Process control of solid-state fermentation

Simultaneous control of temperature and moisture content

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Chapter 1

General Introduction

USE OF SOLID-STATE FERMENTATION

The main task of fungi in nature is to produce hydrolytic enzymes for the breakdown of organic waste materials, plant residues, vegetables and all sorts of waste into inorganic mineral compounds. Bacteria largely do the actual breakdown. To ensure that bacteria do not scavenge all the hydrolysis products, many fungi make antibiotics. These capabilities are the basis of the flourishing antibiotics and enzyme industries. Nowadays, micro-organisms are used in many industrial processes to produce enzymes and other molecules, which are used in peoples everyday life. For example, washing powder now contains enzymes that enable the process to be carried out at a relatively low temperature, and we cannot imagine a life without antibiotics.

In the Western world, the fermentation industry cultivates fungi – and other micro-organisms – in liquid nutrient broth, so-called submerged fermentation (SmF). In Asia, fermentation products are made by cultivating fungi in the absence of free-flowing water on a solid substrate such as cereal grains or beans, so-called solid-state fermentation (SSF). Fungi grow well in solid-state fermentation, because the conditions are similar to their natural habitats, such as organic waste materials, trees and soil. Fungi can withstand limited water availability in the solid substrate, whereas most bacteria cannot. SSF holds tremendous potential for the production of enzymes, which break down macromolecules to smaller molecules that can be further metabolised by bacteria, but also for high added-value pharmaceutical products.

The breakdown or conversion of molecules using enzymes is of interest to almost any industry. These enzymes give a very specific, natural conversion under mild conditions in contrast to chemical conversions, which usually require harsh process conditions that often give undesired side products. In addition to the production of enzymes, SSF can be used to produce a variety of complicated biomolecules, which are almost impossible to synthesize chemically. In the following paragraphs a short overview is given on how SSF is or can be applied in several industries.

Food industry

SSF can be used in the food industry for the production of enzymes, organic acids, aroma compounds and other substances of interest. For the manufacture of soy sauce, enzymes like proteinases are produced industrially by cultivation of *Aspergillus oryzae*

on a mixture of wheat bran and soy flakes. These proteinases convert the insoluble protein in the soy flakes into smaller soluble peptides and amino acids, which make up the soy sauce.
Citric acid is the most important organic acid for the food industry and is currently produced in tonnage by submerged fermentation. However, SSF has great potential to produce citric acid more economically using alternative feedstocks such as agro-industrial residues (VandenBerghe *et al.*, 1999).
Bacteria and fungi are able to synthesize different interesting aroma compounds in SSF. For example, *Ceratocystis fimbriata* cultivated in SSF on coffee husk produces strong pineapple aroma (Soares *et al.*, 2000). Also food additives like pyrazines, which possess a nutty and roasty flavour, can be produced using SSF (Seitz, 1994). L-glutamic acid, a flavour enhancer, is produced by SSF with a bacterial strain of *Brevibacterium* sp. on sugarcane bagasse impregnated with a defined media (Nampoothiri and Pandey, 1996).
Recently, a culture of *Xanthomonas campestris* was used in an SSF-based process for the production of xanthan gum that is used in the food industry as an emulsifier or thickener (Stredansky and Conti, 1999).

Pharmaceutical industry

A new era in medical therapy and the pharmaceutical industry started with the discovery of antibiotics. Penicillin and other secondary products of fungi, actinomycetes and bacteria have provided the most powerful tools to fight bacterial and fungal infections. Several patents (Merck, 1990; 1991) describe the use of SSF for the production of new antibiotics against for example *Cryptococcus* sp., *Candida* sp. and *Streptomyces* sp. Surfactin, a lipopeptide antibiotic, which inhibits fibrin clotting, was produced by cultivating a *Bacillus* sp. on okara, which is a solid waste product of Tofu (bean curd) production. The production in SSF was 4-5 times more effective than in SmF (Nakayama *et al.*, 1997; Ohno *et al.*, 1995).
In January 2001, a lovastatin production facility that uses SSF has been approved by the FDA (www.biocon.com). Statins belong to a group of cholesterol reducing drugs effective in preventing both first and second heart attacks in patients with hypercholesterolemia.

Agro-industry

Fungal biopesticides are an environmentally friendly alternative to chemical pesticides. In literature, a large number of fungi are described as promising candidates for biopesticides (Deshpande, 1999). For example, *Coniothyrium minitans* is a natural antagonist of the fungus *Sclerotinia sclerotiorum*, a widespread plant pathogen affecting more than 360 plant species. This biopesticide is commercially available and is produced by solid-state fermentation on cereal grains (www.prophyta.com). SSF is preferred for the production of this biopesticide as spore yields are higher compared to submerged fermentation (McQuilken *et al.*, 1997). This applies to many fungal biopesticides.

Biological detoxification

Certain agro-industrial residues contain toxic (anti-physiological and anti-nutritional) compounds such as hydrogen cyanide, caffeine, tannins, aflatoxins, etc. that pose difficulties in their effective utilisation. Their disposal is a problem for the processing industries as it leads to serious environmental burdens. SSF has been recently applied to detoxify residues such as cassava peels, rapeseed meal, canola meal, coffee husk, coffee pulp, etc. For example, SSF of cassava peels showed a 95% reduction in hydrogen cyanide level and a 42% reduction in soluble-tannin levels (Ofuya and Obilor, 1994).

COMMERCIALIZATION OF SSF

In recent years, research on SSF has led to a wide range of applications on lab scale, and comparative studies between SmF and SSF claim higher yields for products made by SSF (Pandey *et al.*, 2000; Pandey *et al.*, 1999). In spite of these examples the commercial application of SSF processes in Western countries remains unusual mainly due to problems associated with scale-up. In the Western world, SmF has become the standard process for microbial products. However, in South-East Asia, where SSF is more common, research attention was directed towards the industrialization of fermented foods like soy sauce, miso, natto, sufu, tempeh, and the like, to meet the growing demand of these products caused by a rapidly increasing population. These

products were all made using SSF and large bioreactors were developed from 1950 onwards (Steinkraus, 1989).

The main problem associated with scale-up of SSF is the removal of heat generated by the metabolic activity of the micro-organisms. Heat removal by conduction in static packed beds is limited due to: (1) the poor heat transfer through the bed (Saucedo-Casteneda *et al.*, 1990); and (2) the lack of heat-exchange surface on a large scale. Evaporative cooling has proven to be more efficient than convection and conduction (Sargantanis *et al.*, 1993). It results, however, in large moisture losses and drying of the solid substrate. Therefore, it is essential to combine temperature and moisture control in large-scale SSF systems. Moisture-content control is achieved by adding water during fermentation. The addition of water has major implications on the design of the bioreactor. Water has to be added homogeneously to the solid substrate, which requires a mixing device inside the bioreactor. Mixing during SSF sets additional requirements for the solid substrate and the fungus. First, the solid substrate should be able to withstand shear forces caused by mixing and should not have the tendency to aggregate to clumps as a result of mixing. Second, mixing could damage the fungus, which could decrease its growth and production rate. This effect could also depend on the bioreactor scale, because the scale affects forces acting on the solid mash.

Besides the question "How to add water?", it is also important how much water should be added for optimum growth of the fungus. For example, the production of enzymes and secondary metabolites like aromas and antibiotics, is influenced by the water activity of the solid substrate (Gervais, 1990; Liu and Tzeng, 1999; Narahara *et al.*, 1982). The water activity of the solid substrate changes as a result of SSF. For example, water is taken up by the fungus in order to synthesize new fungal hyphae. In addition, water evaporates from the solid substrate due to metabolic-heat production and solute concentrations increase as a result of enzymatic degradation. All these processes influence the water activity, which makes it very difficult to control. Besides that, information about the water balance and water activity during solid-state fermentation is scarce.

OUTLINE OF THIS THESIS

The aim of the project described in this thesis was to demonstrate the feasibility of solid-state fermentation with controlled temperature and moisture content on labscale.
At first a model system was chosen based on an agar matrix as solid support in which a defined medium was dissolved. *Rhizopus oligosporus*, having known physiology and kinetic parameters, was chosen as a model organism. Chapter 2 describes the improvements made to this model system with respect to pH control and growth.
It was expected that such a well-defined model system would facilitate research, as the results would be more reproducible. A second advantage of using an agar gel is that it allows direct determination of biomass dry matter and this is almost impossible when a solid substrate is used. However, this model system turned out to be not very practical for research on SSF. The main problem was that the effect of dehydration of agar beads is not comparable to the dehydration that occurs in the majority of SSF applications, due to the high moisture content of agar beads (95% water).
The use of *R. oligosporus* as model organism also had some disadvantages that would hinder the interpretation of data. *R. oligosporus* has the ability to convert sugars anaerobically when oxygen limitation occurs. This would hamper the prediction of biomass production and the relation between heat production and oxygen consumption would become less accurate.
Therefore, we decided to switch to a different SSF system in which *Aspergillus oryzae* was cultivated on moist wheat grains. Like *R. oligosporus*, *A. oryzae* is widely applied in industrial SSF for the production of enzymes and "koji", an intermediate which is used for the production of soy sauce and miso.
The use of mixed bioreactors for SSF was considered the first step in solving scale-up problems with respect to temperature and moisture-content control. Although mixing of the solid substrate offers many advantages, the question remained if these would outweigh possible disadvantages like effects of shear on growth. Chapter 3 shows that mixed SSF can be applied without seriously affecting fungal growth.
In Chapter 4 a model is decribed, which estimates the extracellular (nonfungal) and overall water content of wheat grains during SSF. Model parameters were determined using an experimental membrane-based model system, which mimicked the growth of *A. oryzae* on the wheat grains and permitted direct measurement of the fungal biomass

dry weight and wet weight. The model can be used to calculate the water addition that is required to control the extracellular water content in a mixed solid-state bioreactor.

Chapter 5 describes a control strategy for simultaneous control of the temperature and moisture content during cultivation of *A. oryzae* on wheat in a continuously mixed paddle bioreactor. Evaporative cooling with varying air flow rate was applied to control the temperature of the solid substrate (Chapter 3). The extracellular water content was controlled by adding a fine mist of water droplets to the mixed solid substrate, using a previously described model (Chapter 4) to calculate the required addition.

Chapter 6 describes the use of ^{1}H-NMR imaging as a powerful technique to study solid-state fermentation at the particle level. Gradients inside substrate particles cannot be prevented in solid-state fermentation. In this chapter we describe gradients in moisture and glucose content during cultivation of *A. oryzae* on membrane-covered wheat-dough slices; the gradients were calculated from ^{1}H-NMR images measured *in vivo*.

Finally, in the last chapter two commercial bioreactors are discussed that can facilitate the scale-up and process control of SSF. The koji bioreactor is discussed as a suitable alternative for the production of low to medium-added-value products in the food industry. Bioreactors based on the design of industrial solids mixers are discussed as alternative for the production of pharmaceuticals and other high-added-value products.

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Chapter 2

Improved model system for solid-substrate fermentation

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ABSTRACT

In a model system for solid-substrate cultivation of *Rhizopus oligosporus*, it was observed that without pH control rapid acidification occurred, which inhibited the biomass production. With some degree of pH control, biomass production was improved significantly. Several buffering agents with different initial pH values were tested to find the optimum pH and the most suitable buffer for this model system. Between pH 4 and 6, the biomass-production rate was not significantly influenced by pH and citric acid was the most suitable buffer.

Addition of 5 g/L tryptone to a standard mineral medium increased both the maximum specific growth rate (μ_{max}) and the maximum CO_2-production rate, and strongly shortened the lag phase.

INTRODUCTION

Solid-substrate fermentation (SSF) is generally defined as the growth of micro-organisms - often fungi - on solid substrates in systems with a continuous gas phase and no free-flowing water. In order to develop bioreactors for large-scale SSF, a quantitative analysis of kinetics and stoichiometry of the reaction is needed. This analysis is hampered in most natural SSF systems by experimental difficulties, because the substrate/support is structurally and nutritionally heterogeneous and measurement of biomass dry matter is impossible. Several model systems have been developed to overcome these problems. For example, Raimbault and Alazard (1980) impregnated bagasse with a mineral glucose medium and Mitchell *et al.* (1986; 1988a; 1988b; 1989) used κ-carrageenan gel with a mineral starch medium. The advantage of using a gel over bagasse is that it allows direct determinations of biomass dry weight. Auria *et al.* (1990) used a synthetic resin (Amberlite IRA-900) impregnated with a defined mineral medium, which also allowed direct biomass determination.

In previous work (Rinzema *et al.*, 1997) a model system with agar and a defined glucose medium was used to cultivate *Rhizopus oligosporus*, which is quite similar to the system described by Mitchell *et al.* (1988b). However, glucose rather than starch was used as carbon source, in order to avoid a decrease in reaction rate attributed to diffusion limitation of glucoamylase into the matrix (Mitchell *et al.*, 1991). Two

drawbacks were observed with this model system : (1) the pH change was large and its effect on the growth rate was not clear; (2) the specific growth rate was low compared to that for the natural substrate (soy beans). The present paper describes two attempts to improve the model system: better pH control and enhanced growth rate.

Control of pH could be critical for certain SSF processes (Agosin *et al.*, 1989; Yang and Chiu, 1986), but is difficult in unmixed SSF. Some degree of pH control in both natural and model SSF systems is usually obtained by using different ratios of ammonium salts and urea in the substrate (Raimbault and Alazard, 1980; Yang, 1988; Saucedo-Castañeda *et al.*, 1992). Supplementation with urea is not completely effective in stabilising the pH, which in most cases results in a fluctuation of the pH by more than one pH unit (Prior *et al.*, 1992). Combining the above with the narrow optimal pH range (7-7.5) found for *R. oligosporus* in radial-growth-rate studies (Mitchell *et al.*, 1988b), better pH control is expected to be necessary.

The second improvement of the model system concerns the increase of the growth rate. A high growth rate is essential for studying one of the major scale-up problems in SSF: the removal of reaction enthalpy. Heat removal through the wall is expected to be minimal in large-scale bioreactors due to the relative decrease in wall area versus reactor volume. In research with down-scaled bioreactors aiming at scale-up studies, a high growth rate minimises the ratio between inevitable heat losses over heat production and will therefore help to approach the behaviour of a large-scale bioreactor. The maximum specific growth rate (μ_{max}) measured for *R. oligosporus* on soy beans at 30 °C was 0.46 h^{-1} (De Reu, 1995). In our initial model system (Rinzema *et al.*, 1997) the average value for μ_{max} was only 0.27 h^{-1} (unpublished). However, the purpose is to develop a model system which approaches the specific growth rate found on the natural substrate. For that, the effect of subsequent additions of soya peptone, tryptone or vitamins on μ_{max} is studied.

The first aim of this research was to improve pH control in the model system through optimal use of buffering agents. First, the effect of increasing phosphate-buffer concentrations on the pH decline was investigated. Second, the effect of pH on the maximum specific growth rate was studied. Third, four buffers were compared to find the most suitable buffer. The second aim of this research was to increase the growth rate by subsequent additions of soya peptone, tryptone or vitamins.

MATERIALS AND METHODS

Culture

Rhizopus oligosporus NRRL 5905 was grown for 7 days at 30 °C on malt-extract agar (Oxoid, CM 59, UK). To obtain the sporangiospore suspension, a solution of 1 g neutralised bacteriological peptone (Oxoid, L34, UK), 8.5 g NaCl and 10 ml Tween 80 per 1000 ml distilled water was made. Ten millilitres of this solution was added to the plates and a sporangiospore suspension was obtained by scraping the sporangia off the agar. This suspension was filtered through sterile glass wool to discard the remaining mycelium. This spore suspension was stored at -80 °C in cryo vials (Greiner, Germany) for up to 6-8 months. Each vial contained 800 µl suspension and 300 µl glycerol. The viable count of each cryo vial varied between $2 \cdot 10^5$ - $3 \cdot 10^5$ colony forming units (cfu) per ml.

Inoculum

The sporangiospore suspension in the cryo vial was allowed to defrost at 4°C for 2 hours. Inoculum was prepared by mixing the content of one cryo vial with 9 ml of a solution containing 1 g neutralised bacteriological peptone (Oxoid, L34) and 8.5 g NaCl per 1000 ml distilled water.

Media

All chemicals were obtained from Merck (Germany), unless otherwise stated. The standard medium consisted of 22 g $C_6H_{12}O_6 \cdot H_2O$, 10 g $(NH_4)_2SO_4$, 10 ml filter-sterilised stock solution of trace elements ($MnCl_2 \cdot 4H_2O$ 0.1 g/l, $CoCl_2 \cdot 6H_2O$ 0.03 g/l, $CuSO_4 \cdot 5H_2O$ 0.02 g/l, $Na_2MoO_4 \cdot 2H_2O$ 0.02 g/l, $FeSO_4 \cdot 7H_2O$ 0.5 g/l, $ZnSO_4 \cdot 7H_2O$ 0.2 g/l, EDTA 1 g/l), 1 g $CaCl_2$, 0.5 g $MgCl_2 \cdot 6H_2O$, 2 g K_2HPO_4, 15 g agar (Oxoid, L11) and a buffer per 990 ml distilled water. The phosphate buffer was prepared from equimolar quantities of K_2HPO_4 and NaH_2PO_4. Lactic-acid buffer was prepared by adding 30 g lactic acid (90% DL, Cat. no. 366) and 13.5 g KOH to 1 liter standard medium. Citric-acid buffer was prepared by adding 27.5 g citric acid (Cat. no. 244) and 20 g KOH to 1 liter standard medium. Succinic-acid buffer was prepared by adding 17.25 g succinic acid (Cat. no. 681) and 5.8 g KOH to 1 liter standard medium. All molar buffer concentrations are given in Table 1.

Several additions to the standard medium with 0.14 M citric-acid buffer were done in order to investigate their effect on the growth rate. Vitamins were added using 10 ml of Kao and Michayluk vitamin mixture (Cat. no. K3129, Sigma, USA) and 1 ml Provasoli's vitamin mixture (Cat. no. P9931, Sigma, USA) per 1000 ml medium. Filter-sterilised tryptone (L42, Oxoid, UK) was used in two different concentrations: 1 and 5 g/l. Filter-sterilised soya peptone (L44, Oxoid, UK) was used in a concentration of 1 g/l.

Experimental Set-up

For each series, 20 agar plates with 20 ml medium were inoculated with 100 µl inoculum. Two glass jars were used, equipped with a septum and each containing one agar plate, to ensure a closed environment in which the cumulative CO_2-production and O_2-consumption during growth could be determined in duplicate. Agar plates and jars were placed in an incubator (IKS, The Netherlands) at 30 °C.

Sampling procedure

Two agar plates were removed at regular time intervals. The mycelium could easily be pulled off the agar slab. This gave virtually complete recovery of biomass dry weight, whereas melting and filtration gave significant dry-weight losses. Although no membranes were used in these experiments (except for the experiment shown in figure 2), the agar did not adhere to the mycelium and no significant mycelium fragments could be measured by melting and filtration of the remaining agar. In the experiment shown in figure 2, sterile 0.45 µm membranes (Schleicher & Schuell, OE67, Germany) were used. The mycelium was dried for 2 days at 80 °C, placed in a desiccator with kieselgel to cool down to room temperature, and dry weight was measured on an analytical balance (Mettler, AE260-S, Switzerland).

The agar slab was cut in vertical direction into two equal pieces with a scalpel. One piece was liquefied in a micro-wave oven after which the pH was measured at approximately 50°C with a pH meter (WTW, ph323, Germany). The pH measurement in this way represents an average pH value of the agarslab, which may not be the pH the fungus actually encounters. The second piece of agar slab was weighed and frozen at -20 °C in a sample bottle for further analysis. Before analysis this sample was thawed and 10 ml of distilled water was added. The diluted sample was homogenised

through rotation of the sample bottle for 2 h with three small glass beads (∅ 1 cm). The homogenate was filtered through a 0.45 µm filter (Schleicher and Schuell, FP030/2, Germany) and stored at -20 °C for later assay.

Glucose analysis

Glucose was determined using a Peridochrom glucose kit (Cat. no. 676543, Boehhringer Mannheim, Germany).

Organic-acids analysis

Citric acid and succinic acid were analysed by HPLC using an Aminex HPX-87H stainless-steel column (Biorad) at 37 °C and a refractive-index detector (RM IV, Thermo Separation Products, The Netherlands). The acids were eluted with 5 mM H_2SO_4 (0.6 ml·min^{-1}).

Determination of specific growth rate and lag phase

The specific growth rate of *R. oligosporus* was determined from the accumulation of CO_2 in the head space of the jars. Several authors (Narahara *et al.*, 1982; Nishio et *al.*, 1979; Okazaki and Sugama, 1979; Okazaki *et al.*, 1980) have shown that CO_2 production can be used to determine the specific growth rate during SSF. At regular time intervals 100 µl gas samples were taken from the jars. Samples were analysed on a gas chromatograph (CP9001 TCT/PTI 4001, Chrompack, England) for CO_2 and O_2 concentrations. Maximum specific growth rates were estimated by fitting the logistic-growth equation, modified to allow for a lag phase, to the measured amount of CO_2.
The modified logistic-growth equation reads :

$$\begin{aligned} t \le \lambda: &\qquad P(t) = P_0 \\ t > \lambda: &\qquad P(t) = \frac{P_0 \cdot P_{\max}}{(P_{\max} - P_0) \cdot e^{-\mu_{\max}(t-\lambda)} + P_0} \end{aligned} \tag{1}$$

Where

P_0	=	initial amount of CO_2	[mol]
P_{max}	=	maximum amount of CO_2	[mol]
$P(t)$	=	amount of CO_2	[mol]
μ_{max}	=	maximum specific growth rate	[h^{-1}]
λ	=	lag phase	[h]

The values for μ_{max}, λ, P_0 and P_{max} were determined by using the curve-fitting program TableCurve™ 2D (v2.03, Jandel Scientific Software). Figure 1 shows an example of a curve fit. For this method to be used, the respiration coefficient should be constant and anaerobic fermentation should not occur. Anaerobic conditions were avoided in these experiments by using a low glucose concentration and a thin layer of agar in the petri-dish. Therefore the respiration coefficient remained constant during cultivation, which was verified with oxygen and carbon-dioxide measurements.

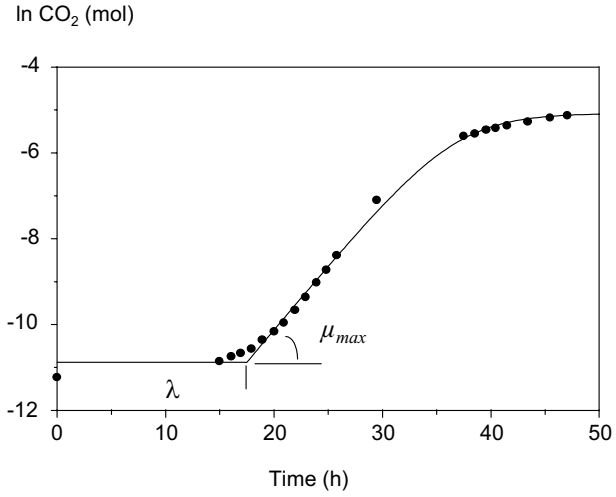

Figure 1. Natural logarithm of the amount of carbon dioxide produced and the fit by the modified logistic equation for growth of *Rhizopus oligosporus* in a model system to determine the maximum specific growth rate (μ_{max}). data ●; Modified logistic equation (——).

Calculation of required buffer concentration

In order to determine the effect of pH on the the specific growth rate, the pH changes should be minimal during cultivation. Different buffers have to be used for different initial pH values in such a way that equal pH changes can be expected. Therefore the buffer concentration was calculated for each buffer from an expected biomass production, a chosen initial pH (pH_0) and pH change (ΔpH). From previous experiments the maximum biomass dry weight for this model system was predicted. In this model system $(NH_4)_2SO_4$ is used as nitrogen source and this is responsible for the pH decrease during cultivation. Through the stoichiometric formula of *R. oligosporus* $CH_{1.88}O_{0.51}N_{0.17}$, the amount of used NH_4^+ (ΔNH_4) is calculated with the assumption that every NH_4^+ used by the fungus releases H^+ into the medium.

The following equation yields the necessary buffer concentration in mol/l to allow for a chosen pH decrease during cultivation:

$$HB_{tot} = \frac{\left[\Delta NH_4 + 10^{-pH_0} - 10^{-(pH_0-\Delta pH)}\right]\cdot\left[10^{-pH_0} + K_a\right]\cdot\left[10^{-(pH_0-\Delta pH)} + K_a\right]}{-\left[10^{-pH_0} - 10^{-(pH_0-\Delta pH)}\right]\cdot K_a} \qquad (2)$$

Where

HB_{tot}	=	total buffer concentration	[M]
pH_0	=	initial pH medium	[-]
ΔpH	=	maximum allowable pH drop	[-]
ΔNH_4	=	ammonium uptake for biomass production	[-]
K_a	=	dissociation constant	[M]

RESULTS & DISCUSSION

Effect of pH

In our model system it was observed that rapid acidification to pH 2.5 occurred with a 0.018 M phosphate buffer. An increase of the phosphate-buffer concentration to 0.12 M gave biomass yields that were more than twice as high (Figure 2).

The pH decline with the 0.12 M phosphate buffer was still rather large (2.6 pH units), and it was unclear from this experiment if the final amount of biomass could be further improved. A further increase of the phosphate-buffer concentration minimised the pH decline during cultivation, but the maximum amount of biomass was the same and heterogeneous biomass distribution over the agar plate was observed.

The fact that the pH dropped to 4 (0.12 M phosphate buffer) and that the biomass production rate did not change significantly (Figure 2), was not in agreement with the narrow optimal pH range found by Mitchell *et al.* (1988b), based on radial-growth rates. Thus the aim of the following experiment was to determine the effect of pH on the maximum specific growth rate and the biomass production rate. Different buffers were used to stabilise the pH at different initial pH values in order to avoid large differences in ionic strength. The concentrations of the various buffers were calculated to give equal buffering capacity.

Table 1. Effect of pH on specific growth rate and maximum biomass production of *Rhizopus oligosporus* on agar plates at 30 °C with the use of different buffering agents.

buffer	mol/l	initial pH	pKa	μ_{max} * (h^{-1})	r_{CO2} * ($\cdot 10^{-4}$ mol/h)	max. biomass dry weight (g)
Phosphate	0.18	6.29	7.21	0.29 (± 0.016)	2.87 (± 0.17)	0.22
Succinic acid	0.15	5.91	5.61	0.31 (± 0.028)	3.17 (± 0.26)	0.17
Citric acid	0.14	4.91	4.77	0.28 (± 0.027)	3.04 (± 0.09)	0.20
Lactic acid	0.14	4.01	3.86	0.23 (± 0.034)	2.33 (± 0.15)	0.11
Lactic acid	0.30	4.40	3.86	0.24 (± 0.007)	3.10 (± 0.36)	0.15

*The values between brackets indicate the ± 95 % confidence intervals

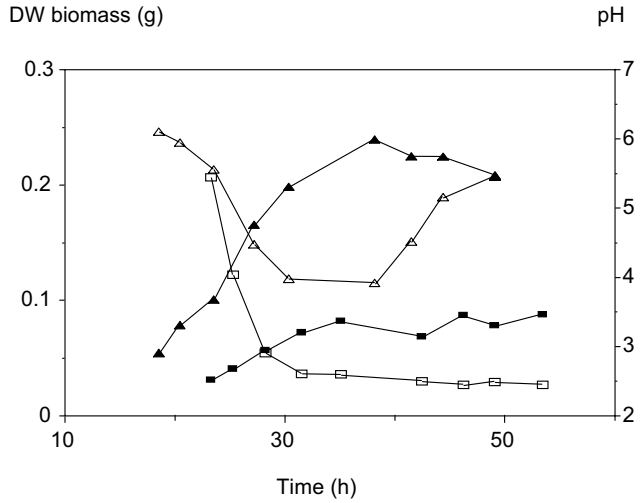

Figure 2. Effect of an increase in phosphate-buffer concentration on the pH profile and biomass development during growth of *Rhizopus oligosporus* using a model system. $[PO_4^{3-}]_{tot}$ = 0.018 M (DW ■, pH □); $[PO_4^{3-}]_{tot}$ = 0.12 M (DW ▲, pH △)

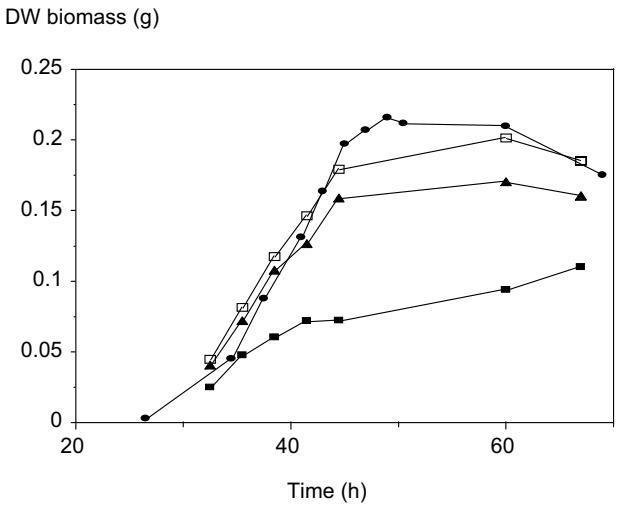

Figure 3. Biomass profiles during growth of *Rhizopus oligosporus* using a model system with different buffer solutions. Phosphate (0.18 M) ●; Succinic acid (0.15 M) □; Citric acid (0.14 M) ▲; Lactic acid (0.14 M) ■.

A broad optimal pH range from 4 - 6.5 was observed for the maximum specific growth rate (μ_{max}) of *Rhizopus oligosporus* in this model system (Table 1). A similar result was also found by Mitchell *et al.* (1991) where pH had little effect on the specific glucose uptake over a range between 5 and 7. These results are not in agreement with the narrow optimal pH range found by Mitchell *et al.* (1988b), for radial-growth rates. This may be due to an effect of pH on the width of the peripheral growth zone. The specific growth rate is only directly related to colony radial growth rate if the peripheral growth zone remains constant, which apparently is not the case for *R. oligosporus* when pH effects are studied.

Biomass profiles (Figure 3) were also considered because μ_{max} is only an indicator of the initial specific growth rate and does not reflect effects over the whole growth curve.

Results showed for the case of the lactic-acid buffer (pH = 4) that although μ_{max} was high (Table 1), the biomass production was lower due to pH values below 3.5 (Figures 3 and 4). This was confirmed with additional experiments using a stronger lactic-acid buffer (0.3 M, initial pH 4.4) which gave better biomass yields and a final pH of 3.9. These results indicated that problems can be expected for growth of *R. oligosporus* at pH values below 4.

At the end of the cultivation an increase in pH was observed of 1-1.5 pH units above the initial values with citric acid or succinic acid as buffer (Figure 4).

In preliminary experiments with these buffers in which glucose was omitted from the medium, it was found that these buffers could not be consumed as sole carbon sources. Nevertheless, it was found that citric acid and succinic acid were consumed when glucose became limiting (Figure 5), which explained the increase in pH towards the end of the cultivation period.

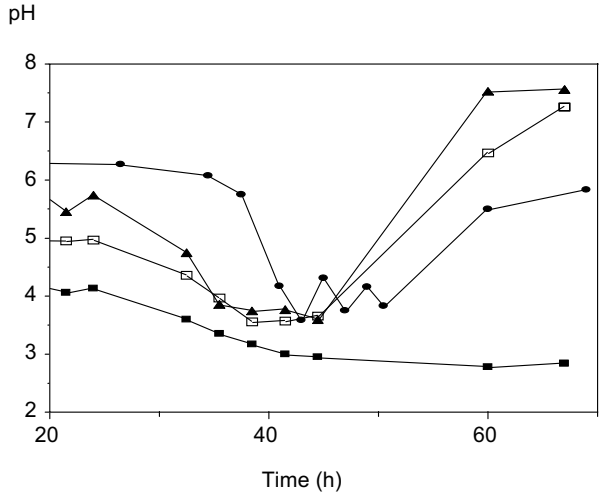

Figure 4. pH profiles during growth of *Rhizopus oligosporus* using a model system with different buffer solutions. Phosphate (0.18 M) ●; Succinic acid (0.15 M) □; Citric acid (0.14 M) ▲; Lactic acid (0.14 M) ■.

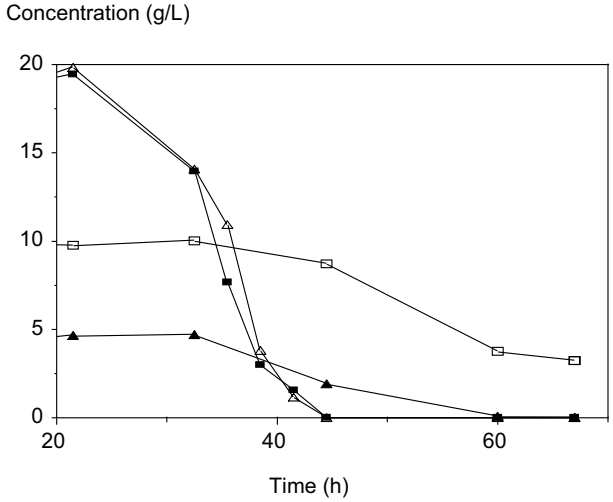

Figure 5. Glucose and buffer concentrations during cultivation of *Rhizopus oligosporus* on media with glucose(■) + citric acid (□) and glucose(△) + succinic acid (▲) respectively.

The increase in pH for the phosphate buffer towards the end of the cultivation period could not be explained directly. In order to find a non-utilised buffer in a slightly acidic environment, phthalate was also considered as buffer in this study. Growth did not occur with a 0.15 M phthalate buffer at pH 6, which made this buffer not suitable.
In summary, pH has little influence on growth between pH values of 4 and 6.3, which means that pH dependencies can be omitted from the kinetic description in this range. In order to avoid bacterial growth during fermentation a slightly acidic environment is preferred. Therefore, citric acid is chosen as the most suitable buffer for the model system. The fact that citric acid can be utilised as carbon source holds no complication because in bioreactor studies the period after the primary-carbon-source depletion is not of interest.

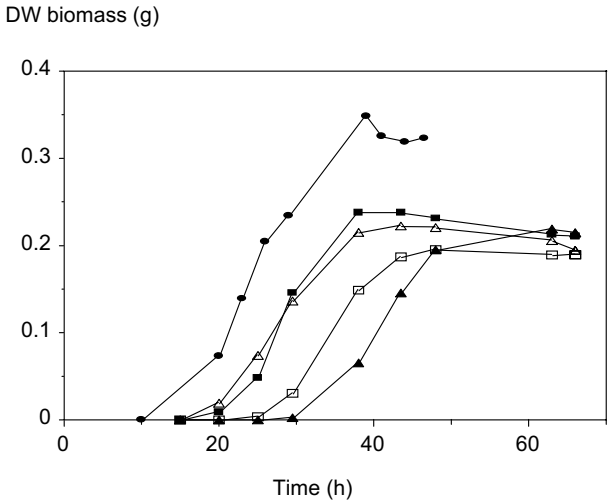

Figure 6. Biomass profiles during cultivation of *Rhizopus oligosporus* using a model system with additions of Tryptone 1 g/l (■), Tryptone 5 g/l (●) Soya peptone 1 g/l (△), Vitamins (□) and no addition (▲).

Effect of nutrient additions

The purpose of this study was to increase the growth rate of *R. oligosporus* for future studies, concerning the removal of metabolic heat in large-scale bioreactors. Vitamins, tryptone or soya peptone were added to the standard mineral medium to increase the growth rate. Since the exponential growth phase was rather short in these experiments (and in SSF in general) and because 65% of the total biomass was produced in the linear part of the growth curve (Figure 6), maximum CO_2-production rate (r_{CO2}) was considered a better indicator for metabolic heat production than μ_{max}.

Nonetheless both parameters were evaluated and the whole cumulative CO_2-production curve was taken to determine μ_{max}, r_{CO2} and the lag phase. μ_{max} and lag phase were calculated from the logistic equation (equation 1, Figure 1) and r_{CO2} was estimated from a linear part of the cumulative CO_2-production curve.

The addition of vitamins did not significantly change μ_{max} or r_{CO2} (Table 2). The addition of soya peptone (1 g/l) or tryptone (1 g/l) increased μ_{max}, but an adverse effect could be observed on r_{CO2}.

The biomass profiles of these two series showed no effect in the biomass production rate (Figure 6). However, the same amount of biomass was obtained more rapidly due to a much shorter lag phase. The short lag phase with these two series is advantageous for the model system, but still the main goal of increasing the heat production was not met.

A further increase in the tryptone concentration to 5 g/l showed a significant increase in both μ_{max} and r_{CO2} compared to all previous series (Table 2). The final amount of biomass was higher compared to the reference, which implies that tryptone was used for biomass production. This means that additional measurements on tryptone conversion are necessary in this model system to obtain correct stoichiometric coefficients during growth. The advantages of a high r_{CO2} and short lag phase were considered more beneficial than the disadvantage of additional measurements.

Table 2. Effect of different additions to a standard synthetic medium on growth of *Rhizopus oligosporus* on agar plates at 30 °C.

additions to standard medium	μ_{max} * (h^{-1})	r_{CO2} * ($\cdot 10^{-4}$ mol/h)	Lag-time * (h)	max. biomass dry weight (g)
none	0.27 (± 0.014)	3.03 (± 0.15)	21.6 (± 0.54)	0.22
Vitamins	0.30 (± 0.020)	3.12 (± 0.37)	17.5 (± 0.52)	0.20
Soya peptone (1 g/l)	0.35 (± 0.037)	2.56 (± 0.09)	10.7 (± 1.58)	0.22
Tryptone (1 g/l)	0.34 (± 0.036)	2.80 (± 0.08)	11.5 (± 0.52)	0.24
Tryptone (5 g/l)	0.42 (± 0.017)	4.31 (± 0.24)	10.8 (± 0.46)	0.35

* The values between brackets indicate the ± 95 % confidence intervals

CONCLUSIONS

Experiments showed that pH control was necessary in this model system when ammonium sulphate was used as nitrogen source. However, the growth rate did not vary much between pH values 4 and 6. Thus only some degree of pH control was necessary (depending on the initial pH) and citric acid was the most suitable buffer for this model system.

Addition of tryptone and soya peptone considerably shortened the lag phase for *Rhizopus oligosporus*. The maximum specific growth rate and maximum CO_2-production rate increased significantly with the addition of 5 g/l of tryptone to a standard mineral medium.

In summary, a model system for solid-substrate fermentation was improved through (1) better pH control by using a citric-acid buffer and (2) an overall shortening of the total fermentation time via the addition of 5 g/l tryptone.

ACKNOWLEDGEMENTS

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Chapter 3

Temperature control in a continuously mixed bioreactor

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Biotechnology and Bioengineering (2001), 72: 219-230

SUMMARY

A continuously mixed, aseptic paddle mixer was used succesfully for solid-state fermentation (SSF) with *Aspergillus oryzae* on whole wheat kernels. Continuous mixing improved temperature control and prevented inhomogeneities in the bed. Respiration rates found in this system were comparable to those in small, isothermal unmixed beds, which showed that continuous mixing did not cause serious damage to the fungus or the wheat kernels. Continuous mixing improves heat transport to the bioreactor wall, which reduces the need for evaporative cooling and thus may help to prevent the desiccation problems that hamper large-scale SSF. However, scale-up calculations for the paddle mixer indicated that wall cooling becomes insufficient at the 2 m^3 scale for a rapidly growing fungus like *A. oryzae*. Consequently, evaporative cooling will remain important in a large-scale mixed system. Experiments showed that water addition will be necessary when evaporative cooling is applied in order to maintain a sufficiently high water activity of the solid substrate. Mixing is necessary to ensure homogeneous water addition in SSF. Automated process control might be achieved using the enthalpy balance. The enthalpy balance for the case of evaporative cooling in the paddle mixer was validated. This work shows that continuous mixing provides promising possibilities for simultaneous control of temperature and moisture content in solid-state fermentation on a large scale.

INTRODUCTION

Solid-state fermentation (SSF), that is, cultivation of micro-organisms on moist solid substrates in the absence of free-flowing water, is an alternative for submerged fermentation (SmF) for the production of biotechnological products. In recent years, research on SSF has led to a wide range of applications on lab scale (Gupte and Madamwar, 1997; Gutierrez-Correa and Tengerdy, 1998; Hang and Woodams, 1998; Kotwal *et al.*, 1998; Sekar and Balaraman, 1998), and comparative studies between SmF and SSF have claimed superior product yields for the latter (Barrios-Gonzalez *et al.*, 1988; Lekha and Lonsane, 1994; Maldona and Strasser de Saad, 1998; Ohno *et al.*, 1992). In spite of these examples the commercial application of SSF processes in Western countries has been limited (Lonsane *et al.*, 1985; Mitchell and Lonsane,

1992), mainly due to problems associated with scale-up (Lonsane *et al.*, 1992; Pandey, 1991). The results presented here are part of a research project aiming to develop scale-up guidelines for SSF.

The main problem associated with scale-up is the removal of heat generated by the metabolic activity of the microorganisms (Saucedo-Castaneda *et al.*, 1990 and 1992). Heat removal by conduction in static packed beds is limited due to : (1) the poor heat transfer through the bed (Saucedo-Castaneda *et al.*, 1990); and (2) the lack of heat-exchange surface on a large scale. Evaporative cooling has proven to be more efficient than convection and conduction (Laukevics *et al.*, 1984, Sargantanis, 1993). It results, however, in large moisture losses and drying of the solid substrate (Narahara *et al.*, 1984, Trevelyan, 1974). Therefore, it is essential to combine temperature and moisture control in large-scale SSF systems (Lonsane *et al.*, 1992; Ryoo *et al.*, 1991; Saucedo-Castaneda, 1992). Evaporative cooling should be combined with spraying of water onto the solid substrate (Barstow *et al.*, 1988). The use of mixed bioreactors is necessary to ensure homogeneous water addition and evaporation. In addition, mixing of the solid substrate offers many advantages, which in many cases outweigh possible disadvantages such aseffects of shear on growth (Stuart *et al.*, 1999; Aidoo *et al.*, 1984). The main advantages of using a mixed SSF system include (1) reduction of inhomogeneity in the bed; (2) uniform distribution of additions during cultivation; and (3) potentially improved gas exchange, evaporation and heat transfer. This will result in better, more stable, reproducible bioreactor performance and process control. In several bioreactors at the pilot scale (100 to 1000 kg wet weight) (Durand and Chereau, 1988; Durand *et al.*, 1996, Fernández *et al.*, 1996) and large scale (25000 kg wet weight or more) (Xue *et al.*, 1992) mixing devices are used for control purposes.

In this article, an aseptic, continuously mixed bioreactor is presented which is suitable for scale-up with respect to heat-removal. A 35-L bioreactor was developed based on the well-known paddle mixer (Aidoo, 1984; Laukevics et al., 1984) in which *Aspergillus oryzae* was grown on whole wheat grains. A rapidly growing fungus *(A. oryzae)* was chosen to assure sufficient heat generation, so that scale-up guidelines would be based on a worst-case with regard to heat-removal problems. In addition, *A. oryzae* is commonly used in SSF for the industrial production of koji (for the production of soy sauce) and for lab-scale production of a variety of enzymes like α-

amylase (Torrado *et al.*, 1998), lipase (Ohnishi *et al.*, 1994) and proteinase (Narahara *et al.*, 1982).

Bioreactor performance of our mixed system was compared to that of nonmixed systems and a packed-bed bioreactor from the literature (Narahara *et al.*, 1984). Scale-up guidelines for temperature control in large-scale mixed bioreactors were developed. Two temperature-control strategies, wall cooling and evaporative cooling, were used separately in this bioreactor for automatic temperature control. Data from these fermentations were used in scale-up calculations to determine the scale at which the capacity of wall cooling would become insufficient for heat removal.

By comparing these two temperature-control strategies we were able to quantify when dehydration affects the growth for the case of evaporative cooling. It was demonstrated that water addition is necessary when evaporative cooling is applied. For large-scale bioreactor design this implies: (1) the necessity for simultaneous control of temperature and moisture content; and (2) the need for agitation devices in SSF systems. Automated control might be achieved using the enthalpy balance. The enthalpy balance during fermentation with evaporative cooling was verified in order to determine the reliability of all measurements in the system. We were able to prove that accurate on-line monitoring of evaporated water is possible, which is an important aspect for automatic moisture-content control.

MATERIALS AND METHODS

Micro-organism and inoculum preparation

Aspergillus oryzae CBS 570.65 was obtained from Centraal Bureau voor Schimmelcultures (Baarn, The Netherlands). A spore suspension was obtained by growing the fungus on malt-extract agar (Oxoid, CM 59) for 7 days at 30 °C and harvesting the spores with 20 mL solution containing 1 g neutralized bacteriological peptone (Oxoid, L34), 8.5 g NaCl and 10 mL Tween 80 per 1000 mL distilled water. This spore suspension was filtered through sterile glass wool to remove the remaining mycelium. Spore suspension (43.5 mL) and 16.5 g glycerol were added to 125 mL-plastic bottles (Nalgene 2105-0004, UK), which were stored at -20 °C for up to 3 months. The spore count of each plastic bottle was approximately $9.7 \cdot 10^6$ spores/mL,

measured by microscope using a Neubauer counting chamber. Inoculum was prepared in a laminar-flow cabinet by dividing the content of one 125-mL bottle spore suspension over two sterile 500-mL pressure tanks each containing 70 mL sterilized water. The inoculation level for the fermentations was $1.3 \cdot 10^5$ spores/g dry weight substrate.

Preparation of the solid substrate

A single batch of wheat grains of commercial origin (Blok, Woerden, The Netherlands), stored at 10°C, was used for all experiments. The initial moisture content of this batch was 0.12 kg water/ kg dry wheat. Five kg of this batch was soaked for 2.5 h in excess water (60°C) for a final moisture content of 0.85 kg water/kg dry wheat. After sieving, the soaked grains were divided over four 25-L bottles (Nalgene 2251-0050,UK) and autoclaved (1.5 h, 121°C).

Fermenter and agitator design

Several successful cultivations were done in the horizontal paddle mixer and, throughout the development phase, this bioreactor was slightly adapted and auxiliary equipment was optimized. The horizontal paddle mixer (Figure 1) had the following dimensions: internal diameter 30 cm, length 50 cm and internal volume 35.3 L. The bioreactor consisted of a glass cylinder (Schott, Germany) with two stainless-steel side plates. Along the central axis, six V-shaped paddles were placed at an equal distance and at a 90° angle relative to the adjacent paddle; two flat rectangular paddles were mounted at each end of the central axis. The central axis was driven by a Watson-Marlow drive (type 505DU, Cornwall, UK) fitted with a factor-10 gearwheels transmission for a sufficient torque on the axis. Air entered the bioreactor via the hollow central axis and came out at the end of each paddle to provide uniform air distribution throughout the bed. An air outlet was situated in the headspace of each side plate. A pressure control valve (Type 1525, Eriks, The Netherlands) was fitted on each side plate to maintain a constant pressure ($1.2 \cdot 10^5$ Pa) in the bioreactor, irrespective of the airflow.

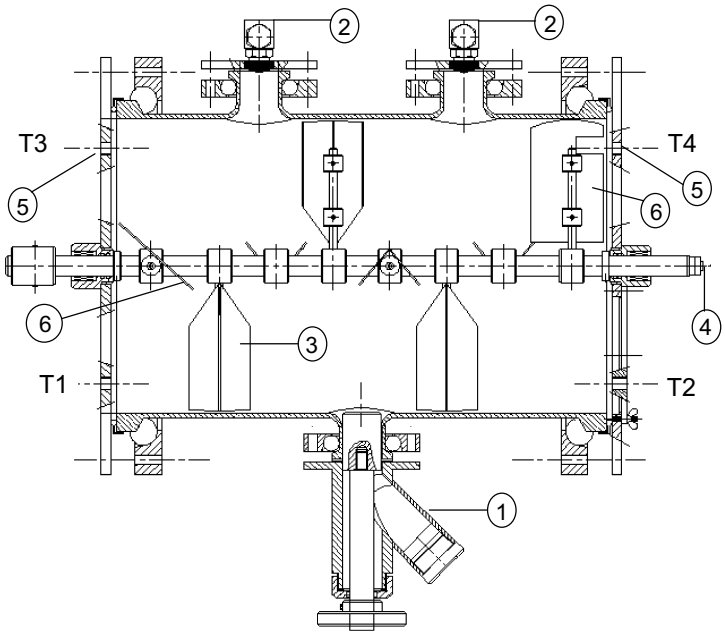

Figure 1. The 35-L horizontal paddle mixer: (1) sampling device; (2) spraying nozzle; (3) V-shaped paddle; (4) air inlet; (5) air outlet; (6) rectangular paddle; (T1 and T2) temperature sensors in the solid substrate bed; and (T3 and T4) temperature sensors in the headspace.

Two full-cone spraying nozzles of the air/water pressure type (Spraying systems, The Netherlands) were mounted onto the glass cylinder in order to spray the spore suspension evenly over the solid substrate. To control the temperature of the wall, a flat rubber tube (Tamson, The Netherlands) was twisted around the glass cylinder and connected to a water bath (Haake type F3, Germany, for the wall cooling experiments; Julabo type F25, Germany, for the evaporative cooling experiments). The temperature of the water bath was controlled based on an external setpoint. In addition, the bioreactor was placed in a temperature-controlled incubator to prevent the disturbing effects of ambient temperature variations.

Air-conditioning system

A controlled vapor delivery system (Bronckhorst Hitec, Veenendaal, The Netherlands) enabled accurate control of the relative humidity of the inlet air (Figure 2). This system was externally controlled and consisted of a mass-flow controller (0-100 L/min, type F202AC), a liquid-flow controller (0-250 g/h, type L2) and a temperature-controlled evaporator (type W303). The moisturised air was filter sterilized using a 0.2-µm hydrophobic membrane (Gellman, type CFF92HP 1700 cm^2, USA). The temperature of the incoming air was equal to the temperature of the cabinet (35°C in all experiments).

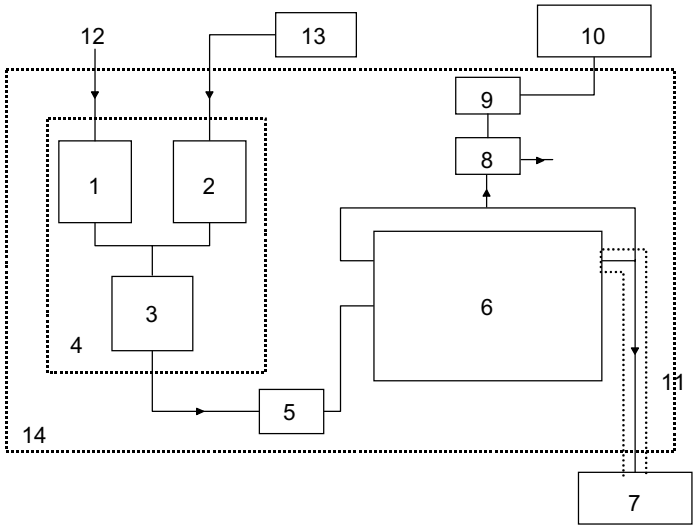

Figure 2. Schematic overview of the air-conditioning system used in the experiments: (1) mass-flow controller; (2) liquid-flow controller; (3) controller-mixed evaporator (CEM); (4) CEM-controlled vapor-delivery system; (5) sterilizing membrane filter; (6) horizontal paddle mixer; (7) cooled-mirror dewpoint-measuring system; (8) bioreactor pressure-control valve; (9) condenser; (10) O_2/CO_2 analyzer; (11) heated coil; (12) air pressure (6 bar); (13) pressure tank for water; and (14) temperature-controlled cabinet.

Fermentation process

The autoclaved and moisturized wheat grains were transferred to the previously autoclaved (1.5 h, 121 °C) and cooled horizontal paddle mixer. The filled reactor was again sterilized for 1.5 h at 121°C in an autoclave. The 500-mL pressure tanks were aseptically connected to the spraying nozzles on top of the bioreactor and the inoculum was sprayed over the continuously mixed (2.5 rpm) wheat grains. During the first 24 hours after inoculation, the wheat grains were discontinuously mixed (55 min with no agitation; 5 min with agitation at 1 rpm) to allow undisturbed germination and to minimize grain damage. During this period the temperature of the wall was set to 34.5 °C and the air flow to 20 L/min (273 K, $1.031 \cdot 10^5$ Pa). After this period continuous mixing (0.5 rpm) was applied. When the temperature of the bed reached 35°C, temperature control was started automatically as explained in what follows.

On-line measurements

Six temperature sensors (Pt-100 Ω Tempcontrol, Voorburg, The Netherlands) were used for on-line temperature readings. Two sensors were placed in the solid substrate and two in the headspace at the positions marked T1 to T4 in Figure 1. Two additional sensors were located outside the fermentor in contact with the glass wall on each side of the bioreactor.

The effluent gas was analyzed for O_2 and CO_2 in a paramagnetic O_2 analyzer (Servomex, Xentra 4100, Servomex Zoetermeer, The Netherlands) and an infrared CO_2 analyzer (Servomex Series 1400). Prior to these analysis, the effluent gas was cooled to 2°C in a condenser. The O_2 analyzer was fitted with a second channel to measure the O_2 concentration in the surrounding air in order to accurately determine O_2 consumption. Both oxygen sensors corrected the readings for pressure fluctuations. CO_2 production was calculated by subtracting a previously measured average of the CO_2 concentration in air from the effluent reading.

The dewpoint of the effluent gas was measured in a cooled-mirror dewpoint-measuring system (DEWMET SD, Michell Instruments Ltd., UK). The effluent gas line to the dewpoint meter was heated with a water-heated coil (50°C) to prevent condensation prior to the measurement. A fit relation, based on literature data (Lide, 1993), was used to convert dewpoint temperature (T in °C) readings to water-vapor pressures:

$$p_{w,sat} = \left(\frac{24.503 + 0.68363 \cdot T + 3.5349 \cdot 10^{-3} \cdot T^2}{1 - 9.935 \cdot 10^{-3} \cdot T + 4.1781 \cdot 10^{-5} \cdot T^2} \right)^2 \quad \text{[Pa]} \qquad (1)$$

To enable automatic process control, all sensors and equipment were linked to a computer-controlled data acquisition system consisting of hardware (RTI 820 boards with 5B modules, Analog Devices Inc., USA), software (VIEWDAC revision 2.10, Keithley Instruments Inc., USA) and a personal computer (80486 PC). The temperature-control strategy (see later) was implemented in a sequence in VIEWDAC and operated fully automatically.

Temperature control strategies

Two temperature control strategies were employed, consisting of wall cooling and evaporative cooling.

In the wall cooling strategy, the temperature of the cooling water circulating in the flat rubber tubes wrapped around the fermentor was lowered to maintain the desired bed temperature. This resulted in a lower wall temperature, which was recorded by a temperature sensor. The air flow rate was manually set at a level that kept the CO_2 concentration between the limits of the analyzer (0 to 24 h, 20 L/min; 24 to 51 h, 40 L/min; 51 h to end, 60 L/min). The humidity of the inlet air was kept constant at 0.0259 mole per mole of dry air, which corresponds to a relative humidity of 45.5% at 35°C and $1.013 \cdot 10^5$ Pa; complete saturation of the incoming air was not possible due to overpressure and subsequent condensation problems before the sterilizing filter. Due to the undersaturation of the incoming gas, evaporation could not be neglected in the enthalpy balance.

In the evaporative cooling strategy, the air flow rate to the fermenter was adjusted to maintain the desired bed temperature. The temperature of the wall was kept at 34.5°C throughout the fermentation to minimize heat transfer through the wall. A constant flow rate of water vapor (13 g/h) was mixed with the inlet air using the liquid-flow controller described above (air-conditioning system). As a consequence of the increasing air flow rate, the inlet relative humidity decreased.

Control algorithm

In both temperature-control strategies, on-line measurements were used to first estimate the control variable from the enthalpy balance over the bioreactor. Then, the estimated control variable was adjusted using a PI controller based on the bed temperature, to correct for setpoint deviations (Figure 3).

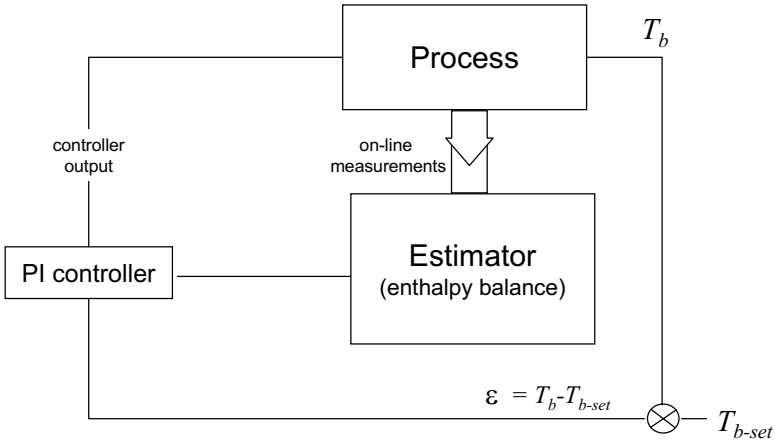

Figure 3. Schematic overview of the temperature-control strategy used during the fermentations to calculate the control variables T_{wall} (wall cooling) and F_{air} (evaporative cooling).

The enthalpy balance used to estimate the control variable is made up of several terms:

$$\frac{dT_b}{dt}\sum_i M_i \cdot C_{p,i} = r_{met} + J_{wall} + J_{evap} \qquad \text{[W]} \qquad (2)$$

in which

J_{evap}	=	heat transfer from evaporation and heating of the air	[W]
r_{met}	=	heat production by micro-organisms	[W]
J_{wall}	=	heat transfer through the wall	[W]
T_b	=	average temperature of the bed (Pt100 1&2, Fig. 1)	[°C]
M_i	=	weight component i (water, starch, protein etc.)	[kg]
$C_{p,i}$	=	heat capacity component i (water, starch, protein etc.)	[J·(kg·°C)$^{-1}$]

The mass accumulation term ($\Sigma dM/dt \cdot T_b \cdot C_p$) in this balance is neglected. For the case of evaporative cooling this term was 2.7 W maximum ($\Sigma dM/dt$ = 0.028 g/s; T_b = 35 °C; C_p = 2.77 J/g °C), which is a minor term compared with the maximum heat production rate of 100 W. In the equation, all losses are regarded as negative production terms. The physical constants used in the equations were taken from literature (Lide, 1993). The different contributions are now examined briefly.
The amount of heat lost by evaporation and heating of the air was calculated by :

$$J_{evap} = -\left(h_o - h_i\right) \cdot F_{air} \cdot \rho_{air} \quad \text{[W]} \qquad (3)$$

with

F_{air}	=	volumetric dry air flow rate at 273 K and $1.013 \cdot 10^5$ Pa	[m^3 dry air·s^{-1}]
ρ_{air}	=	1.293 density dry air at 273 K and $1.013 \cdot 10^5$ Pa	[kg·m^{-3} dry air]
h_o	=	enthalpy of the outcoming air	[J·kg^{-1} dry air]
h_i	=	enthalpy of the incoming air	[J·kg^{-1} dry air]

The enthalpy of the air (h) is:

$$h = C_{p,air} \cdot T_{air} + y_w \cdot \left(H_V + C_{p,V} \cdot T_{air}\right) \quad \text{[J·kg}^{-1}\text{ dry air]} \qquad (4)$$

with

$C_{p,air}$	=	1007	heat capacity dry air	[J·(kg·°C)$^{-1}$]
H_V	=	$2.501 \cdot 10^6$	heat of vaporization of water at 273 K	[J·kg^{-1}]
$C_{p,V}$	=	1870	heat capacity water vapor	[J·(kg·°C)$^{-1}$]
T_{air}	=	temperature of the air		[°C]
y_w	=	moisture content of the air calculated from dewpoint		[kg·kg^{-1} dry air]

The amount of heat that is removed through the wall is calculated from :

$$J_{wall} = -\alpha_{ov} \cdot A \cdot \left(T_b - T_{wall}\right) \quad [W] \quad (5)$$

with

α_{ov}	=	heat transfer coefficient of the wall	[W·(m^2·°C)$^{-1}$]
A	=	heat transfer area of the wall	[m^2]
T_{wall}	=	average temperature inlet and outlet cooling coil	[°C]

Roels (1983) and other authors have shown that for aerobic fermentation the heat production can be related to oxygen consumption:

$$r_{met} = \left(C_{O2,i} - C_{O2,o}\right) \cdot F_{air} \cdot 460 \cdot 10^3 \quad [W] \quad (6)$$

with

$C_{O2,i}$	=	inlet O_2 concentration at 273 K and 1.013·10^5 Pa	[mol·m^{-3} dry air]

The estimator for wall cooling calculates the temperature of the wall ($T_{wall,est}$) necessary to assure temperature control (*i.e.* $dT/dt = 0$). Substitution of equation (5) in equation (2) gives the estimated control variable $T_{wall,est}$:

$$T_{wall,est} = T_b - \frac{r_{met} + J_{evap}}{\alpha_{ov} \cdot A} \quad [°C] \quad (7)$$

where J_{evap} is calculated by equation (3), by using the dew-point measurement in the effluent air and the setpoints of the liquid- and gas-flow controllers.
The estimator for evaporative cooling calculates the air flow rate ($F_{air,est}$) necessary for temperature control (i.e. $dT/dt = 0$). Substitution of equation (3) in equation (2) gives the estimated control variable $F_{air,est}$:

$$F_{air,est} = \frac{r_{met} + J_{wall}}{\left(h_o - h_i\right) \cdot \rho_{air}} \quad [m^3 \cdot s^{-1}] \quad (8)$$

where J_{wall} is calculated with equation (5), by using the measured bed and wall temperatures, and the product of heat transfer coefficient and wall area estimated as explained in what follows.

Wall cooling is minimized in this situation by setting a small temperature difference (0.5°C) between the bioreactor and the wall, but was not neglected in the enthalpy balance.

A PI controller was used for feedback control and to correct for errors in the estimated control variable (O_{est}) as a result of measuring errors, wrong assumptions in the enthalpy balance or incorrect input parameters. The estimated control variable ($T_{wall,est}$) for wall cooling; $F_{air,est}$ for evaporative cooling) was corrected with a proportional-integral algorithm to give the controller output ($O_{control}$):

$$O_{control} = O_{est} + K_c \cdot \varepsilon(t) + \frac{K_c}{\tau_I} \cdot \int_0^t \varepsilon(t) \cdot dt \tag{9}$$

in which

K_c = proportional gain of the controller [-]

τ_I = integral time constant [h]

The input of the controller is the deviation (or error ε) between the actual substrate temperature and the desired setpoint:

$\varepsilon(t) = T_b(t) - T_{b\text{-}set}$

$T_{b\text{-}set}$ = set-point temperature for the bed of solids [°C]

Parameters

The values for K_c and τ_I used in the PI controller were -5 [-] and 1.4 h, respectively, for wall cooling, and 7.5 L·(min·°C)$^{-1}$ and 7.5 h, respectively, for evaporative cooling. The value for $\alpha_{ov} \cdot A$ was estimated using Equation 2 from data of the first 24 hours of the fermentation. The values for $\alpha_{ov} \cdot A$ used in the estimator for wall cooling and evaporative cooling were 6 and 8.5 [W·°C^{-1}], respectively. The difference between both values must be attributed to the rough estimation procedure used and perhaps also to differences in water flow rate through the cooling tubes.

Off-line analysis

Sample Preparation

Twice a day during the fermentation samples (± 30 g) were taken from the bioreactor. Moisture content and water activity were determined immediately after sampling. Approximately 5 g of wheat grains were transferred into a sample bottle and stored at -20°C for analysis of glucosamine.

Moisture Content & Water Activity

The moisture content of the sample was determined in duplicate by drying 6 g of wet grains at 80°C for 2 days in a preweighed dish. The water activity of the sample was determined in a Novasina Thermoconstanter (Type TH200, Switzerland) at 35°C.

Glucosamine

The glucosamine content in the samples was used as an indirect estimate of biomass. Because the starch in the sample interfered with the peak separation during high-performance liquid chromatography (HPLC) analysis, the starch was first removed from the sample with an enzymatic method.

After the sample bottle was thawed, the wheat grains were homogenized with water (grains : water = 1 g : 4 g) in an ultraturrax (Silverson L4R, UK). Three gram of this homogenate was added to 6.5 mL 13 mM phosphate buffer (pH = 4.5) and 20 µL amyloglucosidase (Sigma A-3042, USA) enzyme solution and incubated at 55°C for 1 hour. This suspension was centrifuged (20 min, 5500 rpm, Beckman centrifuge GS-15R), after which the supernatant was discarded. The pellet was washed with 5 mL distilled water, again centrifuged and then dried (50°C, 3 days).

The dried pellet was prehydrolyzed with 0.5 mL 10 M H_2SO_4 for 24 hours and occasionally stirred at room temperature. After prehydrolysis, 4.5 mL distilled water was added and further hydrolysis was continued at 100°C for 3 h in a thermal heating block (Grant QBT4, UK). After hydrolysis, the pH was adjusted to 5 with NaOH and the sample was filtered through a 0.45-µm membrane. The glucosamine content was measured by ion-exchange chromatography (Carbopac™ PA1 column with guard column, Dionex, Sunnyvale, USA) with pulse amperometric detection, at 25°C, using D-(+)-glucosamine hydrochloride (Sigma G-4875, USA) as reference solution and 20 mM NaOH (1 mL/min) was used as eluent.

Measurements of glucosamine content (grams per dry matter sample) are recalculated in order to express the concentration per gram of initial dry matter wheat. For this calculation it was assumed that CO_2 linearly correlates with loss of dry matter. The cumulative CO_2 production at the moment of sampling was used together with total dry-weight loss and total CO_2 production to calculate the total dry matter at the moment of sampling (term above the division sign in Equation 10). Glucosamine measurements are expressed per gram initial dry matter wheat as calculated by:

$$Gm = Gm` \cdot \frac{\left(IDM - CO_2^{\,t_{sample}} \cdot \frac{\left(IDM - DM_{t_{end}} \right)}{CO_2^{\,t_{end}}} \right)}{IDM} \qquad [mg \cdot g^{-1}\ IDW] \quad (10)$$

Gm	=	glucosamine content per gram initial dry matter	[mg·g^{-1} IDW]
$Gm`$	=	glucosamine content sample per gram dry matter	[mg·g^{-1} DW]
IDM	=	initial dry matter in bioreactor	[g]
DM_{tend}	=	measured final dry weight in bioreactor	[g]
$CO_2^{tsample}$	=	cumulative CO_2 production at sampling time	[g]
CO_2^{tend}	=	final cumulative CO_2 production	[g]

Mixing performance paddle mixer

To determine radial and axial mixing in the horizontal paddle mixer, three colored wheat grain fractions were used: red, green, and natural. For radial mixing the fractions were placed in three horizontal layers; for axial mixing the fractions were placed in three equal vertical sections. The hold up and moisture content of grains in the mixing experiments were equal to those in the fermentations. Samples were taken using a long plastic cylinder in opening 2 (Figure 1). The colored grain fractions were counted and calculated as a percentage of the total number per sample. Mixing experiments were done at 0.5 and 1 rpm.

Miniature packed bed and tray

Packed bed: aeration through the bed

The miniature packed bed consisted of a glass cylinder (∅ = 5 cm, h = 15 cm). A stainless-steel wire mesh at 7 cm from the bottom supported one layer of grains (10 g wet weight, moisture content 0.44 kg/kg wet weight). The packed bed was placed in a temperature-controlled cabinet at 35°C. The air flow (24 L dry air/h at 273 K and $1.013 \cdot 10^5$ Pa) was humidified (94.6 % relative humidity) using a 1.5-L bubble column with 0.5-cm Rashig rings. The air was filter sterilized using a 0.2-µm hydrophobic membrane (acros 50, Gellman, USA) before it was passed through the bed. The effluent air was dehumidified using a condenser at 2°C before it was analyzed for CO_2 and O_2 (see "Measurements On-Line" subsection).

Tray: aeration over the bed

A Petri dish (without the lid) containing 30.2 g wet weight grains (moisture content 0.48 kg/kg wet weight) was placed in a closed jar. The air inlet was situated beside the Petri dish. The same cabinet and air-conditioning system were used as described earlier. The air flow was 27 L dry air/h at 273 K and $1.013 \cdot 10^5$ Pa, and the temperature of the cabinet was controlled at 37°C.

Desorption isotherm

For the water desorption isotherm of wheat grains, autoclaved wheat grains were used as described earlier. Portions of wheat grains were transferred to predried and preweighed dishes (Novasina, Switzerland). The dishes were placed in a large vacuum desiccator with saturated LiCl solution (a_w=0.11). At regular time intervals, dishes were removed and sealed. After a few hours at room temperature, the water activity and the moisture content were determined. Water activity was determined in a thermoconstanter (Model TH200, Novasina, Switzerland) at 35°C.

RESULTS AND DISCUSSION

Bioreactor performance

Several mixing experiments were done to determine the radial and axial mixing performance of this bioreactor. Radial mixing is easily accomplished in a paddle mixer. For axial mixing, however, V-shaped paddles had to be used that could push the solid substrate sideways. Axial mixing is important for homogeneous water distribution in the bed, because industrial spraying nozzles usually cannot spray evenly over the total surface of the bed. Adequate radial mixing is achieved after six revolutions (Figure 4), implying a mixing time of 12 min at 0.5 rpm, which was used during the fermentations. Axial-mixing experiments showed that, after 120 revolutions, the bed was well mixed (Figure 4). The number of revolutions needed to achieve radial or axial mixing was the same for both mixing frequencies tested (0.5 and 1 rpm). Although the axial-mixing time was rather long (4 h), it was brief compared with the time needed (30 h) to evaporate all the water in the bed, given a maximum heat generation of 100 W. Furthermore, the spraying frequency was about once every hour and full-cone spraying nozzles were used, and therefore good water distribution could be assured.

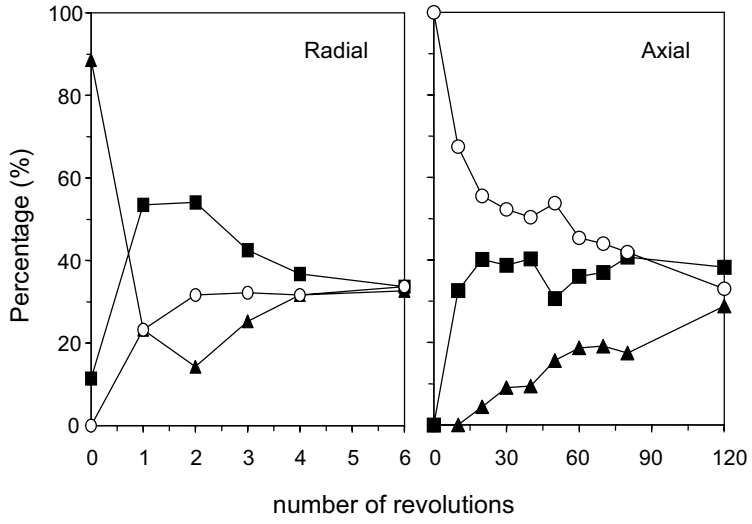

Figure 4. Axial and radial mixing at 1 rpm in the 35-L horizontal paddle mixer. Percentages of colord wheat grains in the sample taken from the position underneath the right nozzle (red ■; green ▲; natural color ○).

The next aim was to measure mixed-bioreactor performance and to compare it with measurements in a packed bed containing one layer of grains and literature data for a 1 kg packed-bed bioreactor. Bioreactor performance was determined from the maximum oxygen consumption rate per kilogram initial dry matter (*IDM*). When measuring the maximum oxygen consumption rate it is important to control the temperature and moisture content in order to allow comparison between different experiments. Wall cooling was applied to control the temperature and to minimize dehydration effects in the paddle mixer. The cultivations were done twice using similar conditions and reproducible results were obtained.

The temperature of the solid substrate could be controlled adequately using wall cooling in the 35-L paddle mixer (Figure 5). The oxygen consumption rate hardly showed an exponential-growth period, but rather a linear increase in consumption rate to 0.17 mol/(h·kg IDM) (Figure 5).

The maximum oxygen consumption rate measured in the miniature packed bed of grains with forced aeration through the bed was 0.20 mol/(h·kg IDM). Although the oxygen-consumption rate in this one-layer packed bed was slightly greater, probably due to ideal circumstances for oxygen transfer, bioreactor performance in our mixed system was satisfactory. Its results are comparable to those of a previously described packed-bed bioreactor with temperature and moisture-content control (Narahara *et al.*, 1984). A maximum oxygen-consumption rate equal to 0.138 mol/(h·kg IDM) was measured in a one-layer tray system when the air was blown over the layer instead of through the layer. This value is lower than the 0.2 mol/(h·kg IDM) measured in the 35-L bioreactor, despite the slightly more favorable temperature in the tray. This is probably attributable to less ideal oxygen transfer caused by the different aeration method. Table 1 summarizes the performance of the SSF systems discussed here.

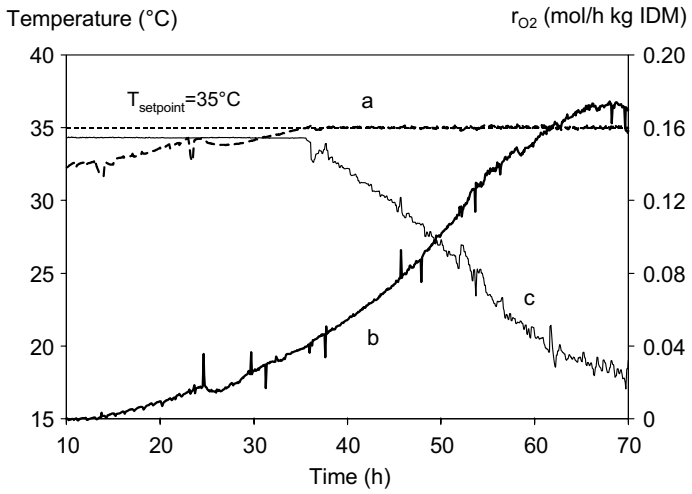

Figure 5. The course of the reactor temperature (dashed line, a), control variable T_{wall} (thin line, c) and oxygen consumption rate (thick line, b) during cultivation of *Aspergillus oryzae* on wheat grains in a 35-L paddle mixer in which the temperature was controlled with wall cooling.

Table 1. Bioreactor performance – expressed as oxygen consumption rate per kilogram initial dry matter (IDM) – of static and mixed solid-state cultivations of *Aspergillus oryzae* with temperature control and little moisture-content variation.

System and wet weight (ww)	Substrate	T [°C]	Water content (kg /kg ww)	Aeration	r_{O2} [mol·h^{-1}·kg IDM^{-1}]
Paddle mixer 8 kg[a]	Wheat grains	35.0	0.46	Forced through	0.172
Packed bed 10 g	Wheat grains	35.4	0.44	Forced through	0.200
Tray system 30 g	Wheat grains	37.0	0.48	Surface	0.138
Packed bed 1 kg	Steamed rice	38.0	0.40	Forced through	0.134[b]

[a] The paddle mixer runs in wall-cooling operation mode.

[b] value represents an r_{CO2} [mol·h^{-1}·kg DM^{-1}] measurement (Narahara, 1984); the respiration coefficient was determined as 1 for this substrate and fungus (Narahara, 1982).

The good bioreactor performance of our mixed system indicates good mass and heat transfer and minimal shear effects on the fungus. Microscopic observations of various cross-sections of wheat grains revealed that the fungus grew inside the wheat grain. Microscopic observations of whole grains showed hardly any fungal biomass on the outside. Apparently, continuous mixing forced the fungus to grow inside the wheat grain and it seemed that the fungus was protected by the seed coat. Therefore, shear effects are thought to be minimal in this case. Furthermore, aggregate formation was prevented through continuous mixing. It can be stated that wheat grains are an ideal solid substrate to be used in continuously mixed bioreactors.

Scale-Up of Wall Cooling

During scale-up of geometrically similar drum-type bioreactors, the ratio between wall area and reactor volume will decrease. It can therefore be expected that, given a maximum heat production rate, wall cooling will become insufficient at a certain bioreactor volume. To develop scale-up rules for wall cooling it is necessary to calculate for which bioreactor volume the capacity of wall cooling becomes limiting. For the case of *Aspergillus oryzae* in a mixed bioreactor the ratio between maximum wall-cooling capacity and maximum heat production was calculated as a function of bioreactor volume.

Several assumptions were made in this calculation :

- There is a constant bioreactor temperature.
- There is negligible evaporation of water.
- There is a perfectly mixed solid substrate.
- Heat transfer occurs through the wheat bed only, not through the air in the headspace.
- There is a bed hold-up of 0.5 [m^3 bed·m^{-3} reactor].

The maximum metabolic heat production and the maximum heat transfer capacity for wall cooling as a function of bioreactor volume are then given by (see also Equation 6):

$$r_{met}\left(V_r\right) = -r_{O2}''' \cdot 0.5 \cdot V_r \cdot 460 \cdot 10^3 \qquad \text{[W]} \qquad (11)$$

and

$$J_{wall}(V_r) = -\pi \cdot \left(\frac{V_r}{2 \cdot \pi \cdot C} \right)^{2/3} \cdot (2C+1) \cdot \alpha_{ov} \cdot (T_b - T_{wall}) \qquad [\text{W}] \qquad (12)$$

in which

r'''_{O2}	=	maximum oxygen production rate	[mol·s^{-1}·m^{-3} bed]
V_r	=	bioreactor volume	[m^3]
C	=	ratio of length over diameter of the drum	[-]
α_{ov}	=	overall heat transfer coefficient	[W·m^{-2}·°C^{-1}]

The maximum heat production rate was taken from the previously discussed experiments in the 35-L bioreactor. The overall heat transfer coefficient (α_{ov} = 100 W·m^{-2}·°C^{-1}) was measured in an industrial solid mixer filled with moisturized oats and equipped with a stainless-steel water jacket (data not shown). The temperature difference between the bioreactor contents and the wall was assumed to be 20 °C.

Figure 6 shows that wall cooling becomes insufficient at 2-m^3 reactor volume for a length/diameter (C = L/D) ratio of 1.7, the value for the 35-L bioreactor. Increasing the L/D ratio improves the capacity of wall cooling but it becomes insufficient above 8 m^3, even for L/D = 15, which, from a practical point of view, is a rather unrealistic ratio.

In a bioreactor of 60 m^3 (≈ 22000 kg wet wheat), 30-50% of the maximum heat production can be removed with wall cooling, irrespective of the L/D ratio, due to the asymptotic behavior of the curves in Figure 6. Thus, although evaporative cooling is necessary to achieve temperature control on a large scale, wall cooling always removes a substantial part of the maximum heat production. Clearly, in the beginning and toward the end of the fermentation wall cooling alone has sufficient cooling capacity to control the temperature.

Effect of evaporative cooling

As discussed previously, evaporative cooling is inevitable on a large scale. Therefore, we quantified the effect of evaporative cooling on the growth of *A. oryzae* on wheat. A temperature-controlled fermentation using mainly evaporative cooling was compared with the previous fermentation with wall cooling. Both experiments were repeated and duplicates gave comparable results (data not shown).

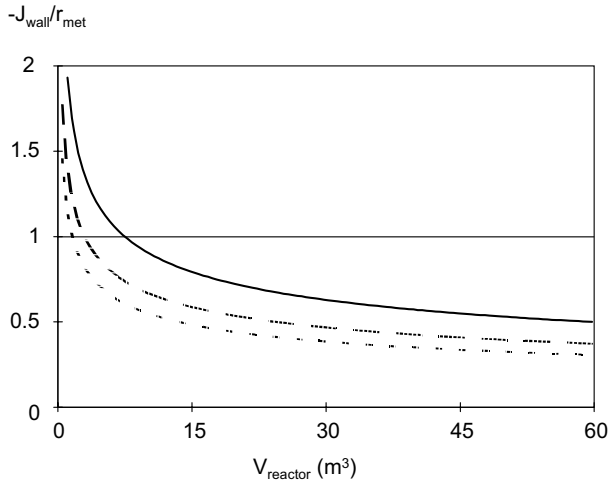

Figure 6. Ratio between maximum heat removal rate through the wall (J_{wall}) and maximum heat production rate (r_{met}) as a function of reactor volume, for three L/D-ratios: 1.7 (dotted line); 5 (dashed line) and 15 (solid line). Parameters used in the calculation are: $\dddot{r}_{O2}$ = 0.0191 mol·s^{-1}·m^{-3}; α_{ov} = 100 W·m^{-2}·C^{-1}; T_b-T_{wall} = 20 ºC.

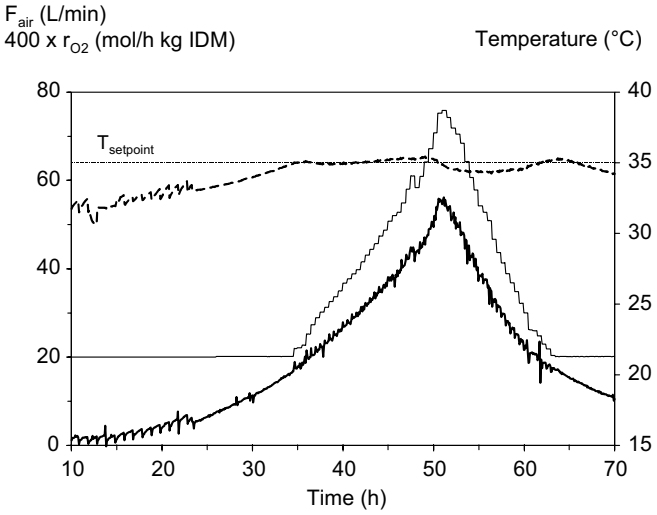

Figure 7. The course of the reactor temperature (dashed line), control variable F_{air} (thin line) and oxygen consumption rate (thick line) during fermentation of *Aspergillus oryzae* on wheat grains in a 35-L paddle mixer in which the temperature was controlled with evaporative cooling.

Figure 7 shows that adequate temperature control was possible in the fermentation with evaporative cooling, with a maximum deviation from the setpoint of 0.6°C. This deviation is acceptable for *A. oryzae* because the maximum specific growth rate is rather constant between 34 and 36 °C (Carlsen *et al.*, 1996). Furthermore, the parameters in the PI controller were not optimized for this purpose, so that better temperature control can be achieved in the future.

A comparison of the oxygen consumption rates for the two fermentations shows that, after a similar development in oxygen consumption rate up to 50 h, a sharp decline occurs when evaporative cooling was applied (Figure 8a).

Similar behavior could be observed with glucosamine measurements, which were used as an indirect estimate for fungal biomass (Figure 8b). These results can be explained by dehydration of the solid substrate caused by evaporative cooling, which eventually leads to a decrease in water activity that affects the growth rate and thus the oxygen consumption rate.

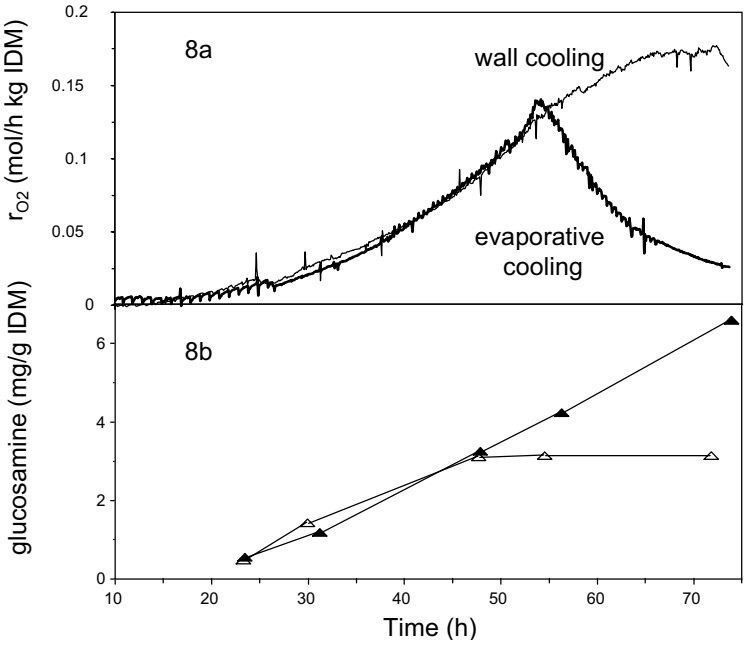

Figure 8. (a)The course of the oxygen consumption rate for a cultivation of *Aspergillus oryzae* with evaporative dooling (thick line) and with wall cooling (thin line). (b) Glucosamine measurements for the fermentation with evaporative cooling (△) and wall cooling (▲).

This is clearly supported by the moisture-content profile of the two fermentations and the measured water activities (Figure 9); that is, after 50-h fermentation, the moisture content dropped below 0.5 kg w/kg DM in the case of evaporative cooling. At that time, the water activity was estimated at 0.95 (linear interpolation between measurements) and it decreased rapidly thereafter. From radial-growth-rate data as a function of water activity for *A. oryzae* (Gibson *et al.*, 1994), the growth rate was estimated to be 70% of its maximum value at a_w=0.95. In other words, the observed decline in oxygen-consumption rate can be explained by a decrease in water activity. For the case of wall cooling, the water content and water activity remained constant ($a_w \approx$ 0.964 to 0.968) between 40- and 80-h fermentation times (Figure 9).

The observation that the oxygen consumption rate is only significantly affected after dehydration below 0.5 kg/kg dry matter (DM) also follows from the water-desorption isotherm of wheat grains. The water-desorption isotherm shows that below 0.5 kg/kg DM, water activity rapidly declines, and that, above 0.5 kg/kg DM, water activity remains almost constant at 0.995 (Figure 10). However, it should be noted that the moisture content of the solid substrate itself during fermentation is probably lower than the measured overall moisture content, because biomass, as part of the wheat grain, probably has a higher moisture content than the overall average (Larroche *et al.*, 1992; Oriol *et al.*, 1988).

In summary, evaporative cooling dehydrates the solid substrate, which eventually leads to a decrease in water activity that affects the growth and thus the oxygen consumption rate. Therefore, water addition becomes necessary when evaporative cooling is applied.

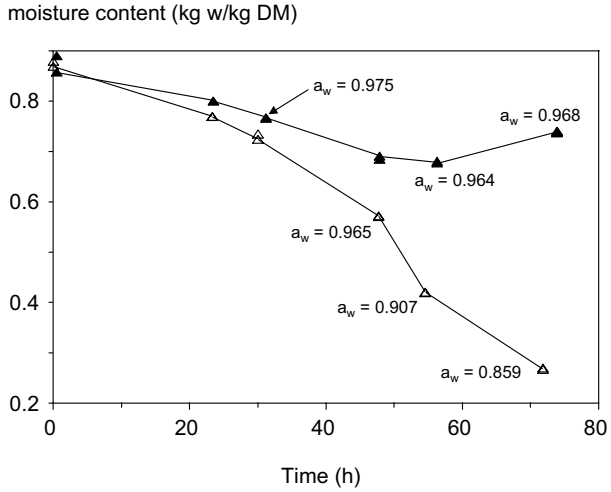

Figure 9. The course of the moisure content of wheat grains during cultivation of *Aspergillus oryzae* with evaporative cooling (△) and with wall cooling (▲).

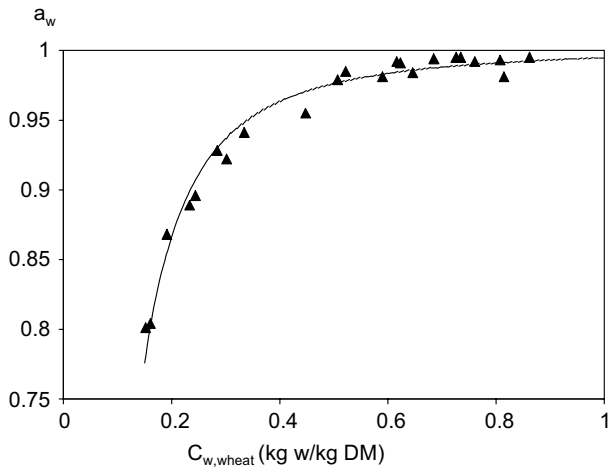

Figure 10. Water-desorption isotherm of moisturized and autoclaved wheat grains at 35ºC.

Validation of the enthalpy balance

Moisture-content control in SSF is difficult as there is no direct on-line measurement for the moisture content of the solid substrate. Therefore, a control strategy should be based on estimating the water balance and dry-weight variations in the system. For these estimations, reliable on-line measurements are a necessity. For example, air flow rate and dewpoint temperature of the effluent air determine the amount of water evaporated, which is the most important term in the water balance but also in the enthalpy balance. The aim was to validate the enthalpy balance in order to check the reliability of all measurements in the system that can be used later for moisture-content control. Furthermore, validation of the enthalpy balance will demonstrate that temperature control can be based on the enthalpy balance, as proposed in this study. The enthalpy balance was validated for the case of evaporative cooling.

On-line measurements and constants from literature were used to calculate the different terms in the enthalpy balance. The only unknown parameter that had to be determined experimentally was $\alpha_{ov} \cdot A$. This parameter (4.72 $W \cdot °C^{-1}$) was determined by fitting all results of the experiment in the 35-L bioreactor in which wall cooling was applied.

Figure 11 shows the absolute values of the individual terms in the enthalpy balance (equation 3). The summation of the individual terms should equal zero from 36 to 46 hours of fermentation in which temperature was adequately controlled, but a small average deviation of 2.4 ± 0.23 W (95% confidence) was found. Despite small deviations, the enthalpy balance is reasonably accurate and the applied on-line measurements can thus be used for moisture-content control. The obtained accuracy is certainly high enough if we take into account the water-desorption isotherm (Figure 10), which shows that a sufficiently high and virtually constant water activity can be maintained over a wide moisture content range above 0.5 kg w/kg dry substrate.

CONCLUSION

Aspergillus oryzae was successfully cultivated under continuous agitation in a new mixed bioreactor for SSF in which temperature could be adequately controlled by evaporative and wall cooling. The bioreactor performance approached the performance

of an ideal one-layer static system and was comparable to a well-controlled, small, packed-bed bioreactor from the literature.

Scale-up calculations for this mixed bioreactor demonstrated the importance of evaporative cooling in addition to wall cooling for cultivating *A. oryzae* on a large scale. Furthermore, it was demonstrated that water addition becomes necessary when evaporative cooling is applied. To assure homogeneous water addition and evaporation, the use of mixed bioreactors is a necessity. This research illustrates that, given a suitable solid substrate, mixing offers advantages, including improved bioreactor performance and facilitated scale-up of solid-state fermentation.

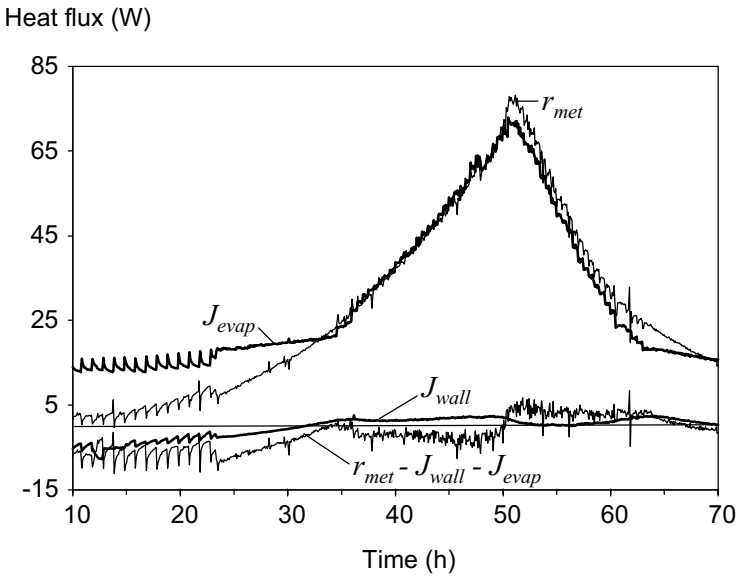

Figure 11. The course of the individual terms in de enthalpy balance during fermentation of *Aspergillus oryzae* on wheat grains in which the temperature was controlled by evaporative cooling.

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Chapter 4

Model for on-line moisture-content control

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Biotechnology and Bioengineering (2001), 72: 231-243

SUMMARY

In this study we describe a model that estimates the extracellular (nonfungal) and overall water contents of wheat grains during solid-state fermentation (SSF) with *Aspergillus oryzae*, using on-line measurements of oxygen, carbon dioxide, and water vapor in the gas phase. The model uses elemental balances to predict substrate dry matter losses from carbon dioxide measurements, and metabolic water production, water used in starch hydrolysis, and water incorporated in new biomass from oxygen measurements. Water losses caused by evaporation were calculated from water vapor measurements. Model parameters were determined using an experimental membrane-based model system, which mimicked the growth of *A. oryzae* on the wheat grains and permitted direct measurement of the fungal biomass dry weight and wet weight. The measured water content of the biomass depended heavily on the moisture content of the solid substrate and was significantly lower than the estimated values reported in the literature. The model accurately predicted the measured overall water content of fermenting solid substrate during fermentations performed in a 1.5-L scraped drum reactor and in a 35-L horizontal paddle mixer, and is therefore considered validated. The model can be used to calculate the water addition required to control the extracellular water content in a mixed solid-state bioreactor for cultivation of *A. oryzae* on wheat.

INTRODUCTION

The growth of microorganisms on solid substrates under conditions of limited water availability is called solid-state fermentation (SSF). It can be used for the production of a wide range of biotechnological products such as spores (Whipps and Gerlagh, 1992), enzymes (Pandey, 1992) and fine chemicals (Shankaranand and Lonsane, 1994). The results presented here are part of a research project aiming to develop large-scale SSF bioreactors with simultaneous control of temperature and moisture content.

One of the main scale-up problems is the removal of heat generated by the metabolic activity of the microorganisms (Saucedo-Castenada *et al.*, 1992). In large-scale mixed bioreactors, evaporative cooling has to be applied due to limited heat removal through the bioreactor wall (Sargantanis, 1993; Nagel *et al.*, 2001). Evaporative cooling results

in large moisture losses and should therefore be combined with water addition to assure moisture-content control (Lonsane *et al.*, 1992; Ryoo *et al.*, 1991). For homogeneous water addition during cultivation, a continuously mixed bioreactor was developed (Nagel *et al.*, 2001). In the present article, a model is presented for automatic moisture-content control during SSF with *Aspergillus oryzae* grown on whole wheat grains. *A. oryzae* is a commonly used fungus for SSF (Pandey, 1992) and wheat grains are an ideal substrate for use in mixed bioreactors (Nagel *et al.*, 2001).

Process design and control for SSF must take into account the availability of water, as was clearly demonstrated in several previous studies (Oriol *et al.*, 1988; Larroche and Gros, 1992; Larroche *et al.*, 1992, Nagel *et al.*, 2001). Besides the evaporation needed for cooling, an important reason is the incorporation of water into new microbial cells. Oriol and coworkers (1988) showed that fungal growth can be hampered by limited water availability, even when the total water content of the fermenting mass increases in time. Their calculations showed that gradually increased water content is present inside the fungal cells and the residual water outside the cells, which determines the water availability or water activity of the substrate matrix, becomes limiting. Literature indicates that fungal mycelium grown in SSF contains approximately 3 kg water per kilogram cell dry weight (Oriol *et al.*, 1988; Larroche *et al.*, 1992). Taking this result, and using the model presented in what follows, we estimated that the water requirement for new cells would be ca. 45% of that for evaporative cooling. Furthermore, Larroche and Gros (1992) reported that the water content of mycelium of *Penicillium roquefortii* varies during cultivation. Therefore, experimental verification was warranted for this important water requirement. Preliminary experiments indicated that the intracellular water content of *A. oryzae* grown on wheat flour varies with the initial water content of the substrate matrix. A membrane model system was developed that mimics the growth conditions on wheat grains, which enabled us to analyze the substrate matrix and the fungal biomass separately.

In the literature, models have been described that predict the moisture content during SSF (Narahara *et al.*, 1984; Sargantanis *et al.*, 1993). These models do not distinguish between intracellular water (fungal biomass) and extracellular water (substrate matrix). In this article, we developed a model that allows calculation of the overall water content as well as the extracellular water content.

This model can be incorporated in an on-line control strategy aimed at maintaining a sufficiently high extracellular water content. Ideally, such a control strategy should aim at maintaining a sufficiently high water activity, but we decided not to include calculations of water activity, for several reasons. First, the desorption isotherm of the wheat used as substrate (Nagel *et al.*, 2001) indicated that the water activity remains very close to a_w=1 as long as the water content of the wheat matrix is > 0.5 kg per kilogram dry matter. Second, water activity may also be influenced by accumulation of monosaccharides, oligosaccharides, or other low-molecular-weight hydrolysis products. Incorporation of their effects in the calculations would require much more detailed information on the kinetics of hydrolysis and uptake reactions than is currently available. This would probably also make the control model too complex for on-line use.

Our model predicts the extracellular water content as well as the overall moisture content from on-line measurements of oxygen, carbon dioxide and moisture content of the air. The water balance in the model incorporates uptake of intracellular water, metabolic water production, water used for hydrolysis of starch, and evaporated water. Several attempts have been described in literature to estimate the intracellular water content of biomass indirectly (Larroche and Gros, 1992; Larroche *et al.*, 1992). We measured the intracellular water content of biomass directly using a model system, that can mimic the growth of *A. oryzae* on wheat grains and allows separate analysis of biomass and substrate matrix. We used elemental balances to estimate metabolic water production and loss of dry solid substrate using three experimentally determined yield coefficients. A systematic method (Wang and Stephanopoulos, 1983) was employed to identify possible gross measurement errors in the measured yield coefficients, and maximum-likelihood techniques were applied to obtain a consistent set of adjustments for the experimental data.

The model was validated using experiments in a 1.5-L scraped-drum reactor (Oostra *et al.*, 2000) and a 35-L horizontal paddle mixer (Nagel *et al.*, 2001). Relatively small deviations were observed between measured and predicted overall moisture contents.

A wide control range was found for the moisture content of the solid substrate. This control range followed from a combination of colony-growth-rate data (Gibson *et al.*, 1994) and the water desorption isotherm for wheat grains (Nagel *et al.*, 2001). In

summary, it may be stated that the proposed model is accurate enough to control the extracellular water content during SSF.

MODEL DEVELOPMENT

Moisture-content control during SSF is difficult because a direct on-line measurement of the overall moisture content is problematic. Furthermore, the extracellular-moisture content cannot be measured off-line because the fungal biomass cannot be separated from the substrate particles. A stoichiometric model is therefore developed that can be used for automatic moisture-content control during SSF. The model uses on-line measurements of O_2, CO_2, and dew-point in the effluent air, to predict the extracellular moisture content and the overall moisture content of the grains (Figure 1). The model is based on elemental balances and does not contain kinetic relations. Kinetics are given by the measured oxygen consumption rate. All yield coefficients in the model were determined using elemental balances and measured yields.

The reaction equation for growth of *Aspergillus oryzae* on glucose and wheat protein is:

$$Y_{S/O2}CH_2O + O_2 + Y_{N/O2}CH_{1.98}O_{0.63}N_{0.26} \rightarrow Y_{X/O2}CH_{1.72}O_{0.52}N_{0.17} + Y_{CO2/O2}CO_2 + Y_{W/O2}H_2O \quad (1)$$

in which

$Y_{S/O2}$	=	yield coefficient for substrate (*S*) on oxygen	[Cmol·mol^{-1} O_2]
$Y_{N/O2}$	=	yield coefficient for protein (*N*) on oxygen	[Cmol·mol^{-1} O_2]
$Y_{X/O2}$	=	yield coefficient for biomass (*X*) on oxygen	[Cmol·mol^{-1} O_2]
$Y_{CO2/O2}$	=	yield coefficient for carbon dioxide on oxygen	[mol·mol^{-1} O_2]
$Y_{W/O2}$	=	yield coefficient for water (*W*) on oxygen	[mol·mol^{-1} O_2]

The elemental composition of the biomass was assumed to be $CH_{1.72}O_{0.52}N_{0.17}$, which is the average biomass composition of *Aspergillus niger* (Nielsen and Villadsen, 1994). The elemental composition of protein ($CH_{1.98}O_{0.63}N_{0.26}$) was calculated from an average amino-acid composition of five different wheat-grain varieties (Shoup *et al.*, 1966). Production of α-amylase by *A. oryzae* was neglected because the ratio between

α-amylase and biomass was reported to be only 0.019 Cmol/Cmol (Agger *et al.*, 1998).

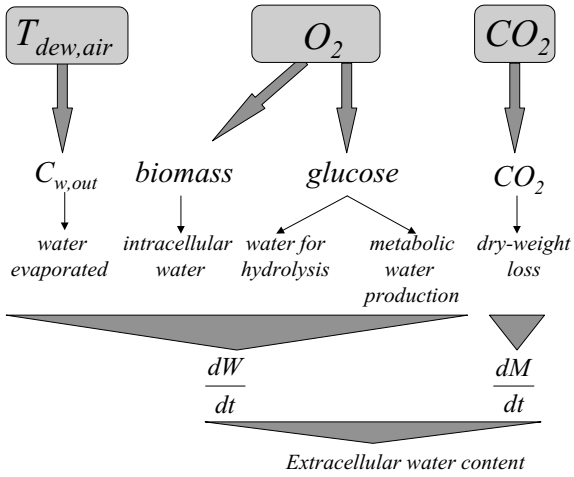


Figure 1. A schematic representation of the model used to estimate the extracellular water content during solid-state fermentation from on-line measurements (oxygen, carbon dioxide, and dewpoint temperature).

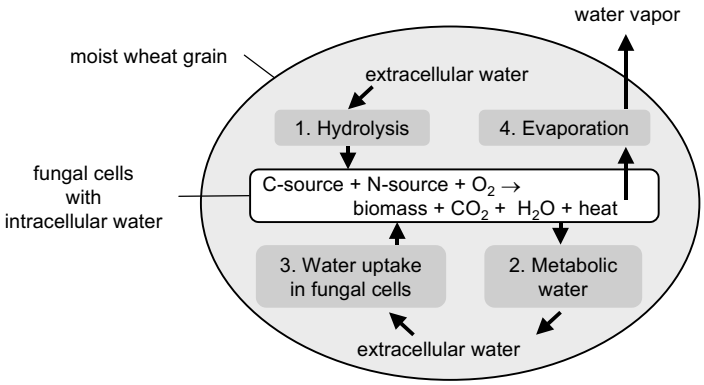


Figure 2. A schematic overview of the four different contributions in the water balance during solid-state fermentation on wheat grains. In order of increasing magnitude: (1) water for starch hydrolysis; (2) metabolic-water production; (3) water uptake in new biomass; and (4) water evaporation.

The water balance has four contributions (Figure 2):

1. Water needed for starch hydrolysis
2. Metabolic water production
3. Uptake of intracellular water during biomass production.
4. Water evaporation as a consequence of metabolic-heat production

All four contributions are taken into account in the mass balance for extracellular water (equation 2). In this balance, biomass production and substrate consumption are calculated from the measured oxygen consumption rate. In the following equations, all rates are production rates; consumption rates have a negative value.
Assuming that the air in the bioreactor is in steady state, the biomass has a constant water content, and accumulation of glucose or oligosaccharides is negligible, the balance for extracellular water is:

$$\frac{dW_{wh}}{dt} = F_{air} \cdot \left(C_{Win} - C_{Wout}\right) + X_{W,X} \cdot Y_{X/O2} \cdot r_{O2} \cdot Mw_X - Y_{W/O2} \cdot r_{O2} \cdot Mw_W + Y_{hyd} \cdot Y_{S/O2} \cdot r_{O2} \qquad [kg \cdot s^{-1}] \qquad (2)$$

in which

W_{wh}	=	weight extracellular water in wheat		[kg]
r_{O2}	=	oxygen production rate		[mol·s^{-1}]
F_{air}	=	volumetric gas flow at 273 K and 1.013·10^5 Pa		[m^3 dry air·s^{-1}]
C_W	=	water concentration in air recalculated to 273 K and 1.013·10^5 Pa		[kg·m^{-3} dry air]
$X_{W,X}$	=	water content of biomass		[kg·kg^{-1} DW]
Mw_w	=	18·10^{-3}	molecular weight water	[kg·mol^{-1}]
Mw_X	=	24.42·10^{-3}	molecular weight biomass	[kg·Cmol^{-1}]
Y_{hyd}	=	0.003	water needed to hydrolyze starch	[kg·Cmol^{-1} S]

The yield coefficients for starch and protein were used to relate dry-weight losses to the measured CO_2 production:

$$\frac{dM_{wh}}{dt} = -\left(\frac{Y_{S/O2}}{Y_{CO2/O2}} \cdot Mw_S + \frac{Y_{N/O2}}{Y_{CO2/O2}} \cdot Mw_N \right) \cdot r_{CO2} \qquad [kg \cdot s^{-1}] \qquad (3)$$

in which

M_{wh}	=	weight of wheat dry matter		[kg]
Mw_N	=	$27.63 \cdot 10^{-3}$	molecular weight protein	[kg·Cmol^{-1} N]
Mw_S	=	$30 \cdot 10^{-3}$	molecular weight starch	[kg·Cmol^{-1} N]
r_{CO2}	=	carbon dioxide production rate		[mol·s^{-1}]

The extracellular water content ($X_{W,wh}$) in time is then given by :

$$X_{W,wh}(t) = \frac{W_{wh}(t)}{M_{wh}(t)} \qquad [kg \cdot kg^{-1} \text{ DM}] \qquad (4)$$

The extracellular water content -that is, water outside the mycelium- can be estimated with the model, but it cannot be measured in the fermentations with grains. To validate the model, a prediction of the overall moisture content is necessary. The amounts of biomass dry weight and intracellular water are needed to calculate the overall moisture content of a fermented wheat grain; these are calculated from:

$$\frac{dM_X}{dt} = -Y_{X/O2} \cdot r_{O2} \cdot Mw_X \qquad [kg \cdot s^{-1}] \qquad (5)$$

$$W_X(t) = X_{W,X} \cdot M_X(t) \qquad [kg] \qquad (6)$$

in which

M_X	=	weight of biomass dry matter	[kg]
W_X	=	weight of intracellular water	[kg]

The overall moisture content of the cultivated wheat grains ($X_{W,ov}$) becomes:

$$X_{W,ov}(t) = \frac{W_{wh}(t) + W_X(t)}{M_{wh}(t) + M_X(t)} \qquad [\text{kg}\cdot\text{kg}^{-1}\ \text{DM}] \qquad (7)$$

MATERIALS AND METHODS

Micro-organism and inoculation

Aspergillus oryzae CBS 570.65 was obtained from Centraal Bureau voor Schimmelcultures (Baarn, NL). The preparation of a sporangiospore suspension has been described previously (Nagel *et al.*, 2001). Glycerol (20% [w/v] final concentration) was added to the spore suspension, which was stored at -80 °C for up to 3 months in 1-mL cryovials (Greiner, Germany) and 125-mL plastic bottles (Model 2105-0004, Nalgene, UK). The 125-mL plastic bottles contained 43.5 mL spore suspension and 16.5 mL of glycerol.

One 125-mL plastic bottle was used to inoculate the horizontal paddle mixer; the inoculation procedure has been described elsewhere (Nagel *et al.*, 2001). For experiments in the scraped drum reactor, three cryovials and 2 mL of peptone physiological salt solution were aseptically sprayed using a tube atomizer (Desaga, Germany) over 300 g of moisturized wheat grains. The contents of two 125-mL plastic bottles and 100 mL of peptone physiological salt solution were used for inoculation of the membrane model system.

Solid substrate

A single batch of wheat grains of commercial origin (Blok, Woerden, NL) was stored at 10°C (initial moisture content 0.12 kg/kg DM) and used for all experiments.

Wheat dough

Whole wheat grains were milled in a mill (Retsch, NL). Milled wheat grains and distilled water were mixed to obtain the desired moisture content of the wheat dough. At moisture contents > 1.5 kg/kg DM wheat, agar (final concentration 0.04 kg/kg total) was used to give sufficient strength to the wheat dough. It was not possible to prepare homogeneous dough below a moisture content of 0.8 kg w/kg DM.

Wheat grains

Whole wheat grains were soaked for 2.5 h in excess water at 60°C to give a final moisture content of 0.87 kg w/kg DM. After sieving, the soaked grains were autoclaved (1.5 h, 121°C).

Cultivation methods

Membrane model system

All parts of the membrane model system (Figure 3) were manufactured by the Mechanical workshop at Wageningen University. The wheat dough was transferred into a sausage maker (Figure 3b), autoclaved (1.5 h, 121 °C) and cooled down to room temperature. Wheat disks (4.5 cm diameter, 0.5 cm height) were prepared in a laminar flow cabinet, using a previously sterilized wire cutter. Each wheat disk was weighed and placed in a previously autoclaved polypropylene (PP) dish (Figure 3a). A sterile 0.45 µm polyamide membrane (Schleicher & Schuell, NL17 ST) was applied on the disk. A glass cylinder was placed on top of the membrane and the cylinder was closed with a lid of a petri dish (Greiner, ø = 4.5 cm).

For each cultivation, 20 PP dishes were prepared and 19 were inoculated by spraying a spore suspension onto the membrane using a moving-belt device (Mechanical workshop, Wageningen University, NL). One PP dish remained uninoculated in order to measure possible evaporation losses and to check for sterility.

Three PP dishes without lids were placed in the middle of a closed jar, which contained a layer of water on the bottom to prevent desiccation, in order to measure O_2 consumption and CO_2 production. The closed jar was placed in a temperature-controlled cabinet at 35°C. The closed jar was aerated (15 L/h at 273 K, $1.013 \cdot 10^5$ Pa) using a mass flow controller (Brooks Instruments BV, NL). To accurately determine CO_2 production, CO_2 was removed from the inlet air using a 1.5 M NaOH solution. The inlet air was further humidified (relative humidity 94.6% at 35°C) using a temperature-controlled water column (height 40 cm; diameter 10 cm) filled with Raschig rings (height 1 cm, diameter 5 mm). The inlet air was filter sterilized using a 0.2 µm hydrophobic membrane (Acros 50, Gelman, USA). The effluent air was first analyzed for dewpoint (DEWMET SD, Michell Instruments Ltd., UK), and then dehumidified (16% relative humidity at 30°C) using a condenser before it was analyzed for CO_2 and O_2 (see later). The remaining PP dishes were placed in a partially

opened box (25L) filled with a layer of water to prevent evaporation losses, which was placed in the same temperature-controlled cabinet as the closed jar. The duration of these cultivations was usually 80 h.

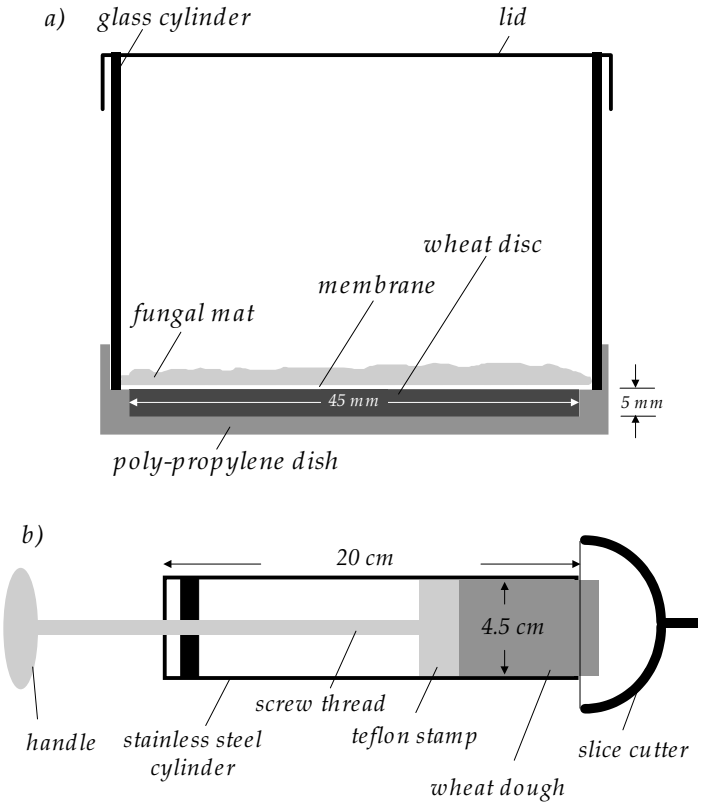


Figure 3. (a) Membrane model system for solid-state fermentation on wheat grains used to measure the moisture content of biomass and all other model parameters. (b) Apparatus used to aseptically prepare wheat disks for the membrane model system.

Scraped Drum Bioreactor (1.5 L)

The Scraped Drum Reactor (SDR, length 20 cm, diameter 5 cm, Figure 4) consisted of a glass cylinder, sealed on both sides with a stainless-steel flange. A hollow rotating shaft was mounted between the flanges. One end of the shaft was connected to the motor; the other end was used for the air inlet. A hollow scraper, mounted in the middle of the shaft, scraped along the wall to achieve radial mixing. Perforations in the scraper allowed sufficient air distribution in the reactor. The SDR was filled with 100 g wet inoculated wheat grains and placed in a temperature-controlled cabinet. The SDR was aerated (90 L/h at 273 K, $1.013 \cdot 10^5$ Pa) using a mass flow controller (Brooks Instruments BV, NL) and mixed continuously (1 rpm). The air-conditioning system for these experiments was the same as described earlier.

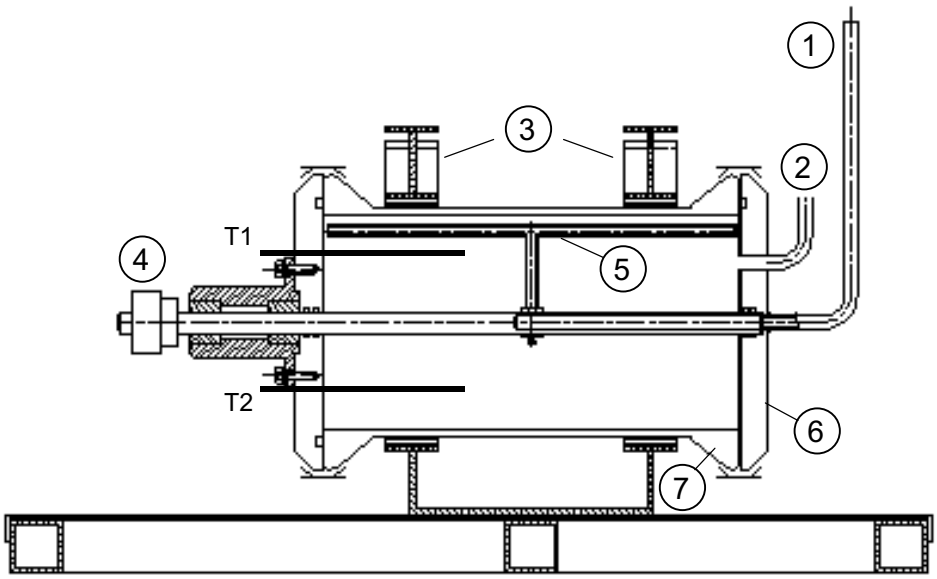


Figure 4. Schematic diagram of 1.5-L scraped drum reactor: (1) air inlet; (2) air outlet; (3) reactor fixings; (4) drive shaft; (5) scraper with fine holes for air distribution; (6) steel side plate; (7) glass cylinder. T1 and T2 are temperature sensors.

Horizontal Paddle Mixer (35 L)

Two cultivations in the horizontal paddle mixer were used to validate the model. A detailed description of the bioreactor and auxiliary equipment is given elsewhere (Nagel *et al.*, 2001). *A. oryzae* was cultivated on 8 kg of moistened wheat grains (moisture content 0.87 kg w/kg DM) for 3 days. The temperature of the solid substrate was maintained at 35°C using evaporative cooling for one cultivation and wall cooling for the other cultivation.

Sampling

Membrane model system

Twice a day, two PP dishes were removed for measurements. The biomass was pulled from the membrane and the membrane was easily peeled from the wheat-dough disk. The wet weights of biomass and wheat disk were recorded. Virtually complete recovery of biomass and dough was possible and virtually nothing adhered to the PP dish or membrane. Two grams of wheat dough from one PP dish was used to determine its water activity before it was transferred into a sample bottle and stored at -20°C for further analysis of glucose. The 2 g of wheat dough from the other PP dish was transferred directly into the sample bottle. The moisture content was determined for the remaining wheat dough and wet biomass.

Scraped Drum Reactor (1.5 L)

Three hunderd grams of inoculated wheat grains was prepared for this cultivation and 100 g of it was used to determine the initial moisture content and the initial concentration of total glucose. This initial moisture content was used to calculate the initial amounts of water and dry matter in the SDR. No samples were taken during cultivation. Instead, the whole bioreactor content was used to determine the final amounts of water and dry matter. Five grams was used to measure the glucosamine content and total glucose concentration.

Horizontal Paddle Mixer (35 L)

Twice a day during the fermentation, a sample (± 30 g) was taken from the bioreactor. Moisture content and water activity were determined immediately after sampling, as

described later. Approximately 5 gram of wheat grains was transferred into a sample bottle and stored (-20 °C) for further analysis of glucosamine and glucose.

Analysis

Moisture content & Water activity

The moisture content of biomass, wheat dough, and wheat grains were calculated from the weight loss after drying the sample at 80°C for 2 days in a preweighed dish. The water activity of the sample was determined in a Novasina Thermoconstanter (Type TH200, Switzerland) at 35°C.

Total and free glucose

After the sample bottle was thawed, the wheat dough or wheat grains were homogenized with water (dough : water = 1 g : 4 g) in an ultraturrax (Model L4R, Silverston, UK). For determination of total glucose, 0.1 g of this homogenate was added to 4.5 mL 13 mM phosphate buffer (pH 4.5) and 202 U amyloglucosidase (Sigma A-7255, 1 U will liberate 1.0 mg of glucose from starch in 3 min at 55 °C, pH 4.5). This mixture was continuously shaken in a water bath (Model 1083, GFL, Germany) at 55°C for 1 h and then filtered using a 0.45-µm membrane filter (Model FP 030/2, Schleicher and Schuell, Germany) after which total glucose was determined. Three milliliters of homogenate was divided into two Eppendorf tubes and centrifuged at 15300 rpm for 20 min (Model GS-15R centrigue, Beckman instruments, Germany) after which the supernatant was used to determine free glucose. Glucose was determined using a Peridochrom Glucose Kit (Cat. no. 676543, Boehringer Mannheim, Germany).

Glucosamine

The glucosamine content of dry biomass was measured over time during cultivation to convert the glucosamine measurements in wheat grains to dry biomass. The procedure for wheat-grain sample preparation and the glucosamine measurement has been described elsewhere (Nagel *et al.*, 2001). To determine the glucosamine content of dry biomass, 0.1 g dry biomass was prehydrolyzed instead of the dried pellet that was used for the glucosamine measurement in wheat grains.

CO_2 and O_2 consumption

Oxygen and carbon dioxide were analyzed using a paramagnetic O_2 analyzer (Xentra 4100, Servomex Zoetermeer, NL) and an infrared CO_2 analyzer (Servomex Series 1400). The oxygen analyzer was fitted with a second channel to measure the O_2 concentration of the outside air, in order to accurately determine O_2 consumption. Both O_2 sensors corrected the readings for pressure fluctuations.

Expression of the results

Measurements of glucose and glucosamine content obtained from the samples taken from cultivations in the membrane model system and SDR were expressed per unit weight of initial dry matter.

RESULTS AND DISCUSSION

Model parameters

Yield coefficients

A biochemical reaction equation (Equation 1) was used together with three measured yield coefficients ($Y_{X/O2}$, $Y_{S/O2}$, $Y_{CO2/O2}$; Table 1) to determine: (1) the metabolic water production ($Y_{W/O2}$), which cannot be measured directly; and (2) the yield coefficient necessary for the calculation of the dry-weight-loss factor ($Y_{N/O2}$). Due to various types of errors in measured yield coefficients, raw measurements rarely form a consistent set of data that satisfies the elemental balance equation (Equation 1). We used a systematic method (Wang and Stephanopoulos, 1983) that: (1) identified measurements that are most likely to contain gross errors; and (2) leads to a consistent set of adjustments on the experimental values. This method was used for our overdetermined system with five unknown yield coefficients, four elemental balances and three measured yield coefficients. Yield coefficients were calculated from the data of two experiments with the membrane model system. Only the data of the three dishes in the closed jars, after approximately 70 h of cultivation, were used to calculate the yield coefficients (Table 1). The measured data were not corrected for maintenance metabolism. Average yield coefficients were calculated from these values and used in the systematic method mentioned earlier. The measurement errors used in this method were estimated by

calculating the average of the two relative standard deviations obtained for each set of three measurements. These errors were 3.9%, 7.3%, 3.4% and 10.7 % for CO_2, O_2, biomass, and glucose, respectively. The value of the test function, 3.17, was compared with the chi-square (χ^2) distribution function for two degrees of freedom (two constraint equations).

Because $\chi^2_{0.9}(2) = 4.61 > 3.17$, the hypothesis test in this method was passed and we could state with 90% confidence that these yield coefficients do not contain gross errors (*i.e.* errors that are much larger than can be reasonably expected based on the measurements errors). The maximum-likelihood estimates for the yield coefficients are given in Table 1.

The yield coefficient for biomass on glucose was 0.8 Cmol/Cmol, which seems very high. It should be noted, however, that protein was also used as carbon source, and the yield of biomass on total carbon source (protein + glucose) was 0.52 Cmol/Cmol, which is in agreement with yield coefficients (0.5 to 0.6) normally found in literature (van 't Riet and Tramper, 1991).

Water content of biomass

The intracellular-water content of biomass ($X_{W,X}$) is an important parameter in the model because it is used to quantify the amounts of intracellular water in biomass and extracellular water in the solid substrate matrix ($X_{W,wh}$). Preliminary experiments indicated that $X_{W,wh}$ influenced $X_{W,X}$. Furthermore, it was uncertain if $X_{W,X}$ remained constant during cultivation. To answer these questions, a membrane model system (Figure 3) was developed that mimics the growth of *Aspergillus oryzae* on wheat grains and that allows direct measurement of $X_{W,X}$ and $X_{W,wh}$. Figure 5 shows the results of four experiments with the model system in which the initial moisture content of the wheat flour discs was 1 kg/kg DM, each with 9 to 12 separate measurements of wet and dry weight of the biomass layer. It shows that $X_{W,X}$ could be determined accurately and was constant during the whole cultivation period (80 h). Linear regression through the origin yielded $X_{W,X} = 2.08 \pm 0.0673$ kg/kg DM (± 95% confidence).

Table 1. Yield coefficients derived from measurements of two experiments (I and II) in a membrane model system. [a]

Yield coefficient	Measurement		Parameter value	Unit
	I	II		
$Y_{X/O2}$	1.06	1.16	1.16	Cmol X·mol O_2^{-1}
$Y_{S/O2}$	1.76	1.55	1.46	Cmol S·mol O_2^{-1}
$Y_{W/O2}$			1.22	mol W·mol O_2^{-1}
$Y_{CO2/O2}$	0.99	1.00	1.06	mol CO_2·mol O_2^{-1}
$Y_{N/O2}$			0.76	Cmol N·mol O_2^{-1}

[a] A systematic method (Wand and Stephanopoulos, 1983) was applied to determine the maximum-likelihood estimates for the yield coefficients used in the model.

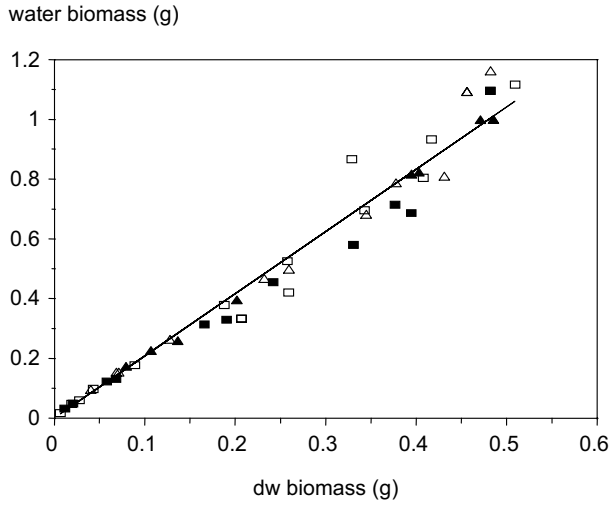


Figure 5. The amount of intracellular water in biomass of *Aspergillus oryzae* plotted against biomass dry weight for four separate experiments ($X_{W,wh}$ = 1 kg /kg DM) (▲, △, □, ■) with the membrane model system. The moisture content of fresh biomass was estimated from the straight line (——), which was determined by linear regression.

Figure 6 shows that the moisture content of the substrate ($X_{W,wh}$) influences $X_{W,X}$, especially at moisture contents > 1 kg/kg DM substrate. The influence of $X_{W,wh}$ on $X_{W,X}$ below 1 kg/kg DM was considered to be insignificant based on the 95% confidence intervals for the average values of $X_{W,X}$. It should be noted that only initial moisture levels of < 1 kg/kg are relevant for cultivation on wheat in our fermentors, because the grains are too quickly damaged by the mixing when the moisture content is higher. Therefore, the water content of biomass ($X_{W,X}$) was taken as a constant in the model for the cultivation of *A. oryzae* on wheat grains; its value was 2.08 kg/kg DM. This constant value is in accordance with results of the four experiments shown in Figure 5, in which $X_{W,wh}$ decreased during cultivation from 1 to 0.8 kg/kg DM, without affecting $X_{W,X}$.

The reasons for this dehydration of wheat dough were the water uptake for biomass production and water evaporation as a result of metabolic heat generation. No water losses were found in the PP dish that was not inoculated. We did not succeed in preparing homogeneous wheat flour discs with water contents of < 0.8 kg/kg. In some of the fermentor experiments presented in what follows, the water content of the wheat matrix dropped below 0.8 kg/kg. In these cases, model predictions were clearly extrapolations.

The value for the intracellular water content of fungal biomass used in our model is lower than the values of around 3 kg/kg DM reported previously for *Aspergillus niger* cultivated on cassava flour (Oriol *et al.*, 1988) and *Penicillium roquefortii* cultivated on buckwheat seeds (Larroche *et al.*, 1992). This may be due to differences in water content or binding of the substrates used; Figure 6 shows that wheat gave similar values. The difference may also be due to differences in measurement methods. Larroche and coworkers (1992) calculated the water content of the mycelium from the protein content and the initial water content of fermented buckwheat samples. Their calculation involved assumptions about the protein content of the biomass, water losses by evaporation and metabolic water production. Although a direct comparison is difficult, due to differences in strain and substrate, we believe that our value is more reliable because it was measured directly.

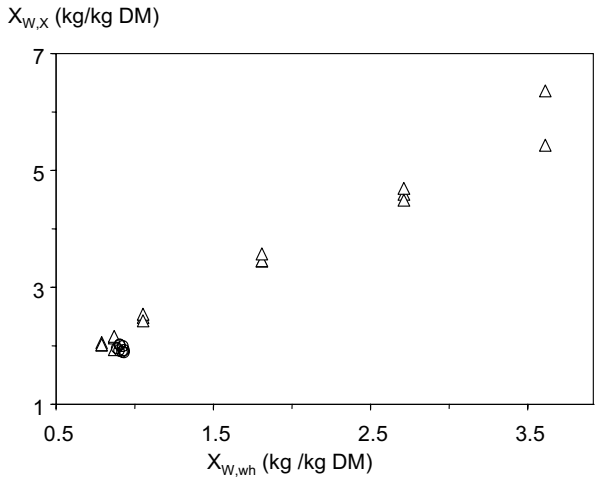


Figure 6. Intracellular moisture content of *Aspergillus oryzae* biomass ($X_{W,X}$) as a function of the moisture content of wheat dough ($X_{W,wh}$) after 60-h cultivation time, measured using a membrane model system. Samples of all four experiments from figure 5 at 60 h cultivation time are also displayed (○).

Dry-weight balance

To predict the extracellular water content $X_{W,wh}$, an accurate description of dry substrate loss is indispensable. Dry substrate loss is simply protein and starch that are consumed as given in Equation 3. For on-line dry substrate estimation, yield coefficients (Table 1) were recalculated based on CO_2:

$$Y_{S/CO2} = Y_{S/O2} / Y_{CO2/O2} = 1.38 \text{ Cmol/mol}$$

$$Y_{N/CO2} = Y_{N/O2} / Y_{CO2/O2} = 0.72 \text{ Cmol/mol}$$

so that the dry-weight loss could be easily estimated from the measured CO_2 production (r_{CO2}). Based on these yield coefficients the value in brackets in Equation 3 amounts to 61.1 g/mol CO_2. With this dry-weight loss factor, the total weight of the wheat matrix can be calculated on-line during cultivation.

Several investigators (Larroche *et al.*, 1998; Smits *et al.*, 1998) characterized dry-weight loss as the overall weight loss of the fermenting matter (substrate and biomass).

Overall dry-weight loss can be measured easily, whereas the weight of dry substrate cannot be measured directly because biomass and substrate are difficult to separate. To check the validity of our approach, the dry-weight loss factor mentioned earlier (61.1 g/mol) was recalculated to give an overall dry-weight loss factor (Equation 8), which was compared with literature data. The overall dry-weight-loss factor was 34.5 g/mol CO_2. Smits (1998) reported a ratio between overall dry-weight loss and CO_2 evolution equal to 32.6 g/mol CO_2 for *Trichoderma reesei* cultivated on wheat bran, which is very similar to our value.

$$f_{DMloss} = Y_{S/CO2} \cdot Mw_S + Y_{N/CO2} \cdot Mw_N - Y_{X/CO2} \cdot Mw_X \qquad [g \cdot mol^{-1}\ CO_2] \quad (8)$$

Our approach is applicable for any type of cultivation if a consistent biochemical reaction equation is available. Furthermore, dry-weight loss can be coupled to any compound (*e.g.* oxygen) present in the biochemical reaction equation. The approach can easily be extended with maintenance metabolism for a better description of the stationary phase.

Glucosamine content of biomass

In order to estimate biomass dry weight in wheat samples taken during cultivation in mixed bioreactors, the glucosamine content was measured. A conversion factor (G_X) is needed to convert the measured glucosamine content to biomass dry matter. Although glucosamine is a widely used indicator for biomass dry weight, two drawbacks are reported in literature: (1) the glucosamine content is dependent on the substrate and cultivation method used (Sakurai *et al.*, 1977); and (2) the glucosamine content is dependent on the age of the mycelium (Sakurai *et al.*, 1977; Arima and Uozumi, 1967). These drawbacks were circumvented by using the membrane model system so that glucosamine and biomass dry weight could be measured simultaneously during cultivation on a substrate with the same composition as that used in bioreactor cultivations. Results of three separate cultivations with the membrane model system showed an increase in the glucosamine content of biomass with cultivation time (Figure 7). The observed increase is best described by a sigmoidal curve fitted through the data:

$$G_X\left(t-\lambda\right)=44.61+\frac{43.65}{\left(1+\exp\left(-\frac{\left(\left(t-\lambda\right)-61.70\right)}{12.34}\right)\right)} \qquad [\text{mg}\cdot\text{g}^{-1}\text{dry biomass}] \quad (9)$$

in which

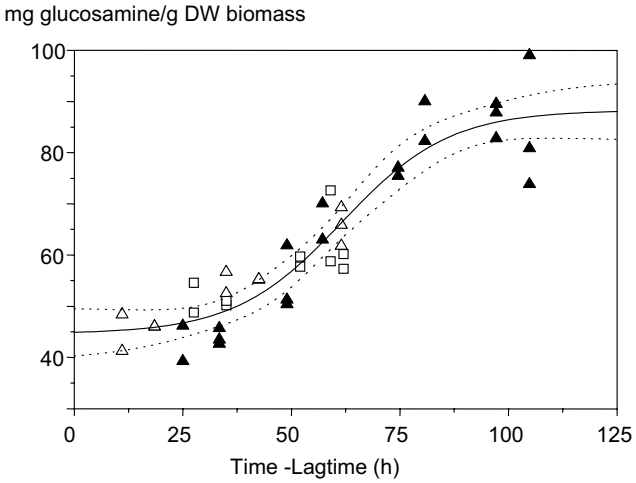
G_X	=	glucosamine content of biomass	[mg·g^{-1} DM]
t	=	time	[h]
λ	=	lag time	[h]

The cultivation time for experiments with the model system was corrected for a lag time so that this relation could be applied for recalculation of glucosamine measurements in the bioreactors, where the lag phase was shorter due to a higher inoculation density. In the model system, we found a linear increase of biomass dry weight in time (results not shown). In the paddle mixer, the glucosamine content of the grain increased linearly (Nagel *et al.*, 2001). The lag time was determined from the intersection of a regression line through the biomass dry matter values or glucosamine values and the time axis. It remains uncertain whether this correction of the cultivation time is legitimate. Nevertheless, this approach is believed to be the best possible way to deal with glucosamine measurements for indirect biomass dry-weight estimation and certainly no worse than other indirect biomass estimates.

Model validation

Although the model predicts both the total water content of the fermented grains and the extracellular water content of the wheat matrix, only the former can be measured. Validation of the predicted extracellular water content can only be achieved indirectly, by checking predictions for biomass and residual starch. Validation experiments were conducted with wheat grains in a scraped drum reactor and a paddle mixer.

We intend to apply a control strategy based on the model developed in this paper in the paddle mixer. The scraped drum was used, because it allows further reduction of water evaporation (more wall area available for cooling) and measurements on the whole amount of fermented grains.



Figuur 7. Glucosamine content of dry-weight biomass as a function of the difference between cultivation and a lag time (time – lag time) in order to estimate biomass from experiments involving whole wheat grains. The different symbols represent three separate experiments. The straight line is the sigmoid fit through all measurements [Eq. (9)]. The dotted line is the 95% confidence interval for the fit.

Scraped drum reactor (1.5 L)

The model was validated with four cultivations in a 1.5-L scraped drum reactor (Table 2). These cultivations used only 100 g wet-weight grains to minimize heat generation and subsequent problems with temperature control. Nevertheless, temperature increased during these cultivations with a maximum of 37.5 °C attained in experiment B. This means that temperature did not limit fungal growth during these fermentations.

Water evaporation was minimized during these fermentations to increase the relative significance of other terms in the water balance; that is, metabolic water production and water used for hydrolysis. In this way, we tried to determine the sum of metabolic water production and water used for hydrolysis (= net water production, Table 2) from the water balance during fermentation, in order to validate the model. No samples were taken during these cultivations and, in the end, the whole bioreactor content was used

to determine final amounts of water and dry weight. A sample was taken in the end to measure glucosamine and glucose.

The model predicted the overall moisture content very well (Table 2). $X_{W,ov}$ increased during these cultivations, despite water evaporation, due to significant dry-weight losses and metabolic water production. Water evaporation could not be prevented completely, mainly due to the increasing substrate temperature during cultivation.

The prediction of metabolic water production was reasonably accurate (Table 2), as indicated by the small difference between measured and predicted net water production. The average deviation between predicted and measured final water amount was 2.1% of the initial amount of water present. Metabolic water production was significant as the amount was 20% of the water initially present after 70 hours of cultivation. The water needed to hydrolyze starch proved to be a minor term in the water balance, as expected.

The prediction of the final amount of dry matter was slightly less accurate. The average deviation between predicted and measured final amounts of dry matter was 4.2% of the initial amount of dry matter. Both deviations between predicted and measured amounts of water and dry matter can lead to a maximum deviation of the predicted moisture content equal to 6.3%.

Significant deviations were observed between model predictions and experimental data for biomass and consumed glucose (Table 2).

Model predictions became more accurate for longer cultivation times (experiments B and C) as more biomass was formed and glucose consumed. Both measurement methods (glucose and glucosamine) contain many steps to prepare the sample, which probably makes them less accurate. In addition, the conversion factor (G_X) is hampered by a large 95% confidence interval (Figure 7), and uncertainty in the lag-time correction, which may lead to a large error in the biomass determination.

Table 2. Validation of a model for the predicton of the extracellular and overall water content during solid-state fermentation of *Aspergillus oryzae* in a 1.5-L scraped drum reactor.[a]

	A (55.2 h)		B (71.1 h)		C (66.4 h)		D (49.4 h)	
Experiment (t_{end})	exp. data	Model	exp. data	Model	exp. data	Model	exp. data	Model
1. $X_{w,ov}$ t_0 [kg/kg DM]	1.02	[c]	0.95	c	1.00	c	0.96	c
2. $X_{w,ov}$ t_{end} [kg/kg DM]	1.23	1.21	1.38	1.23	1.02	0.96	0.98	0.94
3. Water t_0 [g]	53.84	c	50.53	c	52.23	c	50.41	c
4. Water evaporated [g]	2.22	c	13.90	c	22.85	c	8.08	c
5. Water t_{end} [g]	52.59	54.88	46.36	44.98	36.62	36.07	44.62	44.45
6. Metabolic water [g]		+ 4.08		+10.44		+ 8.37		+ 2.60
7. Water hydrolysis [g]		- 0.82		- 2.09		- 1.68		- 0.53
8. Net water production [g]	0.97	3.26	9.73	8.35	7.24	6.69	2.29	2.07
9. *DM* t_0 [g]	52.67	c	53.34	c	52.43	c	52.69	a
10. *DM* t_{end} [g]	42.74	45.20	33.61	36.56	36.06	37.70	45.45	47.24
11. Biomass [b] [g]	8.5 (±1.3)	5.3	11.8 (±1.8)	13.5	8.7 (±1.3)	10.8	4.3 (±0.6)	3.4
12. Substrate loss [g]		12.8		30.3		25.5		8.9
13. *DM* loss [g]	9.9	7.5	19.7	16.8	16.4	14.7	7.2	5.5
14. Glucose consumed [b] [g]	15.2 (±1.9)	8.2	23.2 (±1.7)	20.9	21.7 (±1.8)	16.8	9.9 (±2.2)	5.3
15. Intracellular water [g]		11.0		28.1		22.5		7.1
16. O_2 [g]	9.18	c	21.80	c	18.41	c	6.39	c
17. CO_2 [g]	5.97	c	15.28	c	12.25	c	3.88	c
18. $X_{w,wh}$ t_{end} [kg/kg DM]		1.10		0.73		0.50		0.85

[a] Four batch experiments were each harvested at different timepoints to validate the model.

[b] Values in parentheses indicate absolute measurement error and were calculated using a relative measurement error (15.25% for biomass, 7.33% for glucose) determined from 15 separate duplicate measurements for biomass and glucose.

[c] Experimental data in the left column of every experiment is used as input in the model. Calculations as follows: row 8, (Exp. data and model colomn), net water production = (water t_{end} – water t_0) + water evaporated [row 8= (row 5 – row 3) + row 4]; row 13 (Exp. data and model) column, DM loss = DM t_0 – DM t_{end} [row 13 = row 9 – row 10]; row 15 (Model) column, biomass (row 11) * water content biomass ($X_{W,X}$ = 2.08 kg/kg DM); row 18 (Model) column (water t_{end} – intracellular water)/(DM t_{end} – biomass) [(row 5 – row 15)/(row 10-row 11)].

Horizontal paddle mixer (35 L)

Two cultivations were done in the 35-L horizontal paddle mixer to validate the model: one cultivation in which evaporative cooling was applied and one in which mainly wall cooling was applied (Nagel *et al.*, 2001). This validation differs in two aspects from the aforementioned validation in the SDR. First, one of the cultivations better reflects the situation in which the model will be actually applied; that is temperature-controlled cultivations in which evaporative cooling is applied. Second, samples were taken during cultivation instead of measuring the whole bioreactor content in the end. The model was validated by comparing model predictions for $X_{W,ov}$ with measurements. The model predicted $X_{W,ov}$ very well throughout the whole fermentation for both cultivations (Figure 8).

Figure 9 shows the model prediction of $X_{W,wh}$ during the cultivation in which wall cooling was applied. It is impossible to measure the extracellular water content, but we can estimate such measured values ($X_{W,wh,exp}$) from biomass measurements ($M_{X,exp}$), predicted values of the overall water content ($X_{W,ov}$) and the remaining amount of wheat dry matter (M_{wh}):

$$X_{W,wh,\exp} = \frac{X_{W,ov} - \frac{M_{X,\exp}}{\left(M_{X,\exp} + M_{wh}\right)} \cdot X_{W,X}}{\left(1 - \frac{M_{X,\exp}}{\left(M_{X,\exp} + M_{wh}\right)}\right)} \qquad [\text{kg} \cdot \text{kg}^{-1}\ \text{DM}] \qquad (10)$$

Note that the remaining amount of wheat dry matter cannot be measured directly during the fermentation, and the predicted overall water content agrees well with measured values.

The calculated extracellular water content ($X_{W,wh,exp}$) has a large error, mainly due to the large measurement error (14.9%) for biomass (Table 2). The average deviation between the calculated value for $X_{W,wh,exp}$ and the model prediction for $X_{W,wh}$ between the 30- and 80-h cultivation time was 0.05 kg/kg DM.

To determine the suitability of the model for $X_{W,wh}$ control, the effect of this deviation on the growth rate was examined. For this purpose, a relation between colony growth rate and water activity, as determined by Gibson *et al.* (1994) was combined with a relation between water activity and moisture content (desorption isotherm) for

moisturized wheat grains (Nagel *et al.*, 2001), to give a relation between growth rate and moisture content:

$$u_{col} = \exp\left(-0.3915 + 5.571\sqrt{1-a_w} - 25.16\left(1-a_w\right)\right)$$

$$a_w = -2.917 + \frac{3.919}{\left(1+\left(X_{w,wh}/0.0344\right)^{-1.861}\right)} \quad [-] \qquad (11)$$

in which

u_{col} = colony growth rate of *Aspergillus oryzae* FRR1675 [mm·h^{-1}]

$X_{w,wh}$ = water content wheat grains [kg·kg DM^{-1}]

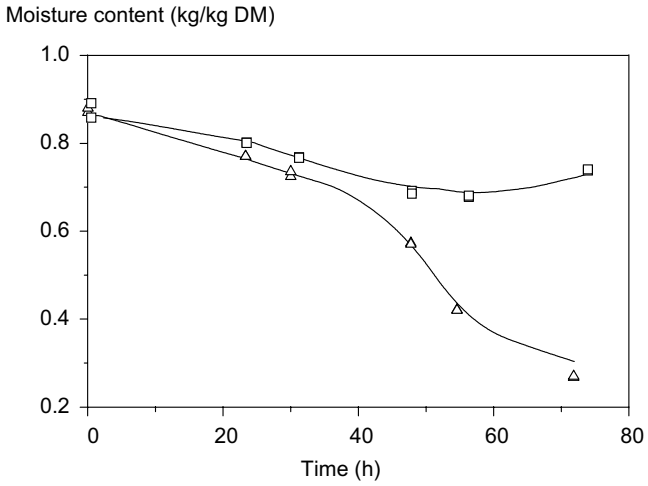


Figure 8. Model validation using two experiments in a 35-L mixed bioreactor with *Aspergillus oryzae*. Solid lines represent model predictions. Symbols represent duplicate measurements of the overall moisture content of wheat grains for an experiment with evaporative cooling (△) and wall cooling (□).

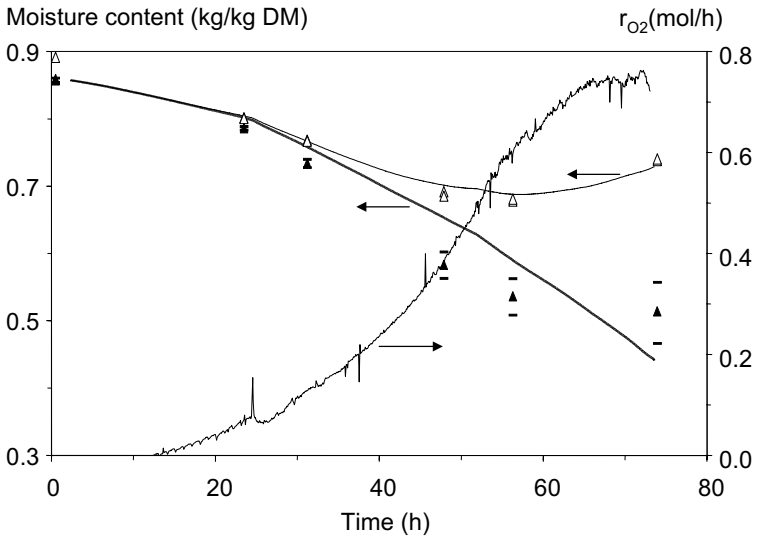


Figure 9. Model predictions of the overall water content (——) and extracellular water content (▲) and measurements of oxygen-consumption rate and overall moisture content $X_{w,ov}$ (△) are given for the cultivation in a horizontal paddle mixer in which wall cooling is applied. The extracellular water content $X_{W,wh}$ (▲) is calculated from Eq. (10). Error bars indicate absolute measurement errors.

Figure 10 shows that when $X_{W,wh}$ is controlled, for example, at 0.8 kg/kg DM, a very broad control range can be applied before the colony growth rate is affected by > 5%. The effect of a decreasing $X_{W,wh}$ on the growth rate can also be seen in Figure 9, as the oxygen-consumption rate leveled off when $X_{W,wh}$ fell below 0.5 kg/kg DM. The measured water activity at that moment was 0.965, which corresponds to 87% of the maximum colony growth rate. Although many assumptions are made when reasoning in this manner, we believe that the model is accurate enough to maintain a sufficiently high growth rate of *A. oryzae* on wheat by controlling $X_{W,wh}$. As mentioned earlier, water evaporation is a measured quantity in the model and, in these cultivations, it is the most important term in the water balance. To demonstrate the relative significance of the various terms in the water and dry-weight balance, a model prediction of absolute quantities is given for the cultivation in which wall cooling is applied (Figure 11). Evaporative cooling could not be prevented in this cultivation because water

condensation and subsequent clogging of the sterile filter had to be avoided. The pressure drop over the sterile air filter therefore limited the inlet air humidification. However, wall cooling prevailed and only about 36% of the total heat production was removed by evaporative cooling. Nevertheless, the total amount of evaporated water was estimated to be 1.5 kg compared to 3.7 kg water initially present, and was therefore the most important term in the water balance. The amount of evaporated water becomes even more important when mainly evaporative cooling is applied in large-scale bioreactors where the capacity of wall cooling is limited (Nagel *et al.*, 2001). The second most important term in the water balance is the amount of intracellular water in biomass (1.2 kg) followed by the metabolic-water production (0.5 kg). Metabolic-water production cannot be neglected (Figure 11) as was done by Oriol *et al.* (1988) and is not balanced by water needed for starch hydrolysis, as was assumed by Narahara *et al.* (1984) in his description of the water balance. The final amount of dry-substrate loss was 1.5 kg compared to the 4.3 kg initially present. Thus, for the prediction of moisture contents, the dry-weight balance is as important as the water balance.

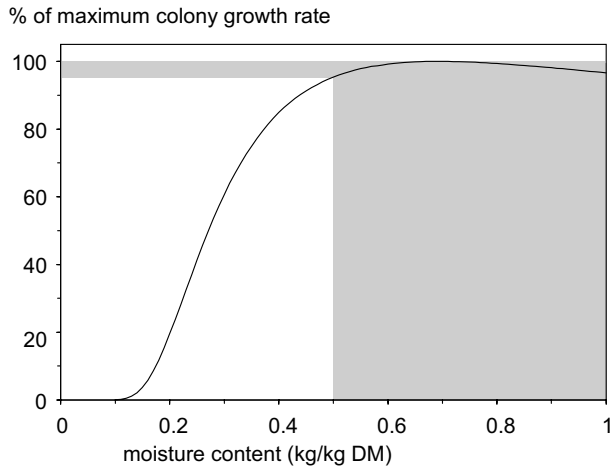


Figure 10. A desorption isotherm for wheat grains is combined with colony-growth-rate data as a function of water activity for *Aspergillus oryzae* to give a relation between colony-growth rate and moisture contents [Eq. (11)]. The gray area represents a suitable control range for the extracellular water content.

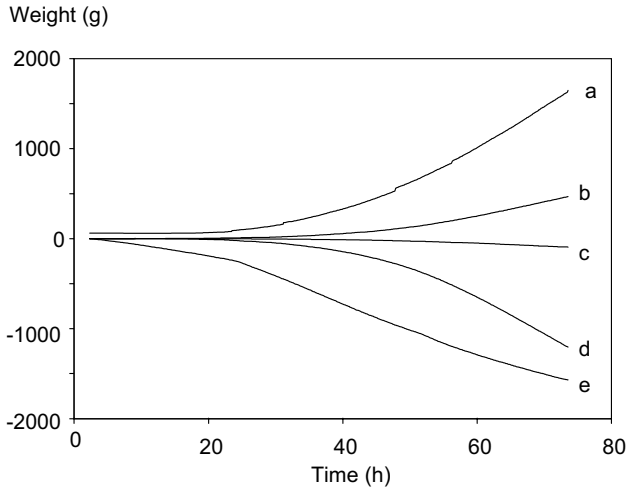


Figure 11. Model prediction of the absolute values of the different terms in the water- and dry-weight balance during an experiment in the 35-L mixed bioreactor in which wall cooling is applied. Curve *a* = dry-weight loss; curve *b* = Metabolic-water production; curve *c* = water for hydrolysis of starch; curve *d* = water in biomass; curve *e* = water evaporated.

Moisture-content control or water-activity control?

Water-activity control is necessary to maintain a sufficiently high growth rate during solid-state fermentation. Direct control of the substrate water activity is difficult as on-line monitoring in the substrate bed remains complicated, especially in a mixed bioreactor. The water activity of the solid substrate can decrease during SSF as a result of two processes: (1) dehydration of the solid substrate; and (2) accumulation of solutes in the substrate (glucose, amino acids etc.). In this article, a model has been developed and validated that can be used to control the extracellular water content, which is what accounts for the dehydration effect. However, the question remains whether it is valid to neglect accumulation of solutes.

We estimated the effect of the free glucose concentrations measured in the wheat grains to get an answer to this question. Free glucose concentrations were measured during all experiments mentioned in this study. The maximum free glucose weight fraction of fermented wheat grains found in the scraped drum reactor and paddle mixer varied between 0.12 and 0.14 kg/kg total DM. Assuming an extracellular water content

equal to 1 kg/kg substrate and a biomass dry-weight content of 0.3 kg/kg total DM (average SDR experiments B and C, Table 2), the glucose concentration can be estimated to be 170 to 200 g/L. According to the data of Bonner (1965), the water activity of a solution with 200 g/L glucose is 0.98. This water activity corresponds to 97% of the maximum colony growth rate for *A. oryzae*, according to data of Gibson *et al.* (1996). For this specific application the free glucose concentration does not seem to limit growth.

In SSF processes, we expect to find more cases in which there is not much free glucose accumulation, and in these cases control of the extracellular water content should keep water activity between acceptable limits. Even when more free glucose accumulates, this will not be important if the organism grows well over a relatively broad range of water activity values (*e.g.* 0.96 to 1.0). However, this does not negate the possibility that glucose, in some cases, accumulates to levels that affect growth significantly. We expect to see this in cases where glucose concentrations reach values of 100 to 200 g/L in the extracellular water, and where the growth of the organism falls significantly below the optimum as the water activity falls to values around 0.98. The proposed model, which aims at control of the extracellular (nonfungal) water content instead of the water activity, is a first step towards improved moisture control in SSF.

However, it may be insufficient; for example, when a microorganism with high amylolytic activity and poor a_w-tolerance has to be cultivated. The model would then have to be extended with hydrolysis and uptake kinetics in order to obtain predictions of the water activity. We expect that intra-particle transport of hyphae, enzymes, and sugars will also have to be taken into account, as this may cause strong glucose concentration gradients in substrate particles (Nagel *et al.*, unpublished). Extension of the model with these elements presents an interesting challenge for future work.

CONCLUSIONS

A model was developed for the prediction of the moisture content of wheat grains during SSF in mixed bioreactors, based on on-line measurements. The model is based on a complete water balance and elemental balances. Parameters for this model were determined from independent experiments using a membrane model system, which mimics the growth of *Aspergillus oryzae* on wheat grains as used in mixed bioreactors. Experiments in 1.5-L and a 35-L mixed bioreactors were used to validate the model. The model was able to predict the experimental moisture contents very well. We expect that the model can be used for automatic moisture-content control of the solid substrate in order to maintain a sufficiently high water activity during SSF in mixed bioreactors.

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