Model for On-Line Moisture-Content Control During Solid-State Fermentation

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Abstract: 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. *Biotechnol Bioeng* **72**: 231–243, 2001.
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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.

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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 (Nagel et al., 2000; Sargantanis, 1993). 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., 2000). 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., 2000).

Process design and control for SSF must take into account the availability of water, as was clearly demonstrated in several previous studies (Larroche and Gros, 1992; Larroche et al., 1992, Nagel et al., 2000; Oriol et al., 1988). Besides the evaporation needed for cooling, an important reason is the incorporation of water into new microbial cells. Oriol and coworkers (1992) 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 (Larroche et al., 1992; Oriol et al., 1988). 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 *P. 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., 2000) 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 allow separate analyses 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., 2000). 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., 2000). 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 has therefore been developed that can be used for automatic moisture-content control during SSF. The model uses on-line measurements of O_2, CO_2, and dewpoint in the effluent air to predict extracellular moisture content and overall moisture content of the grains (Fig. 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 *A. oryzae* on glucose and wheat protein is:

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

in which

Y_{S/O_2}	= yield coefficient for substrate (S) on oxygen	[Cmol · mol^{-1}O$_2$]
Y_{N/O_2}	= yield coefficient for protein (N) on oxygen	[Cmol · mol^{-1}O$_2$]
Y_{X/O_2}	= yield coefficient for biomass (X) on oxygen	[Cmol · mol^{-1}O$_2$]
Y_{CO_2/O_2}	= yield coefficient for carbon dioxide on oxygen	[mol · mol^{-1}O$_2$]
Y_{W/O_2}	= 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 ($CH_{1.98}O_{0.63}N_{0.26}$)

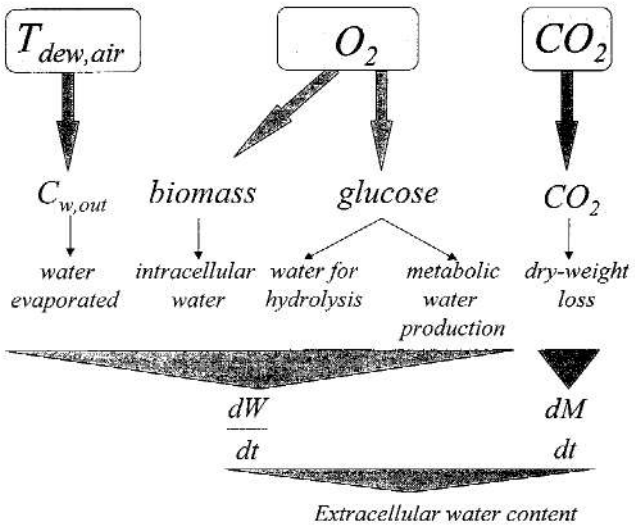

Figure 1. A schematic representation of the model used to estimate the extracellular water content during solid-state fermentation from on-line measurements (oxygen, carbon dioxide, and dewpoint temperature).

was calculated from an average amino-acid composition of five different wheat-grain varieties (Shoup et al., 1966). Production of α-amylase by *Aspergillus oryzae* was neglected because the ratio between α-amylase and biomass was reported to be only 0.019 Cmol/Cmol (Agger et al., 1998).

The water balance has four contributions (Fig. 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 [Eq. (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:

$$\frac{dW_{wh}}{dt} = F_{air} \cdot (C_{W_{in}} - C_{W_{out}}) + X_{W,X} \cdot Y_{X/O_2} \cdot r_{O_2} \cdot Mw_X - Y_{W/O_2} \cdot r_{O_2} \cdot Mw_W + Y_{hyd} \cdot Y_{S/O_2} \cdot r_{O_2} \quad (\text{kg s}^{-1}) \tag{2}$$

in which

W_{wh}	= weight extracellular water in wheat		[kg]
r_{O_2}	= 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} dry weight]
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 starch	water needed to hydrolyze	[kg ·Cmol^{-1} S]

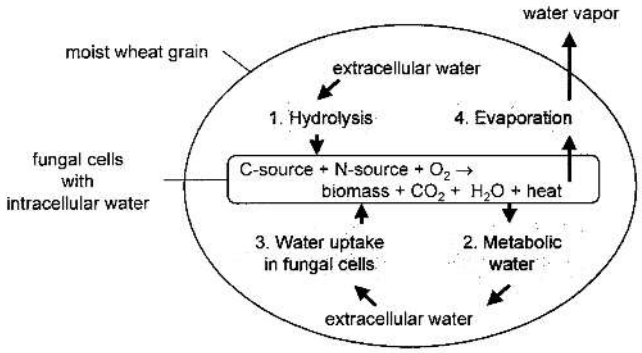

Figure 2. A schematic overview of the four different contributions in the water balance during solid-state fermentation on wheat grains. In order of increasing magnitude: (1) water for starch hydrolysis; (2) metabolic-water production; (3) water uptake in new biomass; and (4) water evaporation.

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

$$\frac{dM_{wh}}{dt} = -\left(\frac{Y_{S/O_2}}{Y_{CO_2/O_2}} \cdot Mw_S + \frac{Y_{N/O_2}}{Y_{CO_2/O_2}} \cdot Mw_N\right) \cdot r_{CO_2} \quad (\text{kg s}^{-1}) \tag{3}$$

in which

M_{wh}	= weight of wheat dry matter		[kg]
Mw_N	= 27.63 · 10^{-3}	molecular weight protein	[kg · Cmol^{-1} N]
Mw_S	= 30 · 10^{-3}	molecular weight starch	[kg · Cmol^{-1} S]
r_{CO_2}	= carbon dioxide production rate		[mol s^{-1}]

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

$$X_{W,wh}(t) = \frac{W_{wh}(t)}{M_{wh}(t)} \quad (\text{kg kg}^{-1}\ \text{DM}) \tag{4}$$

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:

$$\frac{dM_X}{dt} = -Y_{X/O_2} \cdot r_{O_2} \cdot Mw_X \quad (\text{kg s}^{-1}) \tag{5}$$

$$W_X(t) = X_{W,X} \cdot M_X(t) \quad (\text{kg}) \tag{6}$$

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:

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

MATERIALS AND METHODS

Microorganism 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., 2000). 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 of 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., 2000). 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) 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). Milled wheat grains and distilled water were mixed to obtain the desired moisture content of the wheat dough. At moisture contents of >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 (Fig. 3) were manufactured by the Mechanical Workshop at Wageningen University. The wheat dough was transferred into a sausage maker (Fig. 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 (Fig. 3a). A sterile 0.45-μm polyamide membrane (Schleicher & Schuell) 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, ϕ = 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

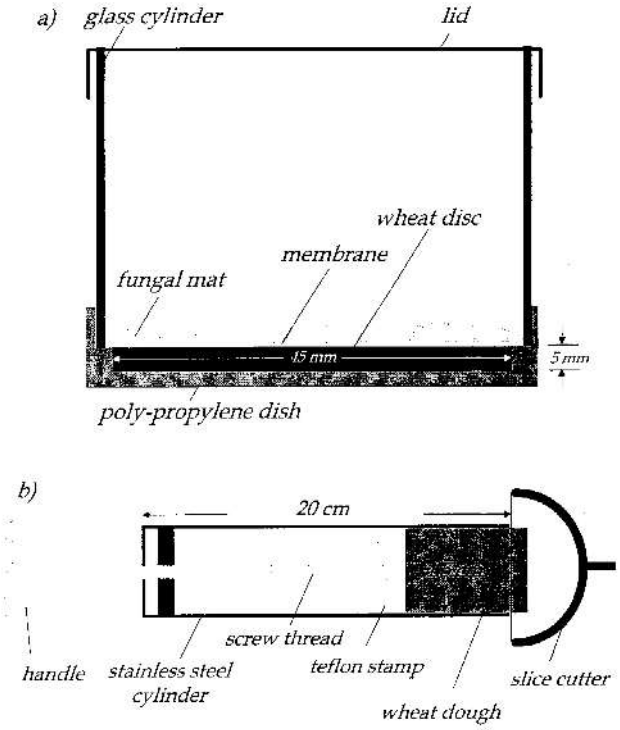

Figure 3. (a) Membrane model system for solid-state fermentation on wheat grains used to measure the moisture content of biomass and all other model parameters. (b) Apparatus used to aseptically prepare wheat disks for the membrane model system.

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). 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). The effluent air was first analyzed for dewpoint (Dewmet SD, Michell Instruments, Ltd.), 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 (25 L) 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.

Scraped Drum Bioreactor (1.5 L)

The scraped drum reactor (SDR; length 20 cm, diameter 5 cm; Fig. 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,

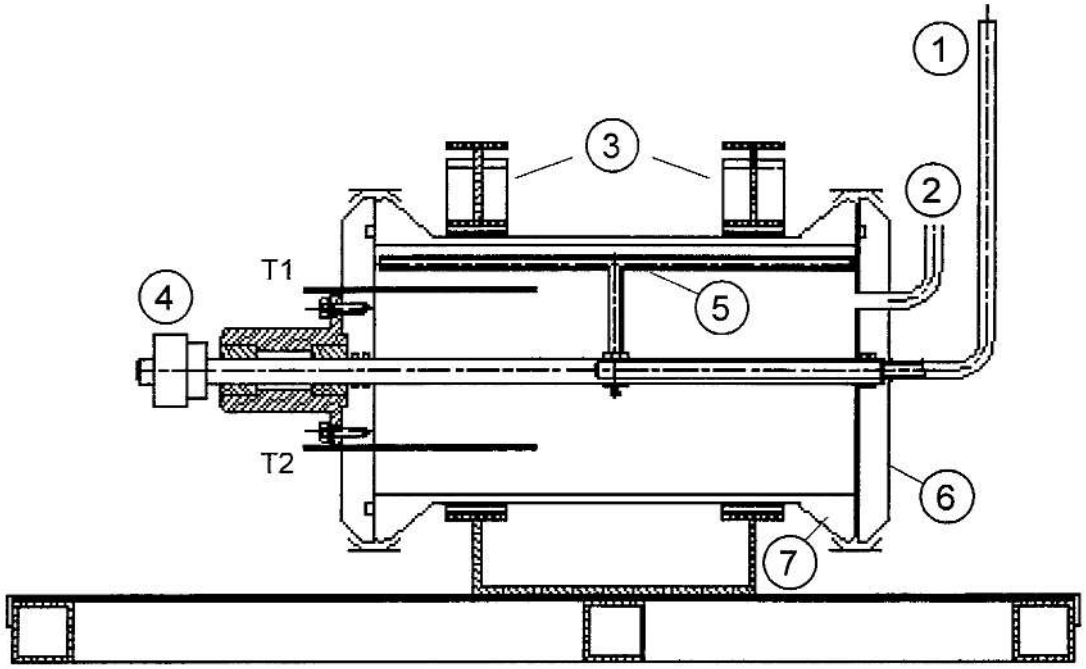

Figure 4. Schematic diagram of 1.5-L scraped drum reactor: 1 — air inlet; 2 — air outlet; 3 — reactor fixings; 4 — drive shaft; 5 — scraper with fine holes for air distribution; 6 — steel side plate; 7 — glass cylinder. T1 and T2 are temperature sensors.

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) and mixed continuously (1 rpm). The air-conditioning system for these experiments was the same as described earlier.

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 has been given elsewhere (Nagel et al., 2000). *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 per 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 hundred grams of inoculated wheat grain 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 g of wheat grain was transferred into a sample bottle and stored (−20°C) for further analysis of glucosamine and glucose.

Analysis

Moisture Content and 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) 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, Silverson). For determination of total glucose, 0.1 g of this homogenate was added to 4.5 mL of 13 m*M* 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 & Schuell), after which total glucose was determined.

Three milliliters of homogenate was divided into two Eppendorf tubes and centrifuged at 15,300 rpm for 20 min (Model GS-15R centrifuge, Beckman Instruments), after which the supernatant was used to determine free glucose. Glucose was determined using a Peridochrom Glucose Kit (Cat. No. 676543, Boehringer Mannheim).

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 glucosamine measurement has been described elsewhere (Nagel et al., 2000). 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

O_2 and CO_2 were analyzed using a paramagnetic O_2 analyzer (Xentra 4100, Servomex Zoetermeer) 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 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 [Eq. (1)] was used together with three measured yield coefficients ($Y_{X/O2}$, $Y_{S/O2}$, $Y_{CO2/O2}$; Table I) 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 [Eq. (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 I). 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)-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 I.

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).

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

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

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

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 (Fig. 3) was developed that mimics the growth of *A. 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 disks 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).

Figure 6 shows that the moisture content of the substrate ($X_{W,wh}$) influences $X_{W,X}$, especially at moisture contents of >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

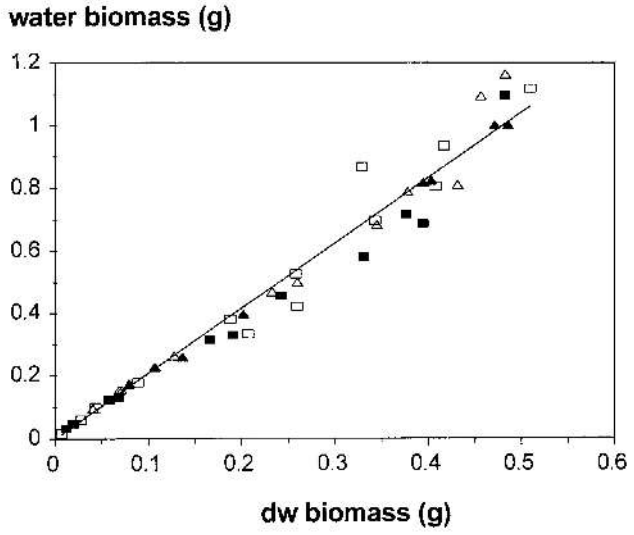

Figure 5. The amount of intracellular water in biomass of *Aspergillus oryzae* plotted against biomass dry weight for four separate experiments ($X_{W,wh}$ = 1 kg/kg DM) (▲, △, □, ■) with the membrane model system. The moisture content of fresh biomass was estimated from the straight line (—), which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

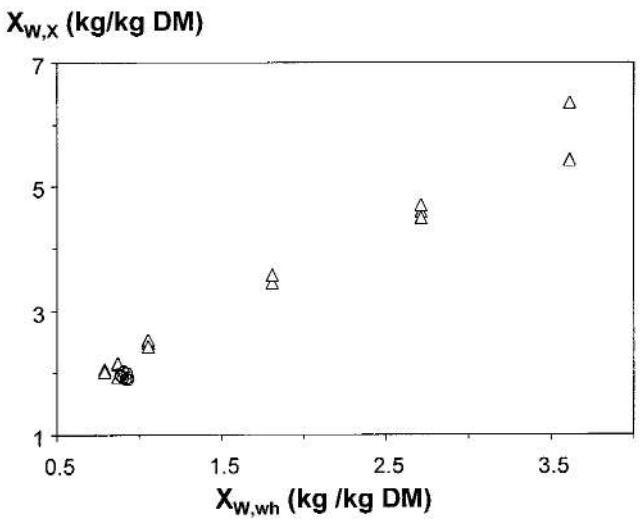

Figure 6. Intracellular moisture content of *Aspergillus oryzae* biomass ($X_{W,X}$) as a function of the moisture content of wheat dough ($X_{W,wh}$) after a 60-h cultivation time, measured using a membrane model system. Samples of all four experiments from Figure 5 at the 60-h cultivation time are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

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 disks 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 was 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.

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 Eq. (3). For on-line dry substrate esti-

mation, yield coefficients (Table I) were recalculated based on CO_2:

$$Y_{S/CO_2} = Y_{S/O_2}/Y_{CO_2/O_2} = 1.38 \text{ Cmol/mol}$$
$$Y_{N/CO_2} = Y_{N/O_2}/Y_{CO_2/O_2} = 0.72 \text{ Cmol/mol}$$

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 Eq. (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 [Eq. (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.

$$f_{DM_{loss}} = Y_{S/CO_2} \cdot Mw_S + Y_{N/CO_2} \cdot Mw_N - Y_{X/CO_2} \cdot Mw_X \quad (\text{g mol}^{-1}\ CO_2) \qquad (8)$$

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 have been reported in the 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 (Arima and Uozumi, 1967; Sakurai et al., 1977). 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 (Fig. 7). The observed

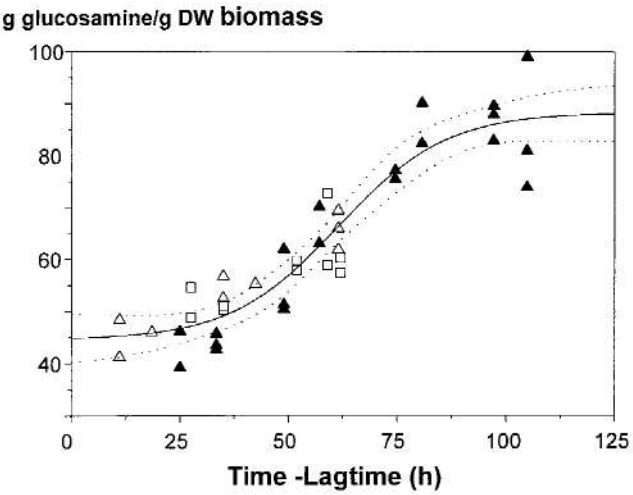

Figure 7. Glucosamine content of dry-weight biomass as a function of the difference between cultivation and lag time (time − lag time) in order to estimate biomass from experiments involving whole wheat grains. The different symbols represent three separate experiments. The straight line is the sigmoid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

increase is best described by a sigmoidal curve fitted through the data:

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

in which

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

where $G_X$is the glucosamine content of biomass (mg g^{-1} DM), t is the time (h), and l is the 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., 2000). 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 study 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 quantity fermented grains.

Scraped Drum Reactor (1.5 L)

The model was validated with four cultivations in a 1.5-L scraped drum reactor (Table II). 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 II) 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 II). $X_{W,ov}$ 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 II), 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 h 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

Table II. Validation of a model for the prediction 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	[c]
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. CO_2 [g]	9.18	[c]	21.80	[c]	18.41	[c]	6.39	[c]
17. O_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 column), 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)].

content equal to 6.3%. Significant deviations were observed between model predictions and experimental data for biomass and consumed glucose (Table II). 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) was hampered by a large 95% confidence interval (Fig. 7), and uncertainty in the lag-time correction, which may lead to a large error in the biomass determination.

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., 2000). 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 (Fig. 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 these values ($X_{W,wh,\mathrm{exp}}$) from biomass measurements ($M_{X,\mathrm{exp}}$), predicted values of the overall water content ($X_{W,ov}$) and the remaining amount of wheat dry matter (M_{wh}):

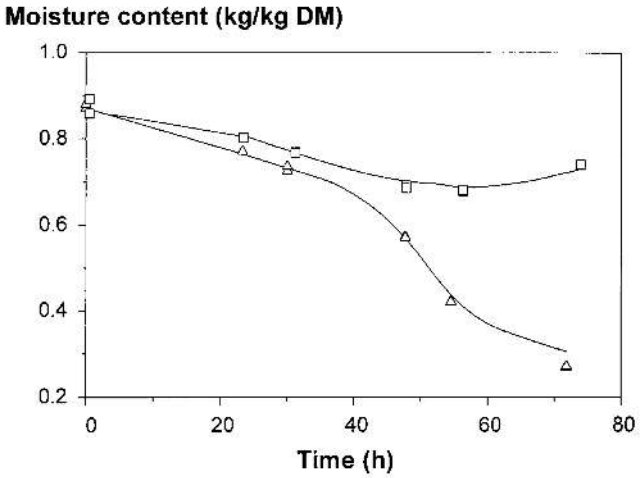

Figure 8. Model validation using two experiments in a 35-L mixed bioreactor with *Aspergillus oryzae*. Solid lines represent model predictions. Symbols represent duplicate measurements of the overall moisture content of wheat grains for an experiment with evaporative cooling (△) and wall cooling (□).

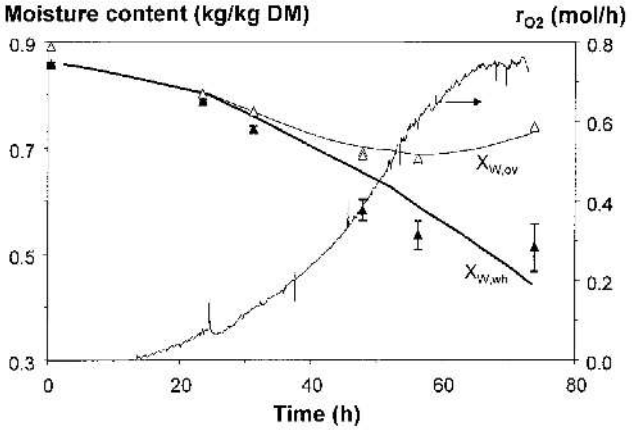

Figure 9. Model predictions of the overall water content (—) and extracellular water content (■) and measurements of oxygen-consumption rate and overall moisture content, $X_{W,ov}$ (△) are given for the cultivation in a horizontal paddle mixer in which wall cooling is applied. The extracellular water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

$$X_{W,wh,\mathrm{exp}} \frac{X_{W,ov} - \dfrac{M_{X,\mathrm{exp}}}{(M_{X,\mathrm{exp}} + M_{wh})} \cdot X_{W,X}}{\left(1 - \dfrac{M_{X,\mathrm{exp}}}{(M_{X,\mathrm{exp}} + M_{wh})}\right)} \quad (\mathrm{kg\ kg^{-1}\ DM}) \tag{10}$$

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 ($X_{W,wh,\mathrm{exp}}$) has a large error, mainly due to the large measurement error (14.9%) for biomass (Table II). The average deviation between the calculated value for $X_{W,wh,\mathrm{exp}}$ 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., 2000) to give a relation between growth rate and moisture content:

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

$$a_w = -2.917 + \frac{3.919}{(1 + (X_{w,wh}/0.0344)^{-1.861})} \tag{11}$$

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 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 can be 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 (Fig. 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 with the 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., 2000). 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 (Fig. 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 loss of dry substrate was 1.5 kg compared with the 4.3 kg initially present. Thus, for the prediction of moisture contents, the dry-weight balance is as important as the water balance.

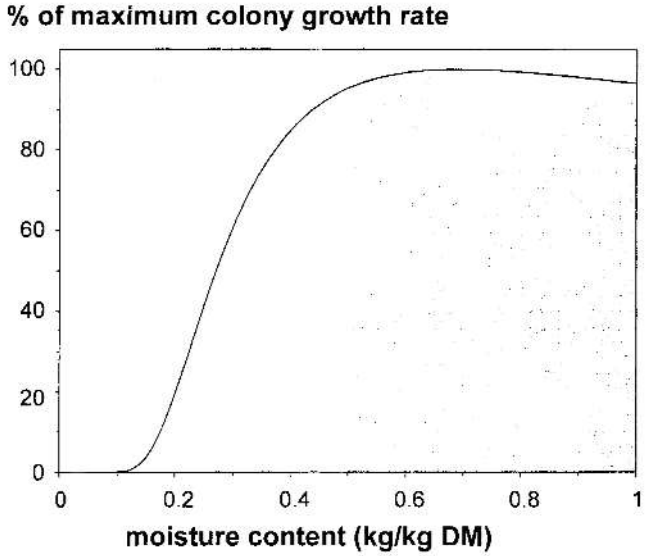

Figure 10. A desorption isotherm for wheat grains is combined with colony-growth-rate data as a function of water activity for *Aspergillus oryzae* to give a relation between colony-growth rate and moisture contents [Eq. (11)]. The gray area represents a suitable control range for the extracellular water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

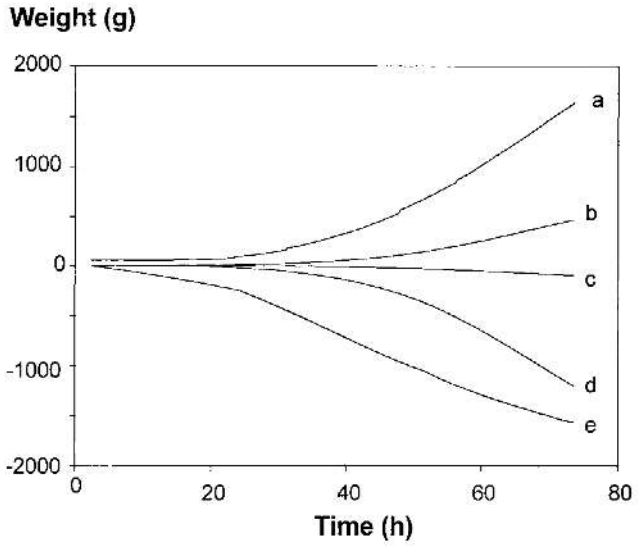

Figure 11. Model prediction of the absolute values of the different terms in the water- and dry-weight balance during an experiment in the 35-L mixed bioreactor in which wall cooling is applied. Curve a = dry-weight loss; curve *b* = Metabolic-water production; curve *c* = water for hydrolysis of starch; 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 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 obtain an answer to this question. Free glucose concentrations were measured dur-

ing 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 in Table II), the glucose concentration can be estimated to be 170 to 200 g/L. According to the data of Bonner and Breazeale (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 *Aspergillus oryzae*, according to data of Gibson et al. (1994). 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 toward 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 intraparticle 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 has been 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- and 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 sufficiently high water activity during SSF in mixed bioreactors.

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