationary phase after 55 hours.

The shape of the increasing micro-colonies was perfectly circular. The experiments with the spore suspension showed a more irregular colony pattern, and therefore it was more difficult to measure the radius of the colony and to determine μ_{rad}.

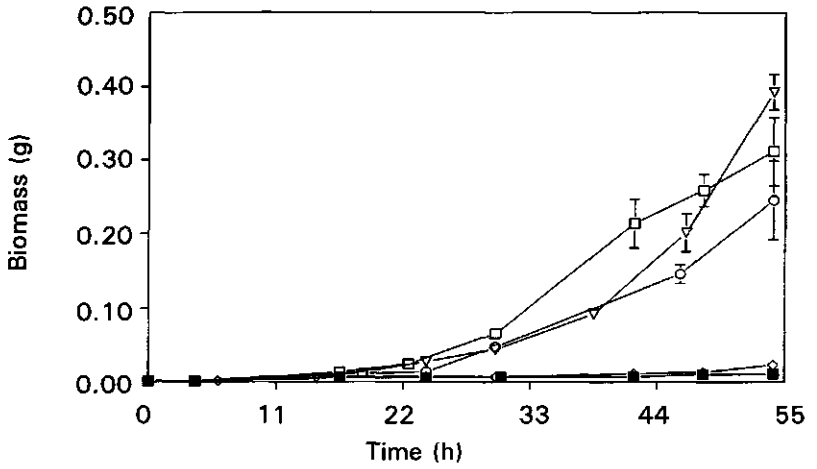

Figure 2. Cumulative biomass dry weight (g) during growth of *Rhizopus oligosporus* on Malt Extract, Soya Peptone and Technical Agar (MESPA1.5) at constant oxygen concentrations at 30 °C. -■- 0.001 % oxygen; -◇- 0.5 % oxygen; -○- 1.0 %; oxygen; -▽- 5.0 % oxygen; -□- 20.9 % oxygen

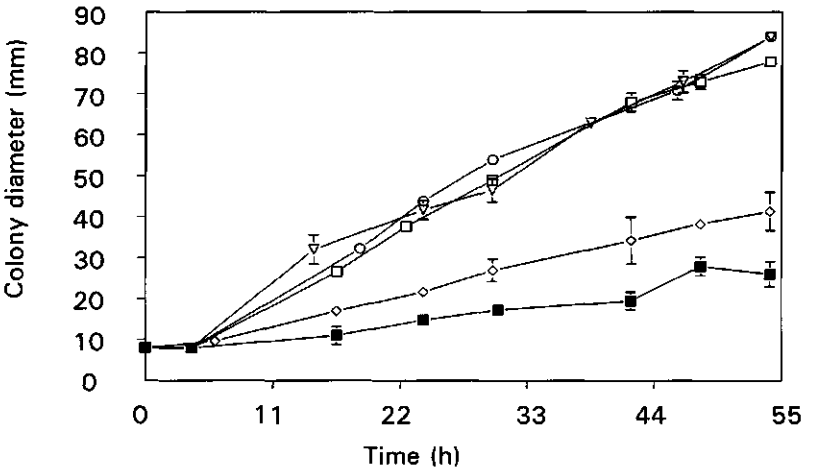

Figure 3. Colony diameters (mm) during growth of *Rhizopus oligosporus* on Malt Extract, Soya Peptone, Technical Agar (MESPA1.5) at constant oxygen concentrations at 30 °C. -■- 0.001 % oxygen; -◇- 0.5 % oxygen; -○- 1.0 %; oxygen; -▽- 5.0 % oxygen; -□- 20.9 % oxygen

The specific growth rate (μ_x, Eq. 2) and the radial growth rate (μ_{rad}, Eq. 1) were determined by linear regression of natural logarithm of the biomass and the colony diameter, respectively. In Table 1, μ_x and μ_{rad} are shown for the experiments with various oxygen concentrations. A decrease in the oxygen concentration from 20.9 % (v/v) to 1 % (v/v) did not result in a significant decrease of the μ_x and μ_{rad}. The growth rates decreased rapidly at oxygen concentrations below 1 % (v/v). Growth at low oxygen concentrations (< 0.001 % (v/v) oxygen) remained possible. Plotting the data in the Lineweaver-Burk plot resulted in: V_m of 0.11 (h^{-1}) and K_m of 0.6 (%O2).

Effect of carbondioxide on growth on MESPA1.5

In Figures 4 and 5, biomass dry weight and colony diameters are shown for the experiments with various amounts of carbon dioxide and 0.5 % (v/v) oxygen.

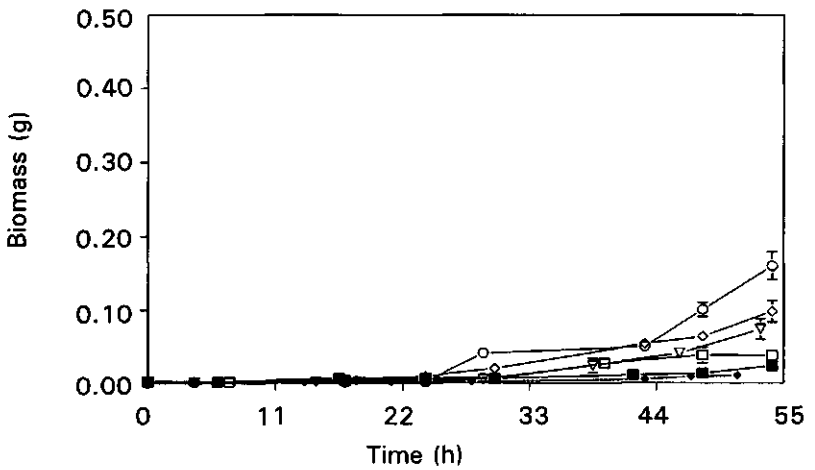

Figure 4. Cumulative biomass dry weight (g) during growth of *Rhizopus oligosporus* on Malt Extract, Soya Peptone, Technical Agar (MESPA1.5) at variuos carbon dioxide concentrations with 0.5 % oxygen at 30 °C, -■- 0.0 % carbon dioxide; -◇- 1.0 % carbon dioxide;-○- 5.0 % carbon dioxide; -▽- 10.0 % carbondioxide; -□- 20.0 % carbon dioxide; -◆- 35 % carbon dioxide

The levels of carbon dioxide varied from 0 to 35 % (v/v) at a limiting oxygen level of 0.5 %. In Figure 4 it is shown that the amount of biomass dry weight increased when the carbon dioxide concentration increased from 0 to 5 % (v/v),

Table 1. Effect of oxygen (without carbon dioxide) and carbon dioxide (mixed with 0.5 % oxygen) on specific growth rate (μ_x in h^{-1}), radial growth rate (μ_{rad} in mm/h) for *Rhizopus oligosporus* on Malt Extract Soya Peptone Agar inoculated with micro-colonies and sporangiospore suspensions and incubated at 30 °C

	Micro-colony				Spore suspension	
Gas (% (v/v))	μ_x (h-1)	95 % Confidence limits	μ_{rad} (mm/h)	95 % Confidence limits	μ_x (h-1)	95 %Confidence limits
Oxygen						
< 0.001	0.016	(0.011 ; 0.022)	0.42	(0.33 ; 0.51)	0.025	(0.011 ; 0.039)
0.5	0.043	(0.021 ; 0.066)	0.67	(0.63 ; 0.70)	0.028	(0.024 ; 0.033)
1	0.115	(0.072 ; 0.158)	1.48	(1.26 ; 1.71)	0.108	(0.086 ; 0.131)
5	0.108	(0.093 ; 0.124)	1.46	(1.26 ; 1.64)	0.095	(0.082 ; 0.107)
20.9	0.135	(0.093 ; 0.176)	1.59	(1.52 ; 1.66)	0.101	(0.091 ; 0.111)
Carbon dioxide						
0	0.043	(0.021 ; 0.066)	0.67	(0.63 ; 0.70)	0.028	(0.024 ; 0.033)
1.0	0.086	(0.072 ; 0.100)	1.13	(1.03 ; 1.23)	0.039	(0.015 ; 0.064)
5.0	0.096	(0.062 ; 0.129)	0.98	(0.89 ; 1.06)	0.094	(0.060 ; 0.128)
10.0	0.096	(0.082 ; 0.110)	1.05	(0.90 ; 1.21)	0.081	(0.061 ; 0.102)
20.0	0.070	(0.054 ; 0.084)	0.94	(0.78 ; 1.09)	0.068	(0.047 ; 0.089)
35.0	0.056	(0.044 ; 0.067)	0.71	(0.64 ; 0.78)	0.054	(0.044 ; 0.065)

whereas further increase from 5 to 35 % of carbon dioxide gave very low amounts of biomass. Similar trends are shown in Figure 5. The differences between the colony diameters at 1, 5, 10 and 20 % of carbon dioxide are small, while the differences in biomass are more pronounced. This implies that the biomass density is more affected by the the increased amount of carbon dioxide than the linear growth. At higher concentrations, carbon dioxide is therefore a growth inhibiting factor.

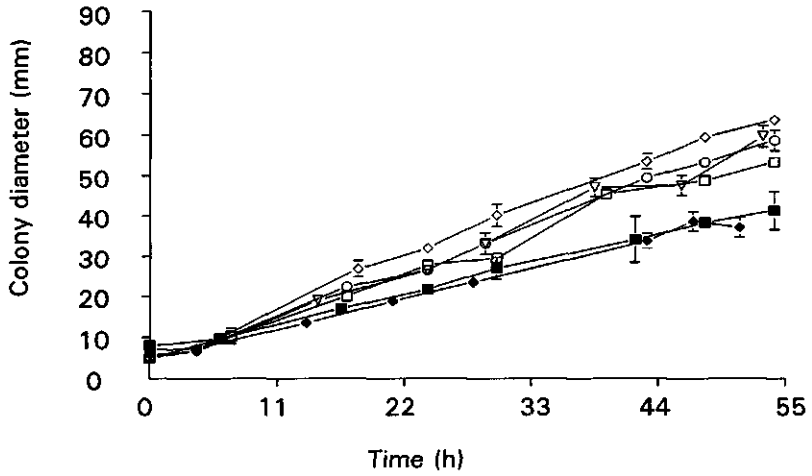

Figure 5. Colony diameters (mm) during growth of *Rhizopus oligosporus* on Malt Extract, Soya Peptone, Technical Agar (MESPA1.5) at various carbon dioxide concentrations with 0.5 % oxygen at 30 °C, -■- 0.0 % carbon dioxide; -◇- 1.0 % carbon dioxide; -○- 5.0 % carbon dioxide; -▽- 10.0 % carbondioxide; -□- 20.0 % carbon dioxide; -◆- 35 % carbon dioxide

Table 1 shows also the effects of various concentrations of carbon dioxide in combination with 0.5 % of oxygen on μ_x and μ_{rad} of micro-colonies and spore suspensions. Increasing the carbon dioxide from 0 to 5 % (v/v) resulted in an increase of μ_x from 0.043 to 0.096 (h^{-1}). When oxygen was not limiting (5 % v/v), carbon dioxide did not stimulate growth as can be seen by comparising μ_{rad} (1.39 vs 1.46 mm/h) and μ_x (0.111 vs 0.109).

Effect of O2 and CO2 on growth and fungal activity in the RDR

Table 2 shows the effects of gas compositions on various fungal activities during fermentation of soya beans with *Rhizopus oligosporus* in the Rotating Drum

Reactor. As it is very difficult to measure biomass directly in SSF in general and in agitated RDR systems in particular, temperature development, pH increase and increase in the degree of protein hydrolysis (as judged from the formol titration) were used as indirect parameters to determine the influence of various gas compositions on the fungal activity. The formol titration gives an indication of the hydrolysis of the proteins, by reacting with free-α-amino groups. The pH increase during this fermentation is based on a combination of on-going processes, such as release of ammonia, release of carbon dioxide, assimilation of organic acids and changes in the buffering capacity of the soya beans.

Table 2. The influence of oxygen and carbon dioxide on the fungal activity of *Rhizopus oligosporus* in the Rotating Drum Reactor.

Oxygen (% v/v)	Carbon dioxide (% v/v)	Number of rotation periods	Slope (°C/h)	formol ml 0.1 N NaOH/g	δpH
0.1	0	0	< 0.1	-0.02	-0.06
0.5	0	0	< 0.1	-0.04	0.90
2.5	0	2	0.6	0.10	1.12
5	0	8	0.7	0.08	1.14
5	10	16	0.8	0.14	1.71
5	15	12	0.5	0.06	1.29
5	35	6	0.6	0.09	1.37
10	0	10	0.8	0.14	1.85
20	0	30	3.5	0.20	1.29
20	10	14	3.5	0.21	2.36
20	15	18	2.0	0.24	1.03
20	35	4	0.9	0.10	1.37
40	0	43	5.5	0.20	1.40

Compared to the results at 20.9 % oxygen (30 rotation periods and a slope of 3.5 °C/h) lower amounts of oxygen had a strong influence on fungal activity which was shown by the lower values for the temperature slope, less pH increase and less increase of the hydrolysis of proteins. Increasing the oxygen concentration from 20.9 till 40 % (v/v) had a significant effect on the heat production but did not result in increased pH and formol values.

When the carbon dioxide concentration increased from 0 to 10 % an

increase in fungal activity was observed as shown by δpH and δformol. A further increase from 15 to 35 % resulted in a lower fungal activity. However, this effect is less strong than decreasing the amount of oxygen from 20 to 2.5 % oxygen.

DISCUSSION

In this study we used micro-colonies to standardize the initial conditions for the growth experiments under various atmosspheric conditions. Using this technique, the effects of the gas composition on the germination of *Rhizopus oligosporus* sporangiospores was excluded. The latter effects were studied during parallel experiments with micro-colonies and spore suspensions in the same incubator under the same gas composition.

Based on the differences in the 95 % confidence limits (Table 1) it is clear that the fitted values for the radial growth rates are more reliable than those for the specific growth rate. With a decreasing amount of oxygen, the band-width of the 95 % confidence limits for the specific growth rate increased. This can be caused by e.g., the inaccuracy of the biomass determination at low values, or the fact that the biomass increase is not yet exponential when oxygen is the limiting factor.

No significant differences were found in biomass dry weight between micro-colonies and sporangiospore suspensions. Therefore the effect of oxygen on the germination of sporangiospores could not be measured using biomass increase. The number of germinated spores might be influenced by the oxygen concentration as reported by Wells and Uota (1969) but this was not monitored in this study.

Once the spores were germinated it was expected that the values for μ_x were similar to those obtained for the micro-colonies. Specific growth rates tend to be lower for the spore suspensions. These differences were small however, and within the 95 % confidence limits.

The increase in specific growth at increased carbon dioxide levels implies that carbon dioxide can stimulate growth when oxygen is limiting. Seaby *et al.* (1988) reported that the colony area increased when the carbon dioxide concentration in a mixture with air was increased from 0 till 5 %. However, this does not automatically imply that the specific growth rate was increased as well. Therefore, it is difficult to compare the data of Seaby with those obtained by us, as we have shown that colonies with the same radius could have a different amount of biomass. Carbon dioxide stimulation of the growth of fungi has been attributed to carbon dioxide fixation to pyruvate by pyruvate carboxylase, yielding oxaloacetate, through which pools of oxoacids in the Krebs cycle are replenished

(Gadd, 1988). This type of metabolism may be significant to some species of fungi when oxygen is growth limiting, and when the carbon dioxide concentrations are not inhibitory. The growth stimulating effect of carbon dioxide was less pronounced when the level of carbon dioxide was increased from 10 to 35 % (v/v). However at 35 % (v/v) carbon dioxide and 0.5 % (v/v) oxygen growth was still observed. This effect was also seen by Paster and Menasherov (1985), who reported that carbon dioxide levels as high as 60 % (v/v) were not completely inhibitory for growth of *Fusarium sporotrichioides* if sufficient oxygen was present.

When the results for the model media are compared with those from the RDR it is clear that biomass formation on model media is less sensitive towards lower amounts of oxygen than fungal activity in the bioreactor. In both systems growth stimulation by carbon dioxide levels between 1 and 10 % (v/v) occurred. High concentrations of carbon dioxide inhibited the growth in both systems.

If non-aerated or non-agitated systems are used in Solid-Substrate Fermentation, the decreasing amount of oxygen and the increasing amount of carbon dioxide might both act as growth limiting factors. The use of forced aeration is a tool to avoid such conditions, but by aeration excessive heat production is also stimulated. That is why, for optimization purposes, a combination of aeration and heat removal are both necessary in large scale Solid-Substrate Bioreactors employing *Rhizopus oligosporus.*

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CHAPTER 7

CHANGES IN SOYA BEAN LIPIDS DURING TEMPE FERMENTATION

ABSTRACT

Soya beans were fermented with pure cultures of *Rhizopus oligosporus* and *Rhizopus oryzae* in perforated petri dishes at 25°, 30° and 37°C during 69 h. During fermentation, samples were taken at different time intervals. Lyophilized samples were analysed for total crude lipid (CL), fatty acids present in glycerides (GFA) and free fatty acids (FFA). With *R. oligosporus*, the level of GFA decreased from 22.3 to 11.5% (w/w, dry matter) after 69 h fermentation at 37°C. In the final product, only 4.3% (w/w, dry matter) of FFA were found, hence the difference of 6.5% (w/w, dry matter) of fatty acids were lost. This difference was attributed to assimilation of fatty acids by *R. oligosporus* as a source of carbon. At 25°C the situation was different as the level of FFA was 1.7 times higher than could be explained on the basis of the decrease of GFA. The distribution pattern of GFA showed a slight increase of C18:1 and C18:2 during the fermentation at the expense of C18:3. Similar results were obtained with *R. oryzae*. With this fungus the strongest effects were observed at 30°C which is close to its optimum temperature for growth. More fatty acids were lost than with *R. oligosporus* and a more pronounced shift in GFA towards saturated fatty acids was observed.

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INTRODUCTION

Tempe is a traditional Indonesian food in which filamentous fungi, particularly *Rhizopus* spp. play an essential role. Yellow-seeded soya beans are the most common and popular raw material. The resulting 'tempe kedele' is usually referred to as 'tempe' (Nout and Rombouts, 1990).
After fermentation, the cake-like mass of soya beans and mould mycelia issliced and either fried or cooked by other means (Sudarmadji and Markakis, 1978).
Rhizopus spp. produce a variety of enzymes, including carbohydrases, lipases, proteases and phytases. This paper presents our investigations on the fate of soya bean crude lipid and individual fatty acids during the tempe fermentation.

Souser and Miller (1977) reported that lipase activity in tempe fermented with *R. oligosporus* was highest after 24 h of incubation. An enzyme was isolated having a molecular mass of > 100,000 Dalton and optimum pH 7, optimum temperature 40°C and which was inactivated after heating at 60°C for 10 minutes. Nahas (1988) reported optimum pH values for *R. oligosporus* growth and lipase production of 5.5 and 6.5, respectively. Temperatures ranging from 35-40°C favoured the growth of *R. oligosporus* whereas its enzyme production was highest at lower temperatures (25°C). The maximum yield of lipase was obtained at 25°C with pH 6.5. In liquid media, maximum *R. oligosporus* lipase activity was obtained after 3 days incubation at 25°C.

Although various aspects of tempe composition have been studied, the knowledge about changes in lipid and fatty acids composition is incomplete. Wagenknecht *et al.* (1961) reported an increase of soya bean free fatty acid (FFA) levels fermented by *R. oryzae* at 37°C: from 0.3 to 8.2 g/100g tempe dry matter. Sudarmadji and Markakis (1978) monitored changes of FFA levels in tempe fermented with *R. oligosporus* at 32°C which was subsequently fried in oil. Frying resulted in increased crude lipid (CL) from 8.7 to 26.5 g/100g tempe and a decrease of all five FFA present in fresh tempe from 4.45 to 1.72 g FFA/100 g tempe dry matter. Hering *et al.* (1991) compared GFA levels in tempe made from various soya bean cultivars with several fungal inocula at different temperatures. They found that the fatty acid composition of tempe is similar to that of soya beans but with higher oleic acid and slightly lower levels of other fatty acids.

The present investigation aims to provide a mass balance of fatty acids occurring in CL, GFA and as FFA in tempe as a function of incubation time and temperature. In addition two strains of the major functional fungi, i.e. *R.*

oligosporus and *R. oryzae* are compared.

MATERIALS AND METHODS

Organisms

Rhizopus microsporus var. *oligosporus* strain LU 575 (NRRL 5905) and *Rhizopus oryzae* strain LU 583 were grown and maintained at 30°C on malt extract agar (Oxoid, CM 59). Sporangiospore suspensions were obtained by scraping off the sporangia from a culture after 7 days incubation at 30°C, and suspending them in sterile distilled water containing 0.1% (v/v) Tween 80 (Merck, Germany). The viable count varied between 5 x 10^5 and 10^6 cfu/ml, when determined on Rose-Bengal Chloramphenicol Agar (Oxoid CM549, England). The sporangiospore suspensions were used immediately after preparation.

Tempe manufacturing process

Dehulled yellow-seeded soya beans (*Glycine max*) were soaked overnight using the accelerated acidification method (Nout *et al.*, 1987). Subsequently, the beans (pH soak water < 4.2) were washed with tap water and boiled for 20 minutes, cooled, superficially dried (15-30 min, at room temperature) and inoculated using a sporangiospore suspension (1% v/w). Petridishes (ϕ 9 cm; 5 perforations/side, ϕ 1 mm) were filled with 60 g inoculated beans, sealed with tape and incubated at 25, 30 or 37°C. Samples were taken at various time intervals and were analysed for moisture content. Remaining sample was frozen at -20°C, lyophilized and stored under vacuum at 4°C in the dark until analysis. Although incubation is stopped after 30 to 48 h under production conditions, the fermentation was continued for up to 70 h to observe the effects of on-going biochemical processes.

Chemical analyses

The lyophilized tempe samples were ground with a mill (Moulinex, Type 32002, France) to a fine powder, and 7.0 g samples were extracted with petroleum-ether (40:60) in Soxhlet extractors. The ether was evaporated, and quantification of the total crude lipid (CL) content was carried out gravimetrically. Free fatty acids (FFA) and glyceride bound fatty acids (GFA) were determined by gas liquid chromatography (GLC) of their respective methyl esters according to Metcalfe and Wang (1981), using a methyl ester of heptadecanoic acid (C17:0, margaric acid) (Merck, Darmstadt, Germany) as an

internal standard. A Perkin Elmer chromatograph (Sigma 3B, Norwalk, Connecticut, USA) with flame inonisation dectector (Hewlett Packard, Geneva, Switzerland) was used. Chromatographic conditions were as follows: column, length 2m, internal diameter 2 mm, external diameter 1/8 ", (Chrompack, Bergen op Zoom, The Netherlands); stationairy phase, 15% CP-Sil 84 on CHROM WHP, 100-200 mesh; injection temperature, 225°C; detector temperature, 250°C; the column temperature, 180°C; carrier gas, N_2.

RESULTS

Total crude lipid content (CL)

Changes of the total crude lipid content are shown in Figures 1(a) and 1(b). The data presented for CL are means of duplicate measurements. For each data point the coefficient of variation (CV) was < 4.6%. With *R. oligosporus* (Fig. 1(a)), CL diminished after the period of most active growth (after 40 h at 25°C, 24 h at 30°C or 16 h at 37°C). This was more pronounced at higher incubation temperatures and reached 30% of the initial value after 69h of fermentation at 37°C.

R. oryzae (Fig. 1(b)) gave a slight increase of CL during most active growth (20-30 h) at all temperatures, which was also followed by a decline. However, with *R. oryzae* the strongest decrease of CL took place at 30°C with 21% of the initial value and was thus less pronounced than with *R. oligosporus*.

Glyceride bound fatty acids (GFA) versus free fatty acids (FFA)

Total GFA and total FFA are shown in Figures 1(a) and 1(b). The data points for GFA and FFA were based on single results, after it had been verified that the CV of the GLC analyses was < 1.9%. In Figure 1(a) it is shown that *R. oligosporus* reduced the GFA with 15.4%, 35.4% or 48% after 69 h of fermentation at 25, 30 or 37°C, respectively. During the same period the FFA increased by 5.7%, 5.9% or 4.3% of dry matter at 25, 30 or 37°C, respectively. When the GFA decreases were compared with FFA increases, an overall loss of fatty acids of 54% of the initial level at 37°C was observed. At 30°C, an overall loss of 21% took place, whereas at 25°C there was an apparent increase of fatty acids of 70% compared to the initial level.

With *R. oryzae* a GFA decrease of 7.9%, 8.6% and 10.2% of the dry matter at 25, 30 and 37°C, respectively, was found. During this period the FFA levels increased with 4.8%, 3.9% and 5.5% at 25, 30 and 37°C, respectively.

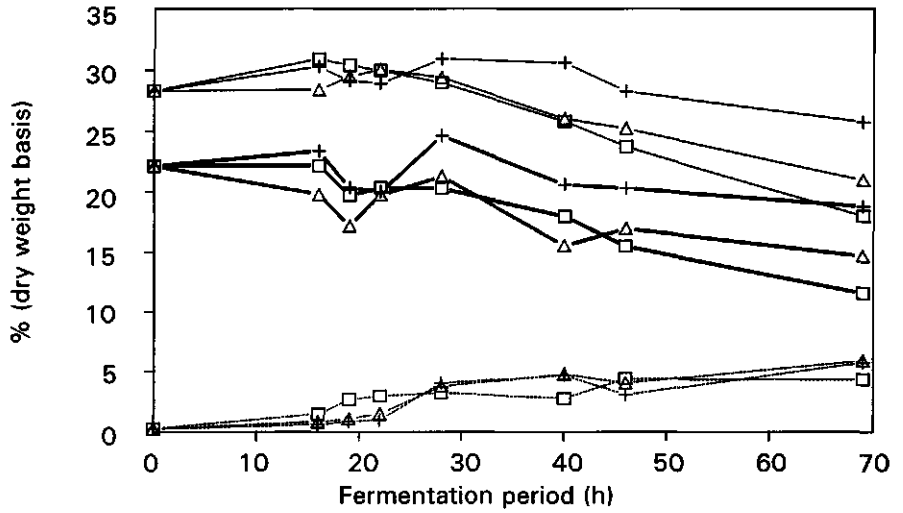

Fig. 1a *Rhizopus oligosporus*

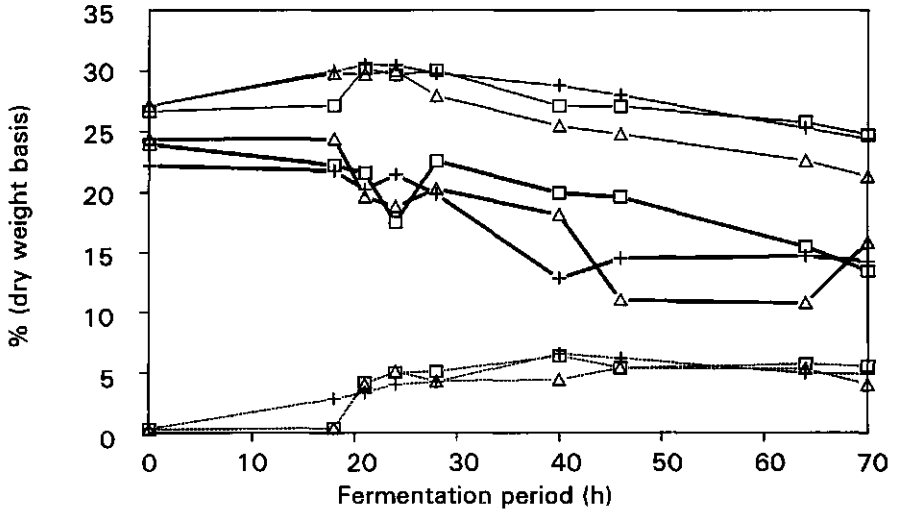

Fig. 1b *Rhizopus oryzae*

Figure 1. Changes of total crude lipid (——), glyceride fatty acids (——) and free fatty acids (-----) during fermentation of soya beans in perforated petridishes at 25°(+), 30°(△) and 37°C (□), with *Rhizopus oligosporus* (Fig. 1a) and *Rhizopus oryzae* (Fig. 1b)

Here again, losses of 39%, 54% and 46% of the initial level of fatty acids occurred at 25, 30 and 37°C, respectively.

GFA patterns

In dehulled soya beans, the following distribution of GFA was found after soaking and cooking: palmitic acid C16:0 (11.2% of total GFA), stearic acid C18:0 (3.3%), oleic acid C18:1 (23.0%), linoleic acid C18:2 (55.6%) and linolenic acid C18:3 (7.0%). In Figure 1(a), total GFA decreased with time and with increasing incubation temperature for *R. oligosporus*.

In Table 1, the individual levels of GFA during fermentation at 30°C are presented. These follow a similar trend as that of total GFA shown in Figure 1a. There was a slight decrease until 19 h followed by a slight increase during the period of most active growth. Beyond 28 h of fermentation, the levels of all GFA decreased. Compared with the initial levels of individual GFA (at time = 0), the minority fatty acids (C16:0, C18:0 and C18:3) decreased faster than the majority fatty acids (C18:1 and C18:2). With *R. oryzae* at 30°C, GFA decreased until 64h and slightly increased again. As can be seen in Figure 1(b), this did not occur at 25° or 37°C. The distribution of the individual GFA was similar as with *R. oligosporus*. The effect of temperature on the GFA pattern is shown in Figures 2a and 2b. With both *Rhizopus* spp. the distribution of GFA developed in a similar pattern at all temperatures, i.e. C18:3 tended to decrease more than the other fatty acids. With *R. oligosporus* this was most pronounced at 37°C and with *R. oryzae* at 30°C.

FFA patterns

As was shown in Figures 1(a) and 1(b), the levels of FFA increased during fermentation. With *R. oligosporus* at 25°C, more FFA was retrieved than expected on the basis of GFA decrease. At 30° and 37°C we found considerably less FFA than expected. Figures 3(a) and 3(b) show the level and distribution pattern of individual FFA expressed as percentage of the expected level. The expected level of FFA equals the decrease of GFA between time = 0 and 46 h of fermentation.

With *R. oligosporus* at 25°C (Fig. 3(a)), about twice as much C18:1 and C18:2 but less C18:3 were found than expected on the basis of GFA decrease. At the higher temperatures, the pattern of FFA shifted towards a higher degree of saturation, but FFA levels were generally lower.

With *R. oryzae* (Fig. 1(b)), total FFA levels were lower than expected

Table 1. Levels of glyceride fatty acids (GFA) and free fatty acids (FFA) during soya bean fermentation at 30°C

Rhizopus oligosporus

Incubation time	C16:0		C18:0		C18:1		C18:2		C18:3		TOTAL		
(h)	GFA[a]	FFA[b]	GFA	FFA	GFA	FFA	GFA	FFA	GFA	FFA	GFA	FFA	FA[c]
0	2.5	0.1	0.7	0.0	5.1	0.1	12.3	0.0	1.6	0.0	22.2	0.3	22.4
16	2.2	0.1	0.6	0.0	4.5	0.1	11.0	0.6	1.4	0.0	19.8	0.8	20.6
19	1.9	0.2	0.6	0.0	3.9	0.1	9.6	0.8	1.2	0.0	17.2	1.0	18.2
22	2.1	0.3	0.6	0.0	4.3	0.2	11.2	1.0	1.4	0.0	19.7	1.5	21.2
28	2.3	0.7	0.6	0.2	5.9	0.7	12.1	2.0	1.4	0.2	21.3	3.8	25.0
40	1.6	0.8	0.5	0.2	3.8	1.0	8.8	2.4	0.9	0.2	15.5	4.8	20.3
46	1.7	0.7	0.5	0.2	4.2	0.9	9.6	2.1	0.9	0.2	16.9	4.0	21.0
69	1.4	0.9	0.4	0.3	3.7	1.4	8.3	3.0	0.9	0.3	14.7	5.9	20.5

Rhizopus oryzae

incubation time	C16:0		C18:0		C18:1		C18:2		C18:3		TOTAL		
(h)	GFA	FFA	GFA	FFA	GFA	FFA	GFA	FFA	GFA	FFA	GFA	FFA	FA
0	2.7	0.1	0.8	0.0	5.4	0.1	13.9	0.0	1.8	0.0	24.4	0.3	24.7
18	2.6	0.1	0.8	0.0	5.5	0.1	13.7	0.1	1.8	0.0	24.4	0.4	24.8
21	1.9	0.9	0.6	0.2	4.5	0.8	11.3	1.9	1.3	0.2	19.6	4.0	23.6
24	1.9	1.1	0.5	0.3	4.3	0.9	10.9	2.4	1.3	0.3	18.8	5.1	23.9
28	1.9	1.0	0.5	0.3	4.7	0.8	11.8	2.0	1.4	0.2	20.3	4.4	24.7
40	1.6	1.0	0.5	0.3	4.2	0.9	10.6	2.0	1.2	0.2	18.2	4.4	22.5
46	1.1	1.0	0.3	0.3	2.6	1.3	6.4	2.5	0.6	0.2	11.0	5.3	16.3
64	1.1	0.9	0.3	0.3	2.6	1.4	6.2	2.5	0.6	0.2	10.7	5.2	16.0
70	1.7	0.6	0.4	0.2	3.7	1.1	9.1	2.0	0.9	0.1	15.8	4.0	19.8

[a] [b] expressed as % of dry matter, [c] FA = fatty acids

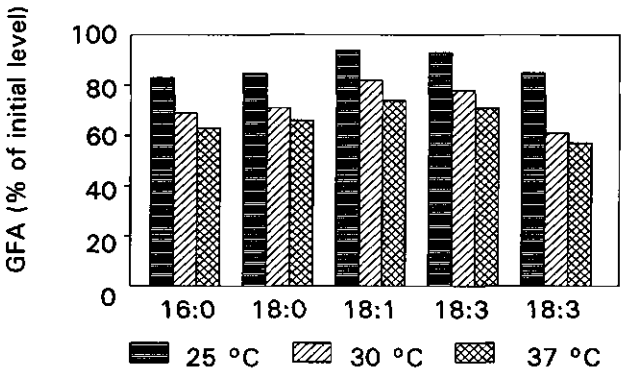

Fig. 2a *Rhizopus oligosporus*

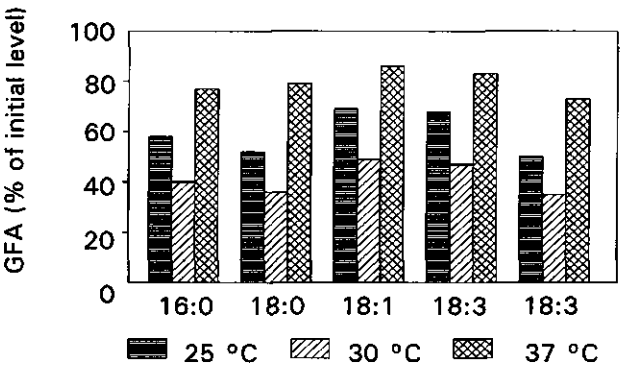

Fig. 2b *Rhizopus oryzae*

Figure 2. Relative changes of GFA distribution after 46 h of fermentation of soya beans in perforated petridishes at 25°, 30° and 37°C, with *Rhizopus oligosporus* (Figure 2a) and *Rhizopus oryzae* (Figure 2b)

from the GFA decrease. However, for the individual FFA there were some remarkable differences between the respective incubation temperatures. At 25°C, there was an increase in C16:0 and C18:0. At 37°C, C18:1 and C18:2 exceeded 100%, and at 30°C the residual FFA's showed a general tendency towards a higher degree of saturation.

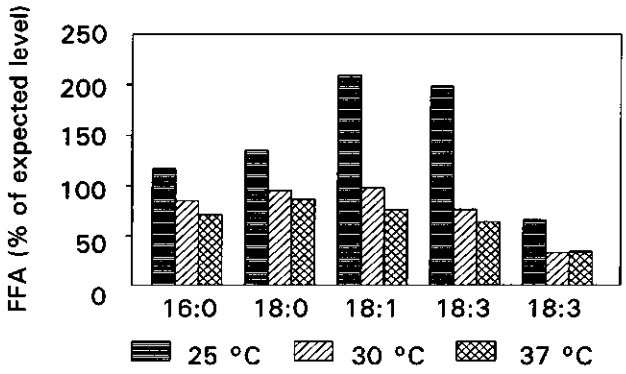

Fig. 3a *Rhizopus oligosporus*

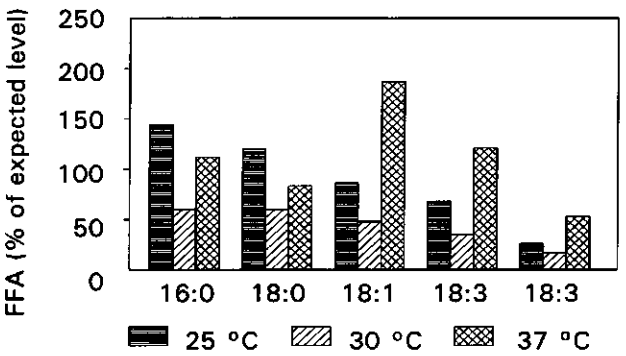

Fig. 3b *Rhizopus oryzae*

Figure 3. Changes of FFA distribution after 46 h of fermentation of soya beans in perforated petridishes at 25°, 30° and 37°C, with *Rhizopus oligosporus* (Fig. 3a) and *Rhizopus oryzae* (Fig. 3b). The expected FFA level corresponds with the quantity of GFA which disappeared during 46 h of fermentation at 25°, 30° or 37°C, respectively

DISCUSSION

During the tempe fermentation, a temperature-related decrease of total crude lipid content was observed. With *R. oligosporus*, strongest CL reduction was

39% of the initial level after 69 h of fermentation at 37°C. *R. oligosporus* has an optimum growth temperature of approx. 37°C. *R. oryzae* has its optimum growth temperature at approximately 30°C. In the experiment with *R. oryzae* at 25° and 37°C we obtained a less pronounced decrease of CL than at 30°C. The data presented here suggest that the decrease of crude lipid is related to the growth rate.

Earlier investigations of CL changes during tempe fermentation did not include the effect of incubation temperature or microbial strain. Van Buren and Schaefer (1972) reported a CL decrease of 18% after 72 h of fermentation at 38°C using a mixed culture of *Rhizopus* spp.. Van Veen (1950) found a CL decrease of 30% with *R. oryzae* at 30°C. Wagenknecht *et al.* (1961) reported that tempe fermented at 37°C with *R. oryzae* had a fairly constant CL level varying from 22.3% to 26.7%. Hering *et al.* (1991) reported that a significant CL loss could not be detected with different *Rhizopus* spp. at 24°, 32° and 36°C. The difference between our results and those obtained by Hering *et al.* (1991) are probably caused by the different methods of acidification. During the accelerated acidification method (Nout *et al.* 1987) profuse growth of lactic acid bacteria occurs. By consequence, a decrease of fermentable carbohydrates takes place. This can cause a quicker depletion of assimilable carbohydrates and hence, a shift towards the use of lipids as a source of energy and carbon during the stage of fungal fermentation. With the chemical acidification procedure used by Hering *et al.* (1991), the fermentable sugars remain available for the fungal fermentation stage. Consequently, less CL will be assimilated during the fermentation.

With *R. oligosporus* grown at 25°C, the apparent increase of CL during the period of most active growth is due to active assimilation of carbohydrates and limited consumption of lipids, causing a shift in the dry matter composition resembling an enrichment of CL.

The results for *R. oligosporus* show that at 30°C and 37°C, the GFA decrease is larger than the FFA increase. This difference is created after the period of most active mould growth (20 - 30h). The disappearance of the liberated FFA indicates that the fungus metabolized the latter as a carbon source. Indeed, *R. oligosporus* was shown to grow on soya oil as a carbon source (Nahas, 1988). The fact that fatty acids disappear after the most active growth period implies that fatty acids are not the preferred carbon source.
Although tempe production usually takes place under uncontrolled conditions, the results presented indicate that it will be possible to influence the fatty acid

pattern by varying the incubation temperature. In order to ensure maximum nutritional value of the product, it will be of interest to optimize bean pre-treatments, incubation temperatures and periods in order to avoid undesirable losses of fatty acids.

ACKNOWLEDGEMENTS

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CHAPTER 8

PROTEIN HYDROLYSIS DURING THE SOYA BEAN TEMPE FERMENTATION WITH *RHIZOPUS OLIGOSPORUS*

ABSTRACT

Hydrolysis of soya protein during fermentation in a Rotating Drum Reactor (RDR) was compared with hydrolysis during the traditional stationary tempe fermentation process. An increase of water-soluble nitrogen was observed. After 72 hours of fermentation, 18.5, 25.4, 27.1 and 34.0 % (w/w of total N) were found for the traditional stationary process at 25°C, 30°C, 37°C and for the RDR at 36 °C, respectively, compared to 2.0 % in cooked soya beans at the start of the fermentation.

In the RDR the fungal activity continued longer than in the traditional process. This was achieved by avoiding heat and mass transfer limitations. In the RDR a higher degree of hydrolysis, as well as higher levels of water-soluble and TCA-soluble nitrogen were found after 72 hours of fermentation compared with the stationary process at 37 °C. Conglycinin was hydrolysed faster than glycinin, which is probably related to the chemical structure; conglycinin is more sensitive towards protease activity.

This chapter has been submitted as:

Protein hydrolysis during the soybean tempe fermentation with *Rhizopus oligosporus*

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INTRODUCTION

Tempe is a traditional Indonesian fungal fermented food made from dehulled, soaked, and cooked soya beans inoculated with a mould, usually of the genus *Rhizopus*. After fermentation has occurred, the soya beans are bound together into a compact cake by dense cottony mycelium. *Rhizopus* spp. produce a variety of carbohydrases, lipases, proteases, phytases and other enzymes, which hydrolyse soya bean constituents and contribute to the development of the desirable texture, flavour, and aroma of tempe. Enzymatic hydrolysis may also decrease or eliminate antinutritional constituents. Consequently, the nutritional quality of the fermented product may be improved (Nout and Rombouts, 1990; Hachmeister and Fung, 1993).

The effect of fermentation on the total nitrogen content is negligible (Nowak and Szebiotko, 1992) but increases of the concentrations of free amino acids take place. The amino acid pattern, as quantified by the essential amino acid index, is not significantly affected during a 24 h fermentation. Longer fermentation results in losses of lysine (25 % lost after 60 h; Winarno and Reddy, 1986; Stillings and Hackler, 1965). Little attention has been paid up till now on the effects of fermentation on the individual subunits of the major soya bean proteins.

The major soya proteins are glycinin and ß-conglycinin. Glycinin is composed of 6 intermediate subunits (A_n-B_n) which are formed of acidic polypeptides (A_1-A_5) and basic polypeptides (B_1-B_4). These acidic and basic components are called subunits and are covalently linked to each other by disulfide bonds, to constitute intermediate subunits. Glycinin intermediate subunits can be separated into distinct groups: I: $A_{1a}B_2$, $A_{1b}B_{1b}$, A_2B_{1a}, and II: A_3B_4, $A_5A_4B_3$. (Yamauchi *et al.*, 1991).

ß-Conglycinin, the other major constituent in soya, is less well defined compared with glycinin. ß-Conglycinin is glycosylated and consists of three subunits combinations of α (Mw 57,000-68,000 Da), α'(Mw 57,000-68,000 Da), ß (Mw 42,000-52,000 Da) giving 7 heterogenities (B_0-B_6). (Yamauchi *et al.*, 1991).

In this study we investigated the influence of incubation temperature and agitation on the proteolysis of soya proteins during the tempe process. The results obtained with fermentation of *Rhizopus oligosporus* at 25°, 30° and 37 °C in the traditional stationary process were compared with those obtained in a Rotated Drum Reactor (RDR) which was developed and described previously (De Reu *et al.*, 1993). In this laboratory system, temperature control during solid-substrate fermentation can be achieved by either discontinuous or continuous rotation and forced evaporation by aeration. As a result of the rotation a more-or-less granular

product is obtained in contrast to the cake-like product resulting from the traditional process.

MATERIALS AND METHODS

Organism

Rhizopus microsporus var. *oligosporus* strain LU 575 (NRRL 5905) was grown and maintained at 30 °C on malt extract agar (Oxoid, CM 59, England). Sporangiospore suspensions were obtained by scraping off the sporangia from a slant culture after 7 days incubation at 30 °C, and suspending them in sterile distilled water containing 0.1% (v/v) Tween 80 (Merck, Germany). The viable count varied between $5*10^5$ and 10^6 cfu/ml, when determined on Rose-Bengal Chloramphenicol Agar (Oxoid CM549, England). The sporangiospore suspensions were used immediately after preparation.

Tempe manufacturing process

Dehulled yellow-seeded soya beans (*Glycine max*) were soaked overnight using the accelerated acidification method (Nout *et al.*, 1987). Subsequently, the beans (pH soak water < 4.2) were washed with tap water and boiled for 20 minutes in tap water (3 l water per kg of soaked beans), cooled, superficially dried (15 - 30 min, at room temperature) and inoculated (1% v/w cooked beans) using a sporangiospore suspension.

Petri dishes (ϕ 9 cm; 5 perforations/side, ϕ 1 mm) were filled with 60 g inoculated beans, sealed with tape and incubated at 25°, 30° or 37 °C. An autoclaved Rotating Drum Reactor vessel (RDR) (De Reu *et al.*, 1993) was filled with 1 kg of cooked and inoculated soya beans. The reactor was placed in a temperature controlled incubator at 30 °C. During the experiments a discontinuous rotating scheme was used to avoid that the substrate temperature would exceed 36 °C. After the substrate temperature reached 36 °C, the vessel was rotated clockwise and counter clockwise during 1 minute at 8 rpm. Due to the rotation, the temperature of the substrate dropped 1.5-2.0 °C. After rotation the reactor vessel stood still until the substrate temperature of 36 °C was reached again. Then the rotation cyclus was repeated. This procedure was repeated continuously resulting in a so called 'saw-tooth' temperature pattern (De Reu *et al.*, 1993).

Samples were taken at various time intervals and immediately frozen with liquid nitrogen. The samples were stored at -20 °C, lyophilized and stored at 4 °C in the dark until analysis. Although incubation would normally be complete after

30-48 h in a commercial production setting, we continued the fermentation up to 72 h to observe the effects of on-going biochemical processes.

Fractionation of nitrogen containing compounds

Lyophilized sample (7 g) was extracted with distilled water (35 ml), in a head-over-tail-rotator during 30 minutes at 4 °C. The suspension was centrifuged (27,000 g) during 10 minutes at 4 °C. The supernatant was filtered through a glass-filter (Whatman, Glass Microfibre, cat.nr. 1820 037). The supernatant was characterized as water-soluble solids (WSS). The residue was washed twice with water (50 ml) and the resulting filtrates were discarded. The residue remaining on the filter was added to the water-insoluble residue and was lyophilized and weighed; this fraction was characterized as water-insoluble solids (WIS).

The WSS-fraction was boiled for 15 min to inactivate enzyme activity, and was then centrifuged for 10 min at 27,000 g. The pellet, heat precipitable water-soluble solids (HPWSS), was washed twice with dist. water and freeze dried. The remaining supernatant was treated with Trichloroacetic acid (TCA) to give a final concentration of 13.6 % (w/v) TCA. After one night storage at 4 °C the suspension was centrifuged for 15 min at 4 °C at 39,100 g. The TCA-pellet was characterized as TCA-insoluble solids (TIS) and the supernatant as TCA-soluble solids (TSS). The TIS and TSS fractions were lyophilized and weighed.

All fractions were analyzed for their nitrogen content using the micro-Kjeldahl technique. The standard deviation of the method was determined analyzing one sample 4 times. This resulted in standard deviation of N ± 0.31 %.
Based on this result and on the total number of data per fermentation condition we decided to analyze the other samples only once.

Electrophoresis

SDS-PAGE was performed using a Protean™ II electrophoresis system (Biorad) essentially according to Laemmli (1970). Homogeneous slab gels (concentration monomers (T) = 12.5 or 15 %, cross-linker concentration (C) = 2.2 %) were used. The gels were stained with Coomassie Brilliant Blue R-250 according to the instruction of the manufacturer.

The optical density of the bands was measured using a laser densitometer (Computing densitometer 300A, Molecular Dynamics, Sunnyvale, CA, USA) equipped with a helium-neon laser (λ 672 nm) using Imagequant 3.15 software. Although band patterns for all the obtained samples were available we elaborated only the data of samples after 22 and 66 h incubation, as this method is very

laborious. The major peaks were identified following the characterization of Wolf *et al.* (1992). Peak 1: α-ß- and α'-ß-conglycinin; peak 2: ß-ß conglycinin; peak 3: A_3-glycinin; peak 4: A_{1a}, A_{1b} and A_2-glycinin; peak 5: B-glycinin.

The peak areas were calculated and expressed as relative intensities. As the protein content in the different lanes can vary, the intensities itself could not be used to compare the various samples.

Degree of hydrolysis

To ± 2.0 gram of sample (approx. 1 g protein) 25 ml of a hot solution (80 °C) of sodium dodecyl sulphate (10 g/l) and 1,4-dithiothreitol (3 g/l) was added. The mixture was placed in a water bath (100 °C) for 1 hour. Next, the solution was cooled to room temperature, with continuous stirring. The solution was adjusted to pH 6, before adding excess (4.5 ml) formaldehyde (37 %, Merck). Finally, the mixture was titrated with 0.1 mol/l sodium hydroxide to pH 9.5. The required volumes for both the fermented sample and the cooked soya beans determine the *degree of hydrolysis (Adler-Nissen, 1986)*:

$$DH = \left(\frac{A}{P} - \frac{B}{Q}\right) * \left(\frac{1000}{7.8}\right)$$

DH	=	degree of hydrolysis (%)
A	=	ml 0.1 mol/l sodium hydroxide per gram of fermented sample
B	=	ml 0.1 mol/l sodium hydroxide per gram of cooked soya beans
P	=	protein content of the fermented sample (% w/w of dry matter)
Q	=	protein content of the cooked soya beans (% w/w of dry matter)
7.8	=	total number of peptide bonds in soya protein (eq/kg protein)

RESULTS

As shown in Table 1, initially 45.7 % (w/w of total N) of the nitrogen was water-soluble in the raw beans. During soaking the water-soluble nitrogen proportion decreased to 7.4 % (w/w of total N). Cooking decreased the water-soluble nitrogen proportion further to 2.0 % (w/w of total N).

During fermentation we observed increases of protein content and pH of the freeze-dried material as shown in Table 2. During soaking the pH of the beans decreased from 6.7 to 4.4. Cooking resulted in a slight increase to 4.5. During fermentation the pH increased to 5.8, 5.8, 6.1 and 5.9 for the traditional process at 25, 30 and 37 °C and the RDR, respectively.

Table 1. Partition of Nitrogen (% w/w of total N) in the different fractions of soya beans

	WIS* (%)	WSS (%)	HPWSS (%)	TIS (%)	TSS (%)
Raw beans	54.3	45.7	29.6	12.36	2.9
Soaked beans	92.6	7.4	1.0	6.10	6.1
Cooked beans	113.6	2.0	0.0	2.54	2.5

*WIS, water-insoluble solids; WSS, water-soluble solids; HPWSS, Heat precipitable water-soluble solids; TIS, TCA-insoluble solids; TSS, TCA-soluble solids

Table 2. Total nitrogen and pH during the fermentation of soya beans with *R. oligosporus* at 25, 30 and 37 °C in petri-dishes and at 36 °C in the RDR after 65 h

Sample	Total (N* 6.25) content (% w/w of dry matter)	pH
Raw beans	44.1	6.7
Soaked beans	46.1	4.4
Cooked beans	45.0	4.5
RDF, 65 h	49.5	5.9
25 °C, 65 h	51.7	5.8
30 °C, 65 h	52.4	5.8
37 °C, 65 h	53.8	6.1

In Figure 1 the proportion of the water-soluble nitrogen (WSS-N) for the different fermentations is shown. The WSS-N proportion increased during fermentation initially in this order: 37 °C, 30 °C, RDR and 25 °C. At 25 °C the increase of the proportion of WSS-N started slowly but still reached 18.7 % (w/w of total N) after 72 hours. Incubation at 30 °C and 37 °C increased the proportion of WSS-N to 25.4 and 27.1 % (w/w of total N), respectively. In the RDR the proportion of WSS-N increased at the same rate as the traditional process at 25 °C until 48 hours, but during the third day of the process the increase was more

pronounced compared to any of the non-agitated fermentations and resulted in a final WSS-N proportion of 34.0 % (w/w of total N) after 72 hours of fermentation. As expected, the WIS nitrogen proportion showed an inverse pattern to the increasing proportion of WSS-N. The nitrogen proportions of the two fractions should add up to give 100 %, in this study (40 samples) an average value of 95.1 ± 4.6 % was obtained.

In Figure 2 the sums of nitrogen present in the fractions HPWSS and TIS expressed as the proportion of N are shown. They formed a minority of the WSS fraction as a maximum of 4 % (w/w of total N) was found in those fractions. The maxima indicate that after 50-60 hours the fractions were hydrolysed further to smaller peptides or amino acids which were soluble in 13.6 % TCA. For the RDR the maximum is reached after 48 hours, and for the traditional process at 25 °C, 30 °C and 37 °C after 65 h, 55 h and 55 h, respectively.

In Figure 3 the proportions of TCA-soluble nitrogen (TSS-N) for the different fermentations are shown. The level of TSS-N increased less at 25 °C than at higher temperatures. The differences between 30 °C and 37°C were small, while after 50 hours the level in the RDR increased rapidly to 34 % (w/w of total N).

In Figure 4 the degree of hydrolysis (DH) of the different fermented samples is shown. It can be seen that the hydrolysis at 25 °C started later and increased less than at 30 °C and 37 °C. Until 48 hours the differences between 30 °C, 37 °C and the RDR were small, but beyond 48 hours the DH increased rapidly to a level of 46 % in the RDR.

To characterize the hydrolysis pattern in tempe, the freeze-dried samples were analyzed by SDS-PAGE. In Figure 5 a typical pattern is shown after 22 hours of fermentation at 25 °C. This pattern was determined by measuring the intensities of the different bands on the gel.

In Table 3 the changes in the ratio between conglycinin (peaks 1 and 2) and glycinin (peaks 3 + 4 + 5) as well as the ratio $\alpha+\alpha'$ subunits of ß-conglycinin (peak 1): ß subunits of ß- conglycinine (peak 2) and the ratio A-subunits of glycinin (peaks 3+4):B-subunits of glycinin (peak 5) are shown during the process. During pre-processing and fermentation conglycinin appears to be hydrolysed to a higher extent than glycinin as the ratio decreased from 0.52 in the raw beans to a minimum of 0.23 after 65 h of fermentation at 25 °C. The α and α' subunits of ß-conglycinin are hydrolysed to a larger extent than the ß subunits of ß-conglycinin, while the ratio A-subunits of glycinin:B-subunits of glycinin decreased in the RDR more rapidly than in the non agitated fermentations.

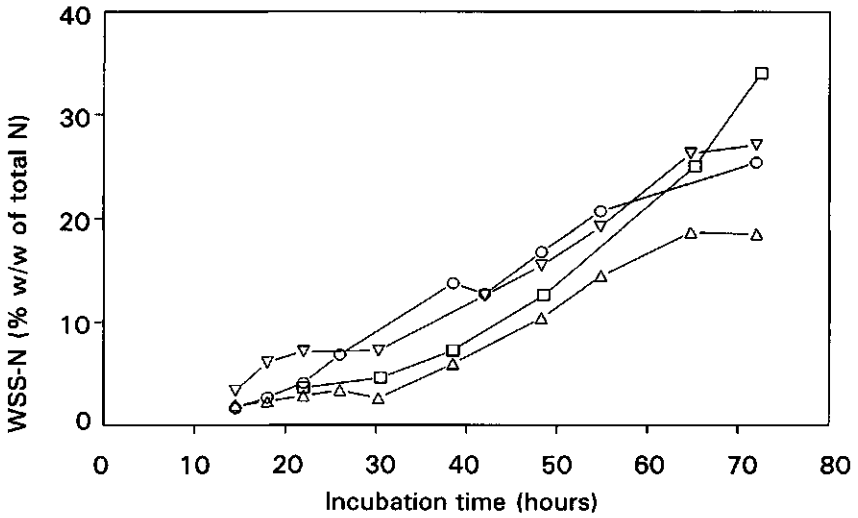

Figure 1. The water-soluble nitrogen content during the fermentation of soya beans with *R. oligosporus* during the traditional process at 25°C (- △ -), 30 °C (- ○ -) and 37 °C (- ▽ -) and with the RDR at 36 °C (- □ -).

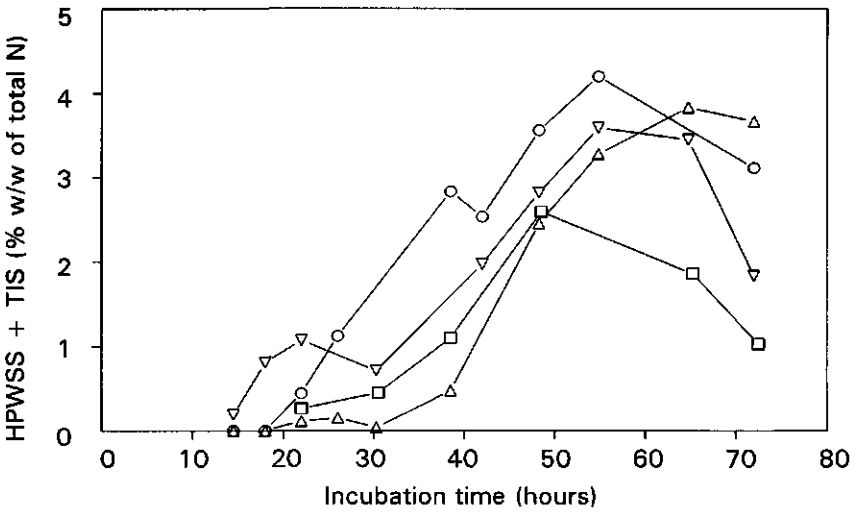

Figure 2. The sum of the residue after boiling the water-soluble fraction (HPWSS) and the TCA-insoluble fraction (TIS) during the fermentation of soya beans with *Rhizopus oligosporus* during the traditional process at 25 °C (- △ -), 30 °C (- ○ -) and 37 °C (- ▽ -) and with the Rotating Drum Fermentor at 36 °C (- □ -).

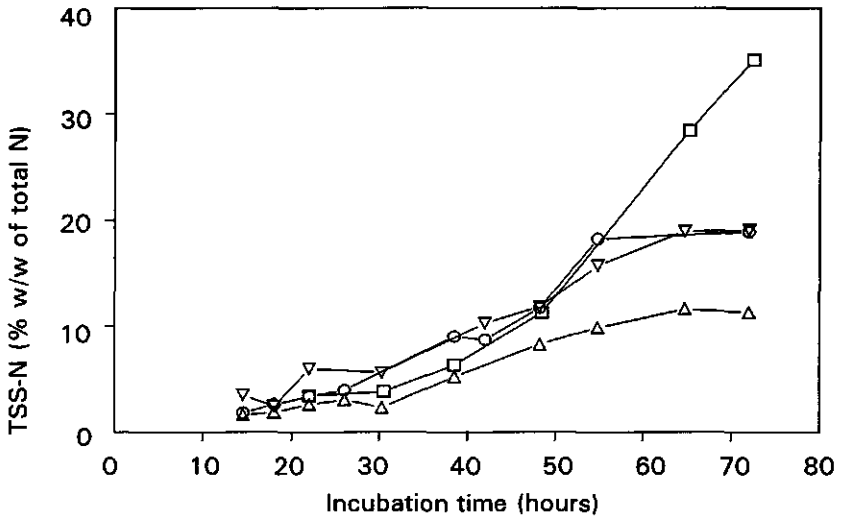

Figure 3. The TCA-soluble nitrogen (TSS) content during the fermentation of soya beans with *R. oligosporus* during the traditional process at 25 ° C (- △ -), 30 °C (- ○ -) and 37 °C (- ▽ -) and with the RDR at 36 °C (- □ -).

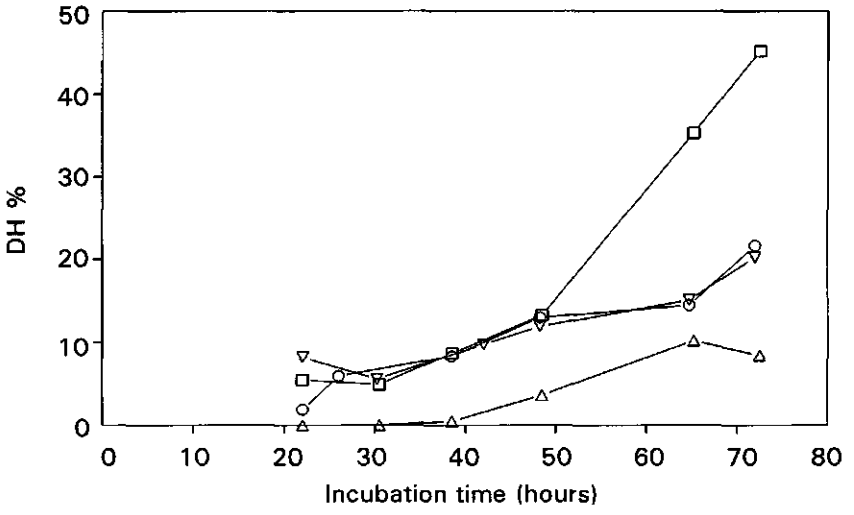

Figure 4. The Degree of hydrolysis (DH) during the fermentation of soya beans with *R. oligosporus* during the traditional process at 25 °C (- △ -), 30 °C (- ○ -) and 37 °C (- ▽ -) and with the RDR at 36 °C(- □ -).

Table 3. The ratio conglycinin:glycinin, α-α'-ß-conglycinin:ß-ß-conglycinin and A-glycinin:B-glycinin in soya beans during the traditional stationary process at 25°, 30° and 37 °C and the agitated process in the RDR at 36 °C

	conglycinin:glycinin			α-α'-ß-conglycinin:ß-ß-conglycinin			A-glycinin:B-glycinin		
		22h	65h		22h	65h		22h	65h
raw	0.52			3.77			1.54		
soaked	0.42			2.86			1.21		
cooked	0.43			1.65			1.33		
RDR		0.32	0.28		1.28	1.11		1.10	0.72
25°C		0.35	0.23		1.77	1.24		1.03	0.94
30°C		0.42	0.30		1.44	1.12		1.10	1.11
37°C		0.39	0.31		1.68	0.77		1.35	1.20

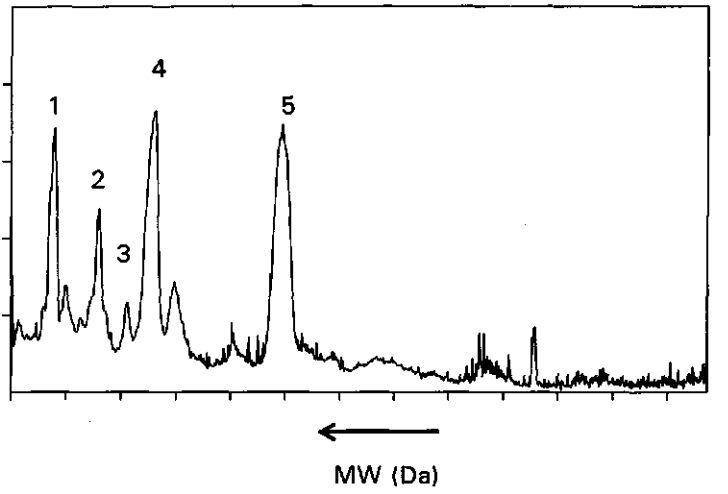

Figure 5. The intensities of bands on SDS-PAGE gel of freeze dried tempe obtained after 22 hours of fermentation at 25 °C.

DISCUSSION

During fermentation a slight increase in total protein content was observed. Several investigators reported on the total protein content during tempe fermentation. Van Buren *et al.* (1972) observed a slight decrease in total protein from 43.2 to 41.6 % (w/w of dry matter). Slight increases were observed by Nowak and Szebiotko (1992) from 45.1 tot 48.8 % (w/w of dry matter), by Van der Riet *et al.* (1987) from 40.1 to 49.3 (w/w of dry matter) and by Murata *et al.* (1967) from 45.7 to 50.6 % (w/w of dry matter). In addition, Baumann *et al.* (1990) concluded that there were no differences in nitrogen content. The most likely explanation of an increase in total protein content is that there is a shift in the composition of the dry matter during fermentation. There is a loss of 10 % dry matter during fermentation (Van der Riet *et al.*, 1987). During fermentation carbohydrates and lipids (De Reu *et al.*, 1994) were used to form biomass, carbon dioxide and water. This will result in a relative increase in protein content. Increases in total protein content are small but seem to be related to the growth rate of *Rhizopus oligosporus*. The optimum temperature for growth is about 37-42 °C. The lower the incubation temperature, the smaller the observed increases in total protein content.

The decrease in the water-solubility during soaking is probably due to leaching into the soak water and more importantly by the drop in pH to 4.2 in the soak water, which has a strong effect on the water-solubility as shown by Lah and Cheryan (1980). During cooking the remaining water-soluble protein became insoluble due to denaturation. In the traditional process the fractions TSS-N in WSS-N were 0.61, 0.74 and 0.70 after 72 hour of incubation at 25 °C, 30 °C and 37 °C, respectively, while in the RDR all WSS-N had turned into TSS-N after the same incubation time. Based on the higher values for the DH and the fact that almost all the WSS-N was soluble in TCA, it can be concluded that in the RDR an exoprotease was active and that the product is hydrolysed to smaller fractions (e.g. amino acids).

In literature little attention has been paid to protease production by *R. oligosporus*, Wang and Hesseltine (1970) characterized the extracellulair proteases as aspartic proteases, having an endo-wise activity. As in the RDR the gradients of oxygen, carbon dioxide and temperature are less than in the traditional system, the activity of exoproteases might be related to the amount of available oxygen or to shear forces during rotating or temperature.

Protease activity in *R. oligosporus* was measured intracellularly (2 U/mg protein after 48 h of incubation), extracellularly (4.1 U/mg after 48 h of incubation) and in the debris (2 U/mg protein) (Baumann, 1992). It was not determined whether the proteases had exo or endo activity. Due to disruption of the hyphae both the extracellular and intracellular enzyme activities as well as some of the activity in the debris might be pooled and the resulting increased level of extracellular activity might be an explanation for the increased degree of hydrolysis in the RDR.

In previous studies we have shown that we are able to control the temperature during the fermentation of soya beans to tempe. In this study it is shown that there are no significant differences of proteolysis between the traditional stationary process and the Rotating Drum Reactor during the first 48 hours of fermentations. But the fungal activity lasted longer in the RDR than in the traditional process as shown by continuing and the higher degree of hydrolysis, the higher content of WSS-N and the differences in TSS-nitrogen. In Figure 3 it is shown that during non-agitated fermentations (at 25°, 30° and 37 °C) the TSS-N levelled off after 48 hours while in the RDR the level still increased. This might be due to the limited heat and mass transfer in the non-agitated systems.

During fermentation we observed a shift in the ratio conglycinin: glycinin from 0.52 of raw beans to a minimum of 0.23 after 65 hours of fermentation at

25 °C. This implies that conglycinin was decomposed faster than glycinin. This phenomenon was reported earlier by Kim *et al.* (1990) for different microbial enzymes and also by Romagnola *et al.* (1990) in the rumen of Holstein cows. The resistance to degradation of glycinin compared to ß-conglycinin is probably associated with its chemical and physical structure.

The shift in the ratio $\alpha+\alpha'$ subunits of ß-conglycinin : ß subunits of ß-conglycine from 3.77 in the raw beans to 0.77 in the fermented product after 65 hours of incubation at 37 °C, implies that the α' and α subunits are more rapidly degraded during fermentation. Qi *et al.* (1992) observed similar patterns due to endogenous soya protease activity (Protease C1) during seed imbibition. It is expected that during the soaking of the soya beans endogenous enzymes could be active, however they will be inactivated during cooking. During the fungal fermentation stage the degradation of the α and α' subunits of ß-conglycinin is prolonged. Instead of endogenous protease activity this is probably caused by fungal proteases.

The shift in the ratio A-subunits of glycinin:B-subunits of glycinin from 1.54 in the raw beans to 0.72 in the RDR after 65 hours of incubation, implies that the A-subunits are decomposed faster than the B-subunits of glycinin, Romagnolo *et al.* (1990) observed a similar pattern for proteolysis in the rumen of Holstein cows.

As in tempe manufacture fermentation is stopped after 30 to 48 h the differences in protein hydrolysis of the traditional product and the RDR-product are small. Protein hydrolysis is an important factor affecting the taste of the final product, which is the subject of further study.

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CHAPTER 9

CONSISTENCY, POLYSACCHARIDASE ACTIVITIES AND NON-STARCH POLYSACCHARIDES CONTENT OF SOYA BEANS DURING THE TEMPE FERMENTATION

ABSTRACT

The relation between consistency of soya beans, polysaccharidase activities and the non-starch polysaccharides (NSP) content of soya beans was investigated during tempe fermentation. The fermentations were carried out in a Rotating Drum Reactor (RDR) as well as in the traditional stationary tempe process.
The firmness of the soya beans decreased rapidly during the first day of incubation. At increased incubation temperatures the hardness of the beans decreased more rapidly.

In the RDR the glycosidase activities became significantly higher beyond 48 hours of incubation compared with the traditional process. This might have resulted from (a) the better control of temperature and gas composition in the RDR and or (b) the agitation in the RDR.

The content of arabinose, galactose and uronic acids in the water-unextractable solids of NSP decreased more rapidly than that of glucose, mannose, xylose and fucose. These results indicate that during enzymatic maceration of soya beans in tempe fermentation, the arabinogalactan and pectin fractions are preferentially solubilized.

This chapter has been submitted as:
Consistency, polysaccharidase activities and non-starch polysaccharides content of soya beans during the tempe fermentation
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INTRODUCTION

Tempe is a traditional Indonesian fermented food which consists of cooked, dehulled soya beans, or other materials, bound into a solid cake by the growth of a mould, most commonly *Rhizopus oligosporus* (Nout and Rombouts 1990). In good quality tempe the bean cotyledons are bound together sufficiently tight to allow the product to be diced or sliced before frying.

Blakeman *et al.* (1988) compared three methods for assessing fungal biomass in tempe. They found that a force required to draw a pin through tempe gave a good indication of the degree of mycelial growth and substrate binding and suggested that this was a more useful measure than direct determinations of fungal biomass after enzymic digestion of the substrate or than assaying for N-acetyl glucosamine.

Nout *et al.* (1985) stated that uncooked tempe should have a consistency corresponding to a force of 1.5-2.5 N required to break a cylinder of tempe 50 mm long and 15 mm in diameter suspended at its extremities. This procedure is only applicable to well developed tempe and cannot be used to distinguish hyphal growth from the changes in consistency of the soya beans.

Arrifin *et al.*, (1994) described a quantitative method for measuring the strength of the hyphae binding the cotyledons together in soya bean tempe. The force to failure and the work to failure both increased from zero values before hyphal development to maximum values after about 30 h of incubation and then declined as the tempe aged and the hyphae started to deteriorate. Although, the authors stated, without experimental evidence, that only the force required to break the hyphae binding the beans together was measured, this method did not exclude changes in consistency of the soya beans.

Sarrette *et al.* (1992) described the production and activity of polysaccharidases, notably polygalacturonase, cellulase and xylanase by *Rhizopus oligosporus* during tempe fermentation. The maximum activity for all polysaccharidases tested occurred 20-30 h after inoculation.

The edible dry matter portion of soya beans consists of 38 % protein, 24 % total lipids, 31 % carbohydrates, 3 % fibre and 4 % ash (MacCarty and Matthew, 1984). The carbohydrate components can be distinguished into monosaccharides (minor quantities), oligosaccharides (10-15 % dm) and polysaccharides (13-22 % dm) (Fennema, 1976). Important components which are related to consistency of the soya beans are the plant cell wall polysaccharides. The cell wall polysaccharides can be divided into water extractable polysaccharides and water-

unextractable polysaccharides.

Several studies have focused on the isolation and characterization of cell wall polysaccharides from soya beans. Arabinose, galactose, glucose and galacturonic acid were prevailing sugar residues, in addition to lesser amounts of fucose, rhamnose, xylose and mannose (Schols *et al.*, 1993; Brillout *et al.*, 1988).

In the present study we investigated the production and activities of polysaccharidases in relation with the consistency of tempe. The fermentation was carried out with *Rhizopus oligosporus* at 25°, 30° and 37 °C in the traditional process. The results were compared with those obtained in a Rotating Drum Reactor (RDR) which was described previously by De Reu *et al.* (1993). In this laboratory-scale bioreactor, temperature control during solid-substrate fermentation can be achieved with discontinuous or continuous rotation and forced evaporation by aeration. As a result of the rotation a granular product is obtained in contrast with the cake-like solid mass resulting from the traditional process.

MATERIALS AND METHODS

Organism

Rhizopus microsporus var. *oligosporus* strain LU 575 (NRRL 5905) was grown and maintained at 30°C on malt extract agar (Oxoid, CM 59). Sporangiospore suspensions were obtained by scraping off the sporangia from a culture after 7 days incubation at 30°C, and suspending them in sterile distilled water containing 0.1% (v/v) Tween 80 (Merck, Germany). The viable count varied between $5*10^5$ and 10^6 cfu/ml, when determined on Rose-Bengal Chloramphenicol Agar (Oxoid CM549, England). The sporangiospore suspensions were used immediately after preparation.

Tempe manufacturing process

Dehulled yellow-seeded soya beans (*Glycine max*) were soaked overnight using the accelerated acidification method (Nout *et al.*, 1987). Subsequently, the beans (pH soak water < 4.2) were washed with tap water and boiled for 20 minutes, cooled, superficially dried (15 - 30 min, at room temperature) and inoculated using a sporangiospore suspension (1% v/w).

Petri dishes (ϕ 9 cm; 6 perforations/side, ϕ 1 mm) were filled with 60 g inoculated beans, sealed with tape and incubated at 25°, 30° or 37°C. An autoclaved Rotating Drum Reactor (RDR) (De Reu *et al.*, 1993) was filled with 1 kg of cooked and inoculated soya beans. The reactor was placed in a temperature

controlled incubator at 30 °C. During the experiments a discontinuous rotating scheme was used to avoid that the substrate temperature increased beyond 36 °C. After the substrate temperature reached 36°C, the reactor vessel was rotated clockwise and counter clockwise during 1 minute at 8 r.p.m. Due to the rotation, the temperature of the substrate dropped 1.5-2.0 °C. After rotation the reactor vessel stood still until the substrate temperature of 36 °C was reached again. Then the rotation cycle was repeated. This procedure was repeated continuously resulting in a saw-tooth temperature pattern (De Reu *et al.*, 1993).

Samples for biochemical analysis were taken at various time intervals and immediately frozen with liquid nitrogen. The samples were stored at -20°C, lyophilized and stored at -20 °C in the dark until analysis. Although incubation is stopped after 30 to 48 h under production conditions, we continued the fermentation up to 144 h to observe the effects of on-going biochemical processes. After 72 h of incubation the material inside the RDR, was removed and stored at 37 °C.

Consistency

To determine the consistency of the soya beans, 10 to 15 cotyledons were dissected carefully from the fermenting cake and placed in a Mulder-device (Mulder, 1946). With this system the force required to decrease the height of the individual cotyledons with 50 % was determined. The Student t-test (Owen, 1962) was used to determine whether differences were significant.

Enzyme extraction

The tempe samples were ground and frozen with liquid nitrogen, and stored at -80 °C until analysis. Enzymes were extracted by adding 9.5 or 9.0 ml water to 0.5 or 1.0 g of tempe, respectively, depending on the pre-determined enzyme level.

Enzyme assays

Activities of enzymes were assayed by determination of reducing sugars released by the hydrolysis of polygalacturonic acid (cat. nr. 102711, ICN pharmaceuticals, USA) for polygalacturonase activity; linear araban (NOVO) for arabinase activity; birch xylan (Roth GmbH & Co., Germany) for xylanase activity and carboxymethylcellulose (Akucell AF 0305, AKZO, the Netherlands) for endocellulase activity. A mixture of 250 μl, containing 50 μl substrate (0.5 % w/v in H_2O), 25 μl test sample and 175 μl 50 mM sodium acetate buffer, pH 5.0, was incubated for 1 h at 30 °C. The release of reducing sugars was determined with

the Nelson Somogyi method (Spiro, 1966) using a Beckman DU-64 spectrophotometer at 520 nm.

Activities of α-D-xylopyranosidase, ß-D-xylopyranosidase, α-D-glucopyranosidase, ß-D-glucopyranosidase, α-D-galactopyranosidase, ß-D-galactopyranosidase, α-D-mannopyranosidase (Koch-Light, Colnbrook Bucks, England; cat. nrs. 14308, 14303, 14279, 14280, 14301, 14277, 14285, respectively), α-L-arabinopyranosidase and α-L-arabinofuranosidase (Sigma, USA, cat. nr. N3512, N3641, respectively) were measured using their corresponding p-nitrophenyl (PNP) derivatives as substrates. An aliquot of 100 µl test sample was added to 25 µl PNP-substrate (0.1 % w/v in 50 mM sodium acetate buffer, pH 5.0) and incubated for 1 h at 30 °C. The reaction was stopped by the addition of 50 mM glycine buffer, pH 9.0 and 2 mM EDTA. The released PNP was measured at 405 nm, and quantified using a molar absorption coefficient of 13700.
All activities were expressed in international units (U). Measurements of enzyme activities were carried out in duplicate.

Non-starch polysaccharides

Total non-starch polysaccharides (NSP) content in the water-unextractable solids (WUS) fraction was estimated by adding the uronic acids content and the neutral sugar content.

Uronic acid content

The anhydro-uronic acid content of the WUS fraction was determined by the automated *meta*-hydroxydiphenyl assay (Thibault, 1979).

Neutral Sugars

Neutral sugars in the WUS fraction of tempe were analyzed as non-starch polysaccharides (Englyst and Cummings, 1984) using inositol as internal standard and pretreatment with 72 % (w/w) H_2SO_4 for 1 hour at 30 °C prior to hydrolysis with 1M H_2SO_4 for 3 h at 100 °C. After starch hydrolysis, extraction and washing of the lyophilized tempe, according to Englyst *et al.* (1984, 1992), WUS was obtained. The WUS fraction was hydrolysed. Alditol-acetates were obtained after a reduction and acetylation according to the method described by Englyst and Cummings (1984). Alditol acetates were separated using a DB1701 column (30 m, 0.32 mm ϕ) in Carlo Erba HRGC 5160 GC operated equipped with a Flame Ionisation Detector. Chromatography conditions were as follows: sample size, 1 µl; carrier gas, Helium; pressure, 100 kPa; the temperature was programmed from

80-180 °C at a rate of 2 °C/min and from 180-270 °C at a rate of 1.5 °C/min and was kept for 3 minutes at 270 °C.

RESULTS

Consistency

In Figure 1 the effects of incubation temperatures and the incubation period on the consistency of soya beans is shown. After soaking a force of 28.9 ± 5.0 N was required to reduce the height of the cotyledons with 45-50 %; subsequent cooking reduced the required force to 25.5 ± 4.9 N. During the first day of incubation the required force decreased rapidly from 25.5 ± 4.9 N to 13.6 ± 4.6 N, 11.0 ± 4.6 N, 10.1 ± 3.2 N and 7.3 ± 1.4 N at 25°, 30,° 37° and in the RDR (36 °C), respectively. During further incubation this sequence was maintained. The decrease in the required forces levelled off after the first day.

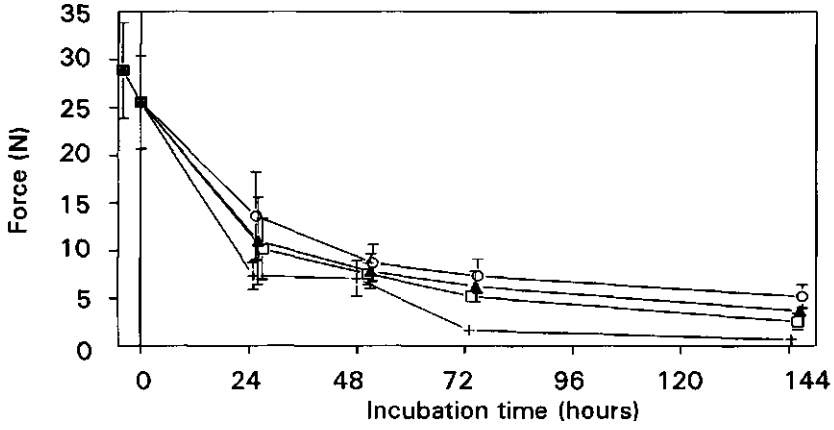

Figure 1. The consistency of tempe as function of the incubation time. Fermentations took place in the traditional system at 25° (-○-), 30° (-▲-) and 37 °C (-□-) and in the Rotating Drum Reactor (-+-) at 36 °C. The results on the y-axis represent means and standard deviation (n = 10-15). T = -4 h represents soaked beans. T = 0 h represents cooked soya beans after inoculation.

The firmness of the RDR-fermented material was significantly less ($p < 0.05$) than that of the traditionally fermented product, except after 48 hours of incubation. Between 25° and 30°C, no significant differences ($p > 0.05$) were observed after 1,2, and 3 days of incubation. The consistency of the beans

fermented at 30°C and 37°C became significantly different ($p < 0.05$) beyond 72 hours of incubation.

Enzyme activity

In Table 1 the enzyme activities during the fermentation of soya beans with *R. oligosporus* are shown for the traditional fermentation at 25°, 30° or 37 °C and for the agitated process in the RDR. The total enzyme activity (TEA) in the RDR and at 37 °C were similar after 48 h of incubation. During the third day of incubation the TEA increased in the RDR to 79 U/g lyophilized tempe, whereas the TEA at 37 °C decreased from 56 to 47 U/g lyophilized tempe.

If the TEA is distinguished into enzyme activity on polymers and that on glycosides some interesting differences are observed. In the traditional non-agitated fermentations the ratio depolymerases : glycosidases increased rapidly until 72 hours of incubation, in other words, in this system activity on polysaccharides became more important than glycosidase activity. If the incubation temperatures were compared, a significant increase was observed at 37 °C compared with 25° or 30 °C. When the individual depolymerases or glycosidases were compared, only small differences could be detected.

Non-starch polysaccharides

In Table 2 the neutral sugars and the uronic acid content of soya beans is shown during the fermentation with *R. oligosporus* at 25°, 30° or 37 °C in the traditional stationary system, as well as in the agitated RDR. In time a drop in total NSP was observed. The higher the incubation temperature the lower the level of NSP in the WUS fraction, at the end of the incubation. The differences in NSP content between the RDR and the traditional system at 37°C were very small.

Consistency versus enzyme activities and NSP content

In Figure 2 the relation between total polysaccharidase activities and the force (N) required to give a reduction of 50 % in height is shown. The higher the enzyme activity the less force is required to achieve a 50 % reduction in height of the cotyledons. Compared to the firmness at $t = 0$ (cooked inoculated beans) a significant decrease of force required resulted already from very low enzyme activities. A r^2-value of 0.48 was obtained when linear regression was used to fit the data.

Table 1. Enzyme activities (U/g lyophilized tempe) during the fermentation of soya beans in the stationary traditional process at 25°, 30° and 37 °C and in the agitated Rotating Drum Reactor (RDR) at 36 °C

	RDR (36 °C)				25°C			30°C			37°C		
Incubation time (h):	0	24	48	72	24	48	72	24	48	72	24	48	72
activity on polymers (I):													
arabinase	1	-1*	5	0	1	2	6	0	1	2	-2	10	10
cellulase	1	0	5	2	0	2	1	1	1	3	3	8	3
polygalacturonase	1	6	5	5	2	2	3	1	1	3	7	5	8
xylanase	1	3	4	3	1	1	1	1	1	3	5	7	6
Subtotal:	4	9	19	10	4	7	11	3	4	11	13	30	27
glycosidase activities (II):													
α-galactosidase	1	4	5	11	3	2	2	2	4	2	5	4	3
ß-galactosidase	1	4	4	7	3	2	2	1	3	2	4	3	2
α-xylanase	1	3	4	6	4	2	4	3	4	5	3	3	3
ß-xylanase	1	4	4	7	3	2	2	2	4	2	3	3	2
α-mannosidase	1	2	4	7	3	3	2	1	3	2	3	3	2
α-glucosidase	1	2	4	7	3	2	2	2	4	2	4	3	2
ß-glucosidase	1	3	4	7	3	2	2	2	4	2	4	3	2
arabinofuranosidase	1	3	4	10	3	2	1	1	3	2	3	2	2
arabinopyranosidase	1	3	3	7	3	2	2	1	4	2	3	2	2
Subtotal:	9	28	36	69	28	19	19	15	33	21	32	26	20
Total:	13	37	55	79	32	26	30	18	37	32	45	56	47
Ratio (I)/(II):	0.21	0.29	0.51	0.16	0.12	0.31	0.73	0.19	0.17	0.59	0.44	1.17	1.47

*) negative values are obtained due to correction for blank results

Table 2. Non-starch polysaccharides content (μmol/g water-unextractable solids) during the fermentation of soya beans in the stationary traditional process at 25°, 30° and 37 °C and in the agitated Rotating Drum Reactor (RDR) at 36 °C

	RDR (36°C)				25°C			30°C			37°C		
Incubation time (h):	0	24	48	72	24	48	72	24	48	72	24	48	72
arabinose	339	263	187	194	383	243	263	284	262	220	319	195	163
fucose	48	44	35	36	54	44	50	48	46	43	47	38	34
galactose	625	497	356	350	684	477	518	548	496	429	595	387	350
glucose	420	563	430	405	648	554	613	486	658	546	574	386	358
mannose	43	45	43	43	49	44	45	46	45	42	44	35	33
rhamnose	32	26	18	19	36	24	27	30	25	22	29	20	16
xylose	139	134	94	96	171	123	129	127	140	114	145	91	81
uronic acids	72	16	16	10	57	54	57	69	52	54	46	39	27
Total:	1718	1588	1179	1053	2082	1563	1702	1638	1724	1470	1799	1191	1062

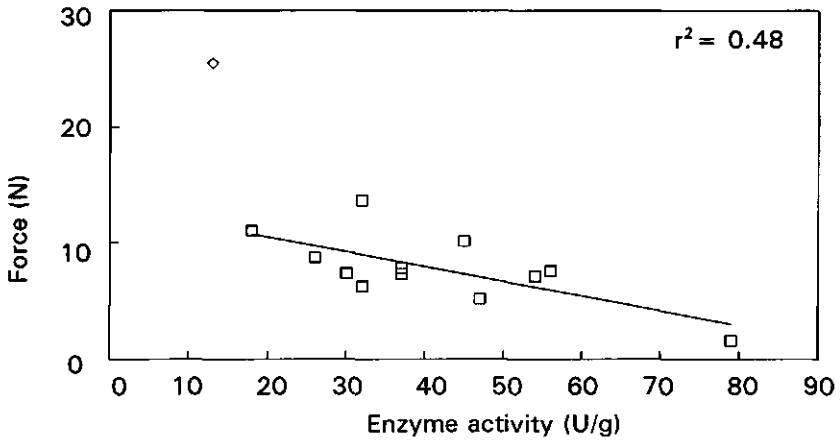

Figure 2. Relationship between total polysaccharidase activities (U/g) and the force (N) required to give a reduction of 50 % in height of the soya cotyledons. Results represent pooled data of experiments carried out in the RDF and the traditional fermentation system. ◇ represents cooked inoculated beans and □ represents fermented samples.

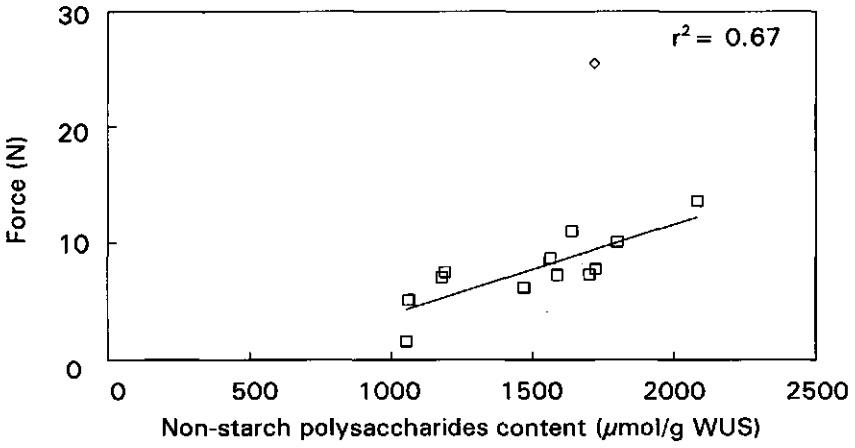

Figure 3. Relationship between Non-starch polysaccharides content of soya beans (μmol/g water-unextractable solids) and the force (N) required to give a reduction of 50 % in height of the soya cotyledons. Results represent pooled data of the experiments carried out in the RDF and the traditional fermentation system. ◇ represents cooked inoculated beans and □ represents fermented samples.

In Figure 3 the relation between the NSP-content (μmol/g WUS) of the soya beans and the force (N) required to obtain a 50 % reduction in height is shown. The r^2 of the fit is 0.67. The lower the NSP content the less force is required, indicating a less firm structure. Increases above the initial value of 1718 μmol NSP/g WUS might be due to shifts in the composition of the WUS fraction, e.g. protein becomes more soluble during fermentation (De Reu *et al.*, Chapter 8).

DISCUSSION

In tempe research little attention has been paid to polysaccharidases and their effects on the consistency of soya beans. It is expected that during fermentation the hyphae may soften the soya beans by enzymatic degradation of cell walls and mechanically pushing the cells apart.

Histological observation of penetration of *R. oligosporus* into soya beans showed that penetration and growth of the hyphae were generally inwardly directed and perpendicular to the surface cells (Jurus and Sundberg, 1976). The degree of distortion caused by the fungus was most severe at or near the cotyledon surface. Portions of the outermost cell layers of the cotyledon were often completely permeated with mycelium, creating an indistinct mass. Walls of these cells were shrivelled, the cytoplasm was very distorted, and frequently tissue was so grossly disrupted that individual cells were no longer recognizable. Fewer hyphae and little or no distortion were observed among inner cellular layers of the cotyledon (Jurus and Sundberg, 1976).

The major reduction in consistency was observed after the first day of incubation, which implies that during the initial growth stages of fungal development mechanical and enzymatic maceration are very effective. During the later stages the measured enzyme activities were higher but had less effect on the consistency. It seems that cell wall polysaccharides which are involved in maintaining the structural integrity of the beans could be hydrolysed easily during the 24 h of incubation, and that after that time the remaining structure is less sensitive for the increased amount of polysaccharidases. Based on the observed reduction in NSP content of the soya beans it seems that penetration of the hyphae into the soya beans is related to hydrolysis of cell wall polysaccharides. After penetration of the outer layers of the cotyledons, the energy sources in the inner tissue, e.g. lipids (De Reu *et al.*, 1994) and proteins become accessible towards hydrolysis by lipases and proteases, respectively.

As described earlier (De Reu *et al.*, 1993) the RDR enables solid-substrate

fermentations to be conducted under controlled conditions of temperature and oxygen supply by avoiding heat and mass transfer limitation problems. As expected for solid-substrate fermentations in the traditional packed bed systems, growth limiting conditions were rapidly reached. After 48 hours of incubation the total enzyme activity increased rapidly in the RDR while in the traditional systems the maximum enzyme activity had already been passed.

In the RDR the consistency changes as a function of both enzyme activity and shear forces during agitation, and therefore it is not surprising that the hardness was less than in the product of stationary fermentation. However, we were not able to distinguish whether this was due to the higher total enzyme activity or to the effect of shear forces or combinations of both.

Schols *et al.* (1993) described the structure of the cell wall polysaccharides of soya. Most arabinose residues were 1,5-linked and 1,3,5-linked, indicating the presence of (highly) branched arabinans, which were mainly attached to other polymers (e.g. galactan or pectin). Alkaline treatment yielded a fraction with solubilized non-esterified polysaccharides mainly composed of arabinose, galactose and galacturonic acid. These polysaccharides were considered to be accessible for degradation by endo-ß-1,4-galactanase, endo-α-1,5-arabinanase, exo-arabinanase and endo-polygalacturonase activities or combinations thereof. The reduction of arabinose, galactose and uronic acids (Table 2) show that during enzymatic maceration predominantly the arabinogalactan and pectin fractions were solubilized.

During tempe fermentation growth of *R. oligosporus* on soya beans resulted in complex biochemical and physical changes of the soya beans. The results might be used to form a link between previous histological observations and cell wall structure studies. The observed changes improve the fundamental knowledge about the tempe process and could be used to optimize the production process, in particular in determining the required incubation time.

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CHAPTER 10

GENERAL DISCUSSION

In this thesis aspects of the solid-substrate fermentation (SSF) of soya beans to tempe were studied. Particular attention was paid to process innovations and product characteristics. The aim of this study was to develop a process for the production of a tempe-like product which could be used as an intermediate product for various food formulations.

Process innovations included the design and evaluation of novel equipment as well as developing new methods. Product characteristics of the traditional product were compared with those of the product obtained with the novel techniques. The latter part of the study was focused on the macro-nutrients, e.g. lipids, proteins and carbohydrates.

In this general discussion, the process innovations and product characteristics are reviewed in relation to the traditional process and their possible impact on the future processing. The discussion is divided into sections which correspond to the different stages of the tempe process, as shown in Chapter 2 (Figure 2).

a) Dehulling

As the mould *Rhizopus oligosporus* cannot grow on whole soya beans, dehulling i.e. removal of the seed-coat is an essential step in the production of tempe. By dehulling, one of the major anti-nutritional factors, viz. phytate is removed to a large extent. Soya beans can be dehulled by either wet or dry processing. Wet dehulling is generally carried out after precooking, which facilitates softening of the seed-coats. At present most wet dehulling is mechanized in the traditional process, using simple electric driven concrete disc dehullers having a sufficiently large gap to avoid damaging cotyledons (Nout and Rombouts, 1990).

Dry dehulling before hydration is a desirable, efficient method providing that suitable mechanical equipment is available. Burr, corn or steel roller mills may be used to crack the hulls (Ko and Hesseltine 1979).

Dry-dehulled soya beans were used throughout this study since they are readily available in this form in The Netherlands.

b) Hydration and fermentative souring

During the traditional process in Indonesia, the soya beans undergo a natural

microbial fermentation during the hydration (soaking) process (Winarno and Reddy, 1986). During this period of 12-48 hours at approx. 28 °C, the micro-flora residing in the beans and the soaking vessels act as a starter for mixed lactic acid/ acetic acid fermentations which lower the pH from 6.5-7.0 to 4.5-5.5. In temperate climates the accelerated acidification method is a powerful method to obtain a vigorous lactic fermentation (Nout *et al.*, 1987). To prevent spoilage of the final product, it is necessary that the beans are properly acidified (pH < 4.2) prior to fungal fermentation. An alternative for fermentative souring is acidification by addition of lactic or acetic acid to the soak water. According to Ashenafi (1991 a,b) growth of *Listeria monocytogenes* and *Bacillus cereus* is inhibited by adding *Lactobacillus plantarum* to the inoculated beans. As was shown in Chapter 5, a disadvantage of accelerated acidification for a period as long as 24 h, is that the formation of acetic acid might lead to levels of undissociated acetic acid (> 0.05 % w/v) that inhibit the germination of *R. oligosporus*, which in turn results in an increase in process time.

To prevent accumulation of excess acetic acid, pure cultures of homofermentative strains of lactic acid bacteria might be used. However, as shown in Chapter 5, even homofermentative lactic acid bacteria strains still produce some acetic acid on semi sterile (10', 121 °C, dry beans) soya beans. The use of pure culture starters of lactic acid bacteria is more expensive compared to the traditional process and the accelerated acidification process. The success of dry pure starter cultures of lactic acid bacteria depends of the hygienic conditions used, which are in general poor at the traditional production sites. Another way to prevent excess acetic acid is to reduce the soaking period from 24 hours to 12-16 hours, using the accelerated acidification method. This is a cheap and easy to adapt method which enables a constant acidification of the soya beans.

Well acidified beans (pH < 4.2) and a short lag phase can be achieved by soaking the soya beans according to the accelerated acidification method during 12-16 hours at 30 °C.

c) Cooking

According to Steinkraus (1983) the purpose of cooking is threefold. It is necessary to destroy contaminating bacteria that could interfere with fermentation, to inactivate antinutritional factors such as protease inhibitors, and to release some of the nutrients required for mould growth. During the cooking process, a heat stable, water soluble mould inhibitor is leached out into the water, which is discarded (Ko and Hesseltine, 1979). Flatulence producing factors such as

stachyose and raffinose are partially removed by soaking and cooking (Winarno and Reddy, 1986).

Cooking also reduces the level of organic acids and increases the pH of the beans. A reduction in the concentration of organics acids of ± 40 % was found when 300 g of soaked beans were boiled in 1.5 l of tap water for 20 minutes. By increasing the volume of water, a further reduction of excess acetic acid might be achieved; but this will increase the amount of energy required for cooking, and cause a pH increase as well.

As a result of the increased pH, the substrate becomes an easy target for growth of unwanted acid-sensitive spoilage bacteria. One should try to find an equilibrium between a short lag phase as a result of the removal of acetic acid, and an acceptable microbial quality of the final product resulting from cooked beans of lower pH.

d) *Draining, cooling*

Traditionally, the boiling water is drained and the cotyledons are spread quickly outdoors on plaited bamboo trays (Nout and Rombouts 1990). Evaporative cooling decreases the temperature of the soya beans rapidly. The cooled cotyledons must have a dull appearance, indicating a relatively dry surface. Shiny, glistening cotyledons are still too wet; as the presence of free water is associated with bacterial spoilage during the incubation phase.

A factor influencing the total process time is the temperature to which the soya beans should be cooled. In Chapter 4 a relation between the temperature of the substrate and the lag phase was shown. Initial bean temperatures between 35-40 °C tended to give a shorter lag phase, resulting in a shorter process time. This observation was confirmed in the experiments which were described in Chapter 5. Incubation at high initial bean temperatures will result in a shorter process time. However, if the fermentation is carried out in traditional tray systems, the incubation temperature should be decreased to approximate 30 °C, otherwise growth limiting conditions are reached rapidly.

e) Inoculum

Traditional techniques for inoculation employ either dried and pulverized tempe of a previous batch ('tempe-to-tempe'), leaf grown inoculum locally referred to as 'usar' or 'laru' (Ko and Hesseltine 1979) or inoculum grown in steamed rice and/or cassava flour inoculated with usar.

Pure fungal culture starters are prepared by growing a pure culture *R.*

oligosporus strain on sterile substrate (e.g. soya beans, wheat or rice) followed by dehydration and pulverizing (Ko and Hesseltine, 1979). Pure fungal culture starters are essential for controlled experimental fermentation, and for this purpose several ways of starter manufacture were developed (Ko, 1985; Shambuyi *et al.*, 1992). As only small amounts of inocula were required in this study, fresh sporangiospore suspensions were used, which enabled us to standardize the age of the culture and the concentration of viable sporangiospores.

f) Fermentation chambers

Banana leaves or other large leaves are known to serve as excellent wrapping for traditionally fermented tempe (Steinkraus, 1983). The leaves can also be used to cover 4-6 cm thick beds of inoculated beans. Steinkraus *et al.* (1965) developed a small factory process in which mesh trays were used. In The Netherlands, perforated hard plastic boxes are used to produce tempe in attractive brick shapes.

The height of the bed is restricted by the access of sufficient oxygen, removal of carbon dioxide and the poor heat conductivity of the soya beans. Thus, increasing the production by increasing the bed height is difficult, if not impossible. To remove the excess heat and to supply sufficient oxygen and to remove carbon dioxide, rotating drum reactors, aerated packed-bed reactors and gas-fluidized bed reactors were tested and compared to the 'conventional' perforated plastic boxes.

In Chapter 3 it was shown that the substrate temperature in a drum reactor can be controlled by discontinuous rotation. But by doing this a granular product is obtained. A major difference between the product of discontinuous rotation and the traditional product, is that the former assumes a granular shape instead of the traditional cake-like mass. This is not necessarily a disadvantage, as the aim of this study was to develop a process for the production of a tempe-like product which could be used as an intermediate product for various food formulations.

Packed-bed reactors (PBR) are widely used in laboratory studies of SSF. In Chapter 4 a PBR was described, which was used to validate a model for the dynamic behaviour of the cultivation of *Rhizopus oligosporus* during the tempe process. In the PBR heat was removed by forced evaporation which also resulted in the removal of water from the substrate. The model fitted the experiments well until the optimum growth temperature was reached. Beyond that temperature, the predicted temperature was higher than the measured temperature. Possible explanations are shrinkage of the substrate and a decrease of T_{max} at lower water activities.

Gas-Fluidized-Bed reactors (GFBR) have been described for some SSF-

processes (Mitchell *et al.*, 1992). In a collaborative study with the Technical University of Berlin it was concluded that cooked/wet soya beans behaved like a moving bed instead of fluidized-bed. Therefore it was not possible to ferment soya beans in a GFBR.

g) Incubation

Once the beans are inoculated it takes about 6-16 hours before CO_2 becomes detectable and 12-24 hours before the temperature increases. These variations in time result from a complex relation between the properties of sporangiospores, substrate and type of incubator as shown in Table 1.

Table 1. Factors influencing the growth of *R. oligosporus* during SSF

SPORANGIOSPORES	SUBSTRATE	INCUBATION
▸ viability	▸ pH	▸ atmospheric temperature
▸ quantity	▸ organic acids	▸ initial substrate temperature
▸ inhibitors	▸ nutrients	▸ oxygen supply
	▸ contamination	▸ relative humidity
	▸ water activity	▸ heat removal

On soya beans it is difficult to study the viability of the sporangiospores. It is expected that viability is influenced by age of the spores, storage conditions, temperature and water activity.

The concentration of viable spores is important, the larger the quantity of viable spores the shorter the lag phase (Peñeloza *et al.*, 1992), while fermentations with lower inoculum densities resulted in increased maximum specific growth rates.

In liquid media it was shown (Chapter 5) that a substance was formed by the germinated spores of *R. oligosporus* which inhibited their germination. The unknown substance is heat-stable and soluble in both water and organic solvents. The self-inhibition was only observed at high sporangiospore densities, viz. approx. $1x10^6$ c.f.u./ml. As the inoculum densities in tempe are significantly lower ($\pm$ $1x10^4$ c.f.u/g cooked beans), it is expected that this inhibitory effect is not important during tempe production. However, Kidby *et al.* (1977) concluded that at $7.8x10^5$ spores per gram dry beans, no lupin tempe was produced due to self-inhibitory activity.

As shown in Chapter 5 the pH, organic acids and the availability of nutrients are also related to germination and growth of *R. oligosporus* sporangiospores. The concentration of undissociated acetic acid appears to be very critical, but its effect can partly be masked by lactic acid. An optimum pH of 4.0 for germination was found.

One problem encountered in this study was the definition of the end-point of the fermentation, in other words: when is tempe a good tempe? Objective quality parameters of fresh tempe include: consistency corresponding to a force of 1.5 - 2.0 N required to break a cylindrical sample of 50 mm length and 15 mm diameter suspended at its extremities; surface colour within the ranges: L = 78-83, b-a = 12-14 (Hunter LAB); and pH 6.0 - 6.6 (Nout *et al.*, 1985). For the granular product obtained with the RDR only the pH could be used of those parameters.

Biochemical changes

Once the spores have germinated, a complex system of substrate uptake, biomass formation and product formation is activated. To obtain sufficient nutrients for mycelium development, the fungi should penetrate into the substrate. To damage and or to weaken the structure of the outer layers of the cotyledons, carbohydrases such as endocellulase, polygalacturonase, endo-arabinase, α- and ß-xylanase, α- and ß-galactosidase, α- and ß-glucosidase and α-mannosidase were formed. The penetration of mycelium into the soya beans was decribed earlier by Jurus and Sundberg (1965). Some of the carbohydrases may release compounds which could serve as carbon and energy source for fungal biomass formation.

As shown in Chapters 7 and 8, significant protease and lipase activities occur in tempe. The lipases hydrolysed the triglycerides to free fatty acids and glycerol. During fermentation a loss of fatty acids was observed, this implies that the fatty acids are used in the metabolism of the mould. The fatty acids form a source for carbon in addition to the carbohydrates. There is also a turnover from the amino acids from the substrate into mycelial biomass initiated by protein hydrolysis by the proteases excreted into the medium. A shift towards a higher level of water and TCA-soluble protein was observed. In the later stages of the fermentation ammonia was found and deaminases were activated as well.

Little attention has been paid in this study to the production of metabolites other than carbon dioxide. But other research groups focused on the formation of vitamins (Keuth *et al.*, 1993) and antioxidant formation (Klus *et al.*, 1993). It is also known that *Rhizopus* spp. are able to form several organic acids, viz. gluconic acid, lactic acid, fumaric acid, malic acid and oxalic acid (Cochrane, 1958).

Nutritional quality and health aspects

The nutritional quality of tempe is an important parameter to focus on. In Table 2 advantages and disadvantages are shown of fermenting cooked soya beans compared with unfermented cooked soya beans.

Table 2. Advantages and disadvantages of the fermentation of soya beans to tempe compared with cooked soya beans

ADVANTAGES	DISADVANTAGES
▸ improved organoleptic qualities	▸ shift towards saturated fatty acids
▸ pre-digested proteins, carbohydrates	▸ digestibility of fungal cell wall ?
▸ removal of ANF's	▸ loss of energy due to fermentation
▸ vitamin B formation	▸ reduced shelf life of tempe
▸ anti-oxidant formation	▸ loss of dry matter
▸ anti-diarrhoea factor	
▸ improved availability of minerals	

The Protein Efficiency Ratio (PER) and digestibility of cooked soya beans were hardly improved by fermentation into tempe (Zamora and Veum 1988; Murata *et al.*, 1971), when studied in healthy rats and pigs. In this study a shift towards more saturated fatty acids during fermentation was observed, which is unfavourable. Due to the proteolytic and carbohydrolytic activity, the soya beans are more or less pre-digested, which might result in a better protein uptake, although this has not been studied in man. On the other hand, during fermentation there is a shift from indigestible plant cell wall material to equally indigestible fungal cell wall material. It is not very well known to what extent other mycelium compounds could be digested by man. In general there is a loss of energy during fermentation, due to the formation of biomass and metabolic heat. Therefore energy sources which could have been used by man are depleted.

However, recovery from diarrhoeal disease was reported to be faster with a tempe-based formula compared with a milk-based formula. A tempe-based formula resulted also in a better weight gain, immunological values and increase of haemoglobin (Karyadi *et al.*, 1990), but it is still unknown which substances are responsible for this tendency.

Knowing the nutritional advantages and disadvantages of tempe, it remains difficult to conclude, as is generally stated, that fermentation is necessary to

convert raw soya beans into a nutritious food. An alternative such as hydrolysed soya protein might be used in Western countries for infants who show allergic reactions towards animal proteins, but it is not an alternative for a cheap basic food such as tempe is now in Indonesia.

Usefulness of reactors

According to Winarno and Reddy (1986) approximate 130,000 people are employed in the approximate 41,000 tempe production sites in Indonesia. The total annual tempe production in Indonesia was estimated on 765,000 tons of tempe. (Karta, 1990). The largest Indonesian factory produces 800 kg tempe/day. In general the investments are low to start a small tempe factory in Indonesia: a dehuller, soaking vessels, cooking pot and wooden trays form the standard equipment. Tempe production in such small factories is labour intensive. From a technological point of view such production sites can be mechanized easily.

In this study we showed that at a laboratory scale RDR could be used to obtain a product with a comparable composition as tempe while avoiding heat and mass transfer problems. Unfortunately, we were not able to increase the production scale of the RDR due to time and financial limitations, so we do not know whether this reactor type is suitable on a larger production scale in practice.

Theoretical studies on a production scale of 500 kg dry matter/h showed that a process line should include the following pieces of equipment: a dehuller, soaking vessels, steam toaster, reactor and a dryer. Several reactor types were studied and special attention was paid to the removal of heat. If the incubation time is set at 40 hours and the dry matter content at 0.4 kg dry matter/kg tempe, it can be concluded that for a tray reactor a surface area of 2000 m^2 is required for a bed height of 5 cm and a packing density of 500 kg/m^3 reactor volume. The size of this reactor is impractable. The aerated packed-bed reactor is excluded for the same reason, the reactor will be too large or the energy cost for aeration to high.

From a theoretical point of view the gas-fluidized-bed reactor is suitable, because heat and mass transfer are less limited compared with the tray reactor. However, we concluded in paragraph f (fermentation chambers) that this type of reactor can not be used due to the inadequate fluidization behaviour of the cooked soya beans.

The size of a suitable Rotated Drum Reactor is estimated at a diameter of 3 m with a length of 20 m. Those sizes are valid for a design were aeration is supplied axially through the substrate.

Finally we can conclude that: 1) The technological feasibility of the scale-up

of the RDR process requires further study. 2) A future implementation of such a process will also depend on a variety of other socio-economic, political, and business-related factors.

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SUMMARY

Solid-substrate fermentation (SSF) systems, involving the growth of microorganisms on moist substrate in the absence of free water, have been used extensively from ancient times in the European, Asian and African countries for the production of fermented foods, starter inocula, mushrooms, dough fermentations, enzymes, flavour components, compost, cheeses and ethanol.

The size of the reactors is restricted by heat- and mass transfer limitations, which might result in unfavourable growth conditions. One way to prevent such conditions is by agitation of the substrate. In this study a Rotating Drum Reactor (RDR) was designed for the fermentation of soya beans with *Rhizopus oligosporus*. The final product, know as 'tempe' is a cheap, protein rich, traditional Indonesian foodstuff. However it might also be used as an intermediate product in various food formulations.

The reactor and the measurement and control system enable an automatic control of the process. The most important process parameters are: rotation speed, substrate temperature, rotation frequency and the relative humidity.

A major disadvantage of RDR that has been cited in literature is the sensitivity of micro-organisms towards agitation. In our study we have shown that the fungal activity in a discontinuous RDR remained high up to 70 hours while in the traditional non-agitated systems fungal activity decreases already after 36 hours of incubation. Unfortunately this feature can not be used in tempe production where incubation is stopped after 30 to 48 hours. If the RDR is used for other purposes, such as enzyme production, the utilization of the substrate might be more effective in the RDR compared to tray or packed-bed reactors. In addition to better temperature control agitation results also in a homogeneous product.

An important variable in the tempe fermentation is the lag phase of the germination of fungal spores. Viability and quantity of the sporagiospore suspension are important factors, as well as pH and temperature of the substrate. The optimum pH for germination was pH 4.0, whereas the highest germination percentages were found at temperatures between 37 and 42 °C.

During soaking of soya beans, according to the accelerated acidification method, lactic acid (2.3 % w/v) and acetic acid (0.3 % w/v) were found in the soak water after 24 hours of incubation at 30 °C. Undissociated acetic acid has an inhibitory effect on germination of *R. oligosporus* sporangiospores at

concentrations exceeding 0.05 % (w/v). As the pH of the beans after soaking is between 4.0 and 4.2 most of the acetic acid is undissociated. The inhibitory effect of acetic acid is reduced by lactic acid, the mechanism underlying this effect still being unknown. A reduction in the lag phase could be achieved if less acetic acid is formed in the soak water. Theoretically this could be done by using homofermentative lactic acid bacteria, but it was observed that on soya beans the homofermentative lactic acid bacteria still produced acetic and propionic acids in addition to lactic acid. A second way to avoid inhibitory levels of acetic acid is to reduce the soaking time in the accelerated acidification method from 24 to 12-16 hours, which might be sufficient to achieve a pH below 4.2.

During SSF in a tray reactor or in a non-aerated packed-bed reactor the oxygen concentration is decreased to < 1 % due to metabolic activity while the carbon dioxide level increased to high levels (± 20 %). It was observed that when *R. oligosporus* was grown on model media, oxygen levels below 1 % were inhibitory to the growth rate. On the other hand levels of 5 - 10 % carbon dioxide had a growth stimulating effect when oxygen was at a growth limiting level. Higher carbon dioxide levels had also an inhibitory effect on the growth rate of *R. oligosporus*. In the RDR fungal activity (estimated on the basis of degree of hydrolysis, pH, and heat production) rapidly fell at oxygen levels below 10 %. High concentrations of carbon dioxide had an inhibitory effect.

During fermentation several enzymes, viz. lipases, proteases, phytases and carbohydrases are formed by *R. oligosporus*. Due to the enzymatic activity changes in the chemical composition of soya beans were observed.

Triglycerides were hydrolysed to free fatty acids and glycerol. At increasing temperatures a decrease in the total fat content was observed. It was also observed that the level of free fatty acids was lower than expected based on the decrease in glyceride bound fatty acids. This might be explained by the fact that *R. oligosporus* used fatty acids as a source of carbon.

A decrease in the amount of water soluble protein in the soya beans was observed during soaking (45.7 → 7.45 % w/w total protein) and cooking (7.4 → 2.0 % w/w total protein) of the soya beans. During fermentation the content of water-soluble protein increased to 34 % in the RDR after 72 hours of incubation. When the degree of hydrolysis (DH) was compared between the RDR and the stationary process (25°, 30° and 37 °C) no significant differences were observed until 48 hours of incubation. Beyond 48 hours the DH increased rapidly in the RDR, which might be the result of the on-going fungal activity. Based on the observation that the entire water-soluble protein fraction was also soluble in 13.6 % TCA it

was concluded that exo-proteases were active in the RDR.

Several active carbohydrases were found: endocellulase, polygalacturonase, endo-arabinase, α- and ß-xylanase, α- and ß-galactosidase, α- and ß-glucosidase and α-mannosidase. Their activity affected the consistency of the soya beans; in the traditional process significant differences were observed between 25°, 30° and 37 °C. The higher the temperature the softer the soya beans became. It was also observed that the firmness of the product in the RDR was significantly less compared to the non-agitated samples.

There might be nutritional benefits from the fermentation step in tempe manufacture through hydrolysis of soya bean cell walls, fats and proteins, making the product more readily to digest.

SAMENVATTING

Vastestoffermentatie (SSF) wordt gekarakteriseerd door de groei van micro-organismen op vaste organische substraten in de afwezigheid van vrij water. Vele vastestoffermentaties worden al eeuwenlang bedreven, met name in het Verre Oosten en Afrika. SSF kan worden toegepast bij de produktie van enzymen, organische zuren, kaas, champignons, aromacomponenten, compost, ethanol en andere gefermenteerde levensmiddelen zoals tempe en sojasaus.

De produktieschaal wordt bepaald door massa- en warmteoverdracht. Vanwege de slechte warmteoverdracht in het substraat kunnen snel ongunstige groeiomstandigheden worden bereikt. Een mogelijke oplossing voor het beheersen van de groeiomstandigheden is het mengen van het substraat waardoor gradiëntvorming wordt voorkomen. In deze studie is de aandacht gericht op de ontwikkeling van een draaiende trommelreactor (RDR). In dit onderzoek is er gewerkt aan de fermentatie van sojabonen met de schimmel *Rhizopus oligosporus*. Het eindprodukt, tempe, is een traditioneel, eiwitrijk, goedkoop, Indonesisch voedingsmiddel. Het kan ook worden toegepast als een bestanddeel in een samengestelde produkten.

De ontwikkelde reactor, inclusief de meet- en regelapparatuur, maakt een volledige automatische besturing van het proces mogelijk. De belangrijkste procesvariabelen zijn: de substraattemperatuur, de rotatiefrequentie, de rotatiesnelheid, de omgevingstemperatuur en de relatieve luchtvochtigheid.

Een in de literatuur beschreven nadeel van roterende trommel reactoren is de gevoeligheid van micro-organismen voor afschuifkrachten tijdens roteren waardoor er minder of geen groei zou optreden. In deze studie is aangetoond dat met behulp van discontinu roteren de fermentatie veel langer actief kan worden bedreven en dat beschadiging van het mycelium derhalve niet desastreus hoeft te zijn. In het traditionele niet-roterende proces stopt de schimmelactiviteit na ± 36 uur, terwijl in de RDR tot 70 uur kan worden doorgegaan. In de praktijk wordt het tempeproces gestopt na 30 tot 48 uur. Het voordeel dat de RDR ten opzichte van het traditionele proces biedt kan dus niet volledig worden benut. Door rotatie verkrijgt men wel een homogener produkt.

Indien men echter de RDR voor andere doeleinden gaat gebruiken, zoals voor de produktie van enzymen, dan zal in vergelijking tot het stationaire proces het substraat beter worden benut in de RDR.

Een belangrijke variabele in het tempeproces is de lagfase, de periode na beënting tot uitgroei van de schimmelsporangiosporen. Naast het aantal sporen en de kiemkracht van de sporen zijn ook externe factoren zoals temperatuur en pH van het substraat belangrijk. De optimum pH voor ontkiemen is 4.0. De hoogste ontkiemingspercentages worden bereikt bij temperaturen tussen 37 en 42 °C.

Tijdens het weken van de bonen, volgens de versnelde verzuringsmethode, vindt men na 24 uur weken 2.3 % (w/v) melkzuur en 0.3 % (w/v) azijnzuur in het weekwater. Van azijnzuur is aangetoond dat het in ongedissocieerde vorm remmend werkt op de ontkieming, bij concentraties boven de 0.05 % (w/v). Daar de pH na weken tussen de 4.0 en 4.2 ligt zal het aanwezige azijnzuur volledig ongedissocieerd zijn.

De remmende werking van azijnzuur kan echter onderdrukt worden door melkzuur, het mechanisme hiervan is onbekend. Een verkorting van de lagfase kan men verkrijgen door te voorkomen dat er een remmende hoeveelheid azijnzuur wordt gevormd. Dit kan op twee manieren. Ten eerste door de bonen te verzuren met homofermentatieve melkzuurbacteriën, echter de praktijk heeft uitgewezen dat er dan door aanwezigheid van zuurstof naast melkzuur toch ook nog azijnzuur en propionzuur gevormd worden. Ten tweede door de methode van versnelde verzuring te verkorten van 24 uur naar 12 à 16 uur. De bonen hebben dan al wel een voldoende lage pH (<4.2), maar de concentratie van azijnzuur is dan nog niet remmend.

Naast de temperatuur is ook onderzocht wat het effect is van verschillende gasconcentraties op de groei van *Rhizopus oligosporus*. Tijdens een niet beluchte fermentatie in een 'tray' of gepakt-bed reactor kan als gevolg van groei de zuurstof concentratie sterk dalen en de kooldioxideconcentratie sterk stijgen. Zuurstof blijkt in concentraties beneden de 1 % remmend te werken bij groei op modelsubstraten. Concentraties van 5-10 % kooldioxide werken stimulerend indien zuurstof limiterend is, terwijl bij hogere kooldioxide concentraties de groeisnelheid sterk daalt. In de RDR neemt de schimmelactiviteit (gemeten als eiwitafbraak, pH toename en warmteproduktie) snel af beneden de 10 % zuurstof. Hoge concentraties kooldioxide vertonen ook hier een remmende werking. Tijdens de fermentatie vormt *Rhizopus oligosporus* diverse enzymen; lipases, proteases, phytases en carbohydrases. Als gevolg van de enzymatische activiteit treden er veranderingen op in de chemische samenstelling van de sojabonen.

Op het gebied van de vetzuren is er aangetoond dat de triglyceriden worden afgebroken tot vrije vetzuren en glycerol. Naarmate de temperatuur dichter bij het optimum lag (37-42 °C) nam de afbraak toe. De vetmassabalans klopte echter niet,

de toename in vrije vetzuren was minder dan de afname van gebonden vetzuren. De vrije vetzuren fungeren zeer waarschijnlijk als koolstofbron voor de schimmel. Tevens is er een verschuiving naar meer verzadigde vetzuren waargenomen.

Op het gebied van soja-eiwit werden de volgende trends gesignaleerd: de hoeveelheid wateroplosbaar eiwit neemt sterk af tijdens het weken (45.7 → 7.45 % w/w totaal stikstof) en het koken (7.4 → 2.0 % w/w totaal stikstof) van de sojabonen. Tijdens de fermentatie stijgt het weer tot ± 34.0 % (w/w totaal stikstof) in de RDR. Als de RDR wordt vergeleken met het traditionele proces (bij 25°, 30° en 37 °C) dan valt op dat er tot 48 uur incuberen weinig verschillen zijn in eiwitafbraak, maar na 48 uur verloopt de eiwitafbraak in de RDR veel sneller dan in het traditionele proces, hetgeen kan worden verklaard door de verlengde schimmelactiviteit in de RDR. Het afbraakpatroon in de RDR geeft aan dat het gevormde protease hoofdzakelijk een exo-protease is, dit omdat de wateroplosbare fractie ook volledig in 13,6 % TCA oplosbaar is.
Het opslageiwit conglycinine werd sneller afgebroken dan glycinine, dit is te verklaren door het verschil in chemische struktuur.

Op het gebied van de carbohydrases zijn activiteiten van de volgende enzymen gemeten: endocellulase, polygalacturonase, endo-arabinase, α- en ß-xylanase, α- en ß-galactosidase, α- en ß-glucosidase en α-mannosidase. Als gevolg van deze enzymactiviteiten worden de sojabonen zacht. Er is aangetoond dat de hardheid van de bonen in de RDR verder daalt dan in het traditionele proces. Dit kan mede veroorzaakt zijn door de afschuifkrachten die in de RDR optreden.

Het is mogelijk dat er voedingskundige voordelen zitten aan de fermentatiestap bij de tempebereiding; door ontsluiting van de boonweefsels, en hydrolyse van vetten en eiwitten zou het produkt lichter verteerbaar kunnen worden.

CURRICULUM VITAE

Johannes Cornelis de Reu werd geboren op 27 mei 1967 te Hoek. Na het behalen van het diploma voorbereidend wetenschappelijk onderwijs aan de Rijksscholengemeenschap Petrus Hondius te Terneuzen in 1985, begon hij aan zijn studie levensmiddelentechnologie aan de toenmalige Landbouwhogeschool. Afstudeervakken tijdens de doctoraalperiode lagen op het vlak van de levensmiddelenmicrobiologie en proceskunde. De stageperiode werd uitgevoerd bij Cargill te Bergen op Zoom en bij Brown Brothers Winery, Milawa te Australië. Het doctoraaldiploma werd hem uitgereikt op 30 november 1990.

Het in dit proefschrift beschreven onderzoek werd uitgevoerd van 1 december 1990 tot 30 november 1994 binnen de secties levensmiddelenchemie en -microbiologie en proceskunde van de vakgroep levensmiddelentechnologie van de Landbouwuniversiteit te Wageningen. Tevens werd een bijdrage geleverd aan diverse onderwijstaken van de vakgroep.

Sinds 1 december 1994 is hij werkzaam als toegevoegd onderzoeker bij de leerstoel geïntegreerde levensmiddelentechnologie van de sectie zuivel en levensmiddelen-natuurkunde aan de Landbouwuniversiteit te Wageningen.