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Highlights

- A dry weight model for fungal growth estimation in solid state has been developed.
- Maximum biomass and specific growth rate of *A. awamori* in wheat was calculated.
- Capacitance was tested as online measurement of growth and to validate the model.
- A metabolic model was also explored and used to validate the dry weight model.

Dry weight model, capacitance and metabolic data as indicators of fungal biomass growth in solid state fermentation

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Abstract

Developing improved industrial bioprocesses has been a driver for the growing research attention to solid state fermentation, in particular involving filamentous fungi. Accurate description of fungal growth in these systems is crucial and certainly needed to enable optimal deployment of subsequent engineering work. This manuscript proposes a model based on total dry weight measurement to describe biomass growth for *Aspergillus Awamori* on wheat grains in two systems: petri dishes and a 1L packed bed bioreactor. The proposed dry weight model can be used not only for identifying growth phases of the fungus but also to calculate key growth parameters such as specific growth rate and maximum biomass concentration. The use of techniques based on capacitance measurements and on metabolic data were also used in order to estimate fungal growth and to validate the proposed model.

Keywords: Solid state fermentation, growth model, *Aspergillus Awamori,* capacitance, metabolic model.

1 Introduction

Solid State Fermentation (SSF) is a biochemical process for culturing microorganisms in solid materials, creating an environment having free water amounts ranging from very low to absent [1]. In specific cases, this low water content confers to SSF systems most of the advantages identified over the last 20 years such as higher product yield and substrate concentration, less energy input and wastewater emission as well as a lower risk of contamination [2]. Better growth, morphological diversity and differentiation are characteristics of SSF systems which provide an environment for growth of microorganisms similar to their natural environment [3]. SSF of particulate substrates mimics the natural environments in soil, foods and others situations where many filamentous fungi thrive, e.g. ascomycetes, basidiomycetes and deuteromycetes [3]. Fungal enzymes, metabolites and other materials have naturally evolved over the eons to work well in moist solid substrates rather than in liquids as in submerged ~~liquid state~~ fermentation (SmF~~LSF~~). Therefore, cultivation of particular microbial species in SSF rather than SmF~~LSF~~ could lead to improved metabolic efficiencies and to higher productivities [4].

In an attempt to elucidate the mechanisms behind the differences between SSF and ~~LSF~~SmF, Biesebeke and collaborators [5] demonstrated that different genes and proteins are transcribed and expressed, respectively, when growing *Aspergillus oryzae* on wheat kernels in SSF and ~~LSF~~SmF. From their physiological studies, it can be concluded that carbon transport and oxygen uptake are mainly performed by aerial hyphae cells, which are uniquely present in SSF. The low free water content and

aerial mycelia empower the fungus with the ability to capture oxygen directly from air by diffusion, so low pressure is involved and therefore, less energy input is required for aeration [6]. Other studies focusing on comparisons between SSF and SmF~~L~~SF systems have examined the production of specific enzymes [7-9], secondary metabolites [10, 11] and/or spores [12].

An accurate description of fungal growth during SSF is crucial to enable the production of information on a variety of engineering aspects. These range from nutrient consumption to heat transfer and from the production of CO_2 to valuable growth dependent products. However, measuring fungal biomass in SSF remains extremely challenging due to its complexity, heterogeneity and the intimate interaction between the microorganism and the substrate, making their separation impractical. These are limitations that render online measurement of fungal biomass concentration impossible and make even offline measurement problematic.

There is a plethora of indirect methods to estimate microbial biomass concentration, in SSF, based on correlations with the concentration of certain cellular biomolecules such as glucosamine, chitin, ergosterol, protein, or nucleic acids [13-15]. A recent review of these methods showing their limitations has been carried out by Rodriguez-Leon et.al. [16]. They noticed as a drawback the large variability in correlations between these cellular biomolecules and the weight of the mycelium due to age, morphology and the environment in which the fungus grows. Other limitations include lengthy analytical procedures, laborious sample preparations and possible interference from substrate components with the assays, to cite just a few [17].

Despite the lack of direct methods to measure fungal biomass in SSF, many quantitative models describing growth on solid media exist. Nopharatana and collaborators [18] modelled fungal growth in time and space at the substrate surface for a simple system mimicking SSF. Ferret et al. [19] developed a microscopic model taking into account steric interactions between hyphae and hyphal tips. Laszlo and Silman [20] studied the use of cellular automata based on empirically derived rules of logic as an alternative method of modelling fungal growth. More recently, Wang and collaborators [21] reviewed the roles of porous characteristics in fungal growth and solid matrix degradation in SSF. There are other types of model that have been reviewed by Mitchell and co-workers [22]. However, until now, these models were developed using artificial solid matrices unrepresentative of natural substrates. In addition, these models rely on indirect measurements and are impossible to validate due to their dependence on specific analyte biomolecules and the difficulty in separating the fungus from the substrate.

In this manuscript a model based on simple dry weight of fungi growing in natural substrates is proposed for predicting fungal biomass concentration. The use of a technique based on capacitance measurements to assess cell biomass was also explored. Finally, metabolic data were also used in order to estimate fungal growth and to validate the proposed model.

2 Theory and Mathematical modelling

2.1 Dry weight model for estimation of fungal growth

Herein is presented a novel indirect modelling method based on dry weight measurements to describe the growth of *Aspergillus awamori* on whole wheat grains during solid state fermentation. The method includes a mathematical model which involves a simple mass balance and a Luedeking–Piret based model to describe product formation kinetics. Using a numerical method to solve the equation, a relationship between biomass and the change in dry weight was obtained. The description of the model, the assumptions considered and the parameters used are described below.

In the SSF bioreactor, the total dry weight of solids can be expressed as:

$$W = I + S + X + P \qquad \textbf{Equation 1}$$

Where,

W is the dry weight of total solids in the fermentation medium, (g)
I is the dry weight of inert components of the solid matrix, (g)
S is the dry weight of substrate, (g)
X is the dry weight of cell biomass, (g)
P is the dry weight of the products, (g)

Since the inert components of the solid matrix do not change during fermentation, the overall rate of change in *W* is therefore the sum of the rates of change for biomass, substrate and products, *i.e*.

$$\frac{dW}{dt} = \frac{dS}{dt} + \frac{dX}{dt} + \frac{dP}{dt} \qquad \textbf{Equation 2}$$

Assuming constant biomass growth yields, the rate of substrate consumption can be related to growth rate and the rate of product formation by the following equation:

$$\frac{dS}{dt} = -\frac{1}{Y_{XS}}\frac{dX}{dt} - m_s X - \frac{1}{Y_{PS}}\frac{dP}{dt} \qquad \textbf{Equation 3}$$

Where,

Y_{XS} is the true fungal growth yield related to substrate (g biomass g^{-1} substrate)
m_s is the substrate maintenance coefficient (g substrate g^{-1} biomass h^{-1})

Y_{PS} is the yield of product formation related to substrate (g product g^{-1} substrate)

Assuming the rate of product formation depends on biomass concentration, the Luedeking and Piret model [23] can be applied to describe product formation kinetics by combining two types of kinetics: specific growth rate (growth-associated) and maintenance related activity (non growth-associated):

$$\frac{dP}{dt} = Y_{PX}\frac{dX}{dt} + m_P X \qquad \textit{Equation 4}$$

Where,

Y_{PX} is the yield of product formation related to fungal growth (g product g^{-1} biomass)

m_P is the product maintenance coefficient, (g product g^{-1} biomass h^{-1})

Combining Equations 2, 3 and 4, the change in dry weight can then be written in terms of biomass using the following equation:

$$\frac{dW}{dt} = \alpha\frac{dX}{dt} + \beta X \qquad \textit{Equation 5}$$

Where, α is a parameter related to the various yield coefficients:

$$\alpha = 1 - \frac{1}{Y_{XS}} + Y_{PX}\left(1 - \frac{1}{Y_{PS}}\right) \qquad \textit{Equation 6}$$

β is a parameter related to the maintenance coefficients and the yield of product formation:

$$\beta = m_P\left(1 - \frac{1}{Y_{PS}}\right) - m_s \qquad \textit{Equation 7}$$

Equation 5 can be rearranged to give:

$$\frac{1}{\alpha}\frac{dW}{dt} = \frac{\alpha}{\beta}\frac{dX}{dt} + X \qquad \textit{Equation 8}$$

Equation 8 can be integrated by using an integration factor, $e^{\frac{\beta}{\alpha}t}$, to obtain the following expression:

$$X = X_0 e^{-\frac{\beta}{\alpha}t} + \frac{1}{\alpha} e^{-\frac{\beta}{\alpha}t} \int e^{\frac{\beta}{\alpha}t} \frac{dW}{dt} dt \qquad \textit{Equation 9}$$

Equation 9 can be used for describing fungal biomass, X, after calculating parameters α and β as follows.

a) **Correction of dry weight**: in the two SSF systems carried out during this project, the two main fermentation products leaving the bioreaction systems were CO_2 and glucose, which was extracted for purposes beyond the scope of this work. This extraction implies that the measured dry weight had to be corrected by adding the mass of glucose, resulting in corrected values of *W*. Given this correction, CO_2 was then assumed to be the only product leaving the reactor and therefore the only product taken into account in the model. Given this, Y_{PS} is YCO_2s.

b) **Determination of parameters α and β:** both were calculated from values of yields and maintenance taken from the literature, using Equations 6 & 7. No values were available for the exact system being studied (ie: *Asperillus awamori* and wheat grains) so values were selected for systems that most closely resembled it. Yields were taken from data, of *Aspergillus oryzae* growing on wheat grains, reported by Nagel et al. [24]. They determined the yields in independent experiments by applying a membrane model (Y_{XS} = 0.51 g biomass g^{-1} substrate, YCO_2s = 0.72 g CO_2 g^{-1} substrate). The maintenance coefficients were taken from the estimated values reported by Lareo and collaborators [25, 26] for *Mucor bacilliformis* on an inert solid support (mCO_2 = 0.007 g CO_2 g^{-1} biomass h^{-1}, m_s = 0.0013 g substrate g^{-1} biomass h^{-1}).

c) **Calculation of *X*:** equation 9 was solved graphically in a software (OriginPro 7.0), for which the time course of fungal biomass accumulation was calculated for the reactor systems studied during this project: Petri dishes and bed bioreactor with intermittent water trickling.

The biomass values, *X*, obtained using Equation 9 were fitted using a logistic equation (Equation 10), which is a model frequently used to describe the kinetics of fungal growth in solid bioprocessing systems [25, 27-31]. This model does not give insight into what controls growth, a topic beyond the scope of this work. However, equation 10 advantageously contains only one term that adequately approximates the whole growth cycle, including the lag phase and, growth cessation in the final stages of fermentation [22]. Equation 10 conceptualizes SSF as a process limited by available surface area for contact since the rate of biomass growth depends on maximal biomass (X_M). An assumption in this equation is that during the initial exponential growth phase the specific growth rate (μ) is independent of substrate concentration [32].

$$X = \frac{X_M}{1 + \left(\frac{X_M}{X_0} - 1\right) e^{-\mu t}}$$ ***Equation 10***

Where ,

X is the fungal biomass, (g)

X_M is the maximum possible fungal biomass, (g)

X_0 is the initial biomass, (g)

μ is the specific growth rate (h^{-1})

After fitting the biomass values obtained from the dry weight model to the logistic equation (Equation 10), the values of maximum biomass concentration (C_M) and specific growth rate (μ) were calculated for the different reactor systems (Table 1). To verify the reliability of the proposed dry weight model, the estimated biomass values from the logistic equation were then used to re-calculate the dry weight using Equation 5. Taking into account the assumptions and the paraments used for the development of the model described above, the application of this model is limited to filamentous fungi growing aerobically in solid substrate which weight change and moisture content can be easily measured and which only product leaving the reactor is CO_2.

2.2 Capacitance or dielectric permittivity as a potential measurement of cell biomass in solid state fermentation

Capacitance or dielectric permittivity is a measure of the number of electrons that a system can hold under a given electrical potential. Under this condition, two metallic plates separated by a non-conducting substance between them make a capacitor. By analogy, cells act as small capacitors. When subjected to an electric field between two electrodes, ions migrate toward the electrodes. However, the cell plasma membrane is non-conductive and therefore polarises on both sides as happens in a capacitor [33]. When the electric field ceases, ion relaxation induces capacitance that can be measured and correlated to cell biomass concentration: an increase in cell concentration relates to an increase in the amount of cell plasma membranes, polarization and thus capacitance [34, 35].

In the present study, capacitance measurements were used to estimate fungal growth in solid state at different growth phases, and the results were compared with spore concentrations and the dry weight model presented above.

2.3 Metabolic data for estimation of fungal growth

Another indirect method to estimate fungal growth explored during this project was the use of CO_2 evolution rate (CER). This metabolic measurement can be used to estimate indirectly fungal growth by applying a mathematical model that correlates these two variables [36, 37]. Predominantly, CO_2 production results from two aspects of fungal metabolism: growth and maintenance. Therefore, yield and maintenance coefficients have to be known to predict fungal growth.

Koutinas et al. [26] developed a method for the determination of fungal concentration during liquid fermentation on whole wheat flour based on on-line measurements of CO_2 evolution. The authors calculated initial values for yield (Y_{XCO_2}) and maintenance (m_{CO_2}) coefficients from fermentations on synthetic media and then the values were correlated with estimated ones from fermentations on whole wheat flour, predicting fungal growth.

A similar model to the one described by Koutinas et al [26] was used for the estimation of fungal growth from on-line measurements of CO_2 evolution in SSF. However, in the work reported herein, the values of yield and maintenance used were the same as previously chosen for the dry weight model [25]. The description of this metabolic model and the assumptions considered are described below.

The CO_2 analyser measures the concentration of CO_2 in the gas at the outlet, and the CER is then calculated assuming that CO_2 behaves as an ideal gas:

$$CER(t) = \frac{F\left[\left(A - A_0\right)/100\right]}{BV} MW \qquad \textbf{Equation 11}$$

Where,

CER is the carbon dioxide evolution rate (g L^{-1} h^{-1}),

F is the aeration rate (L h^{-1}),

A is the percentage of CO_2 in the exit gas (on a volume basis),

A_0 is the percentage of CO_2 in the inlet gas (on a volume basis),

MW is the molecular weight of CO_2 (44),

V is the working volume of the bioreactor (1 L)

B is the volume (22.4 L) occupied by 1 mol of CO_2.

Since CO_2 is a metabolic product, the Luedeking–Piret model can be expressed as follows:

$$CER(t) = \alpha' \frac{dX}{dt} + \beta' X \qquad \textbf{Equation 12}$$

Where,α' is a coefficient equalling the inverse yield of cells with respect to CO_2:

$$\alpha' = \frac{1}{Y_{XCO_2}} \qquad \textit{Equation 13}$$

β' is the maintenance coefficient:

$$\beta' = m_{CO_2} \qquad \textit{Equation 14}$$

Equation 10-14 can be regrouped to give:

$$\frac{1}{\alpha'} CER = \frac{\alpha'}{\beta'} \frac{dX}{dt} + X \qquad \textit{Equation 15}$$

Using as integration factor $e^{\frac{\beta'}{\alpha'}t}$, a general solution of Equation 10-15 can be expressed as follows:

$$X = X_0 e^{-\frac{\beta'}{\alpha'}t} + \frac{1}{\alpha} e^{-\frac{\beta'}{\alpha'}t} \int e^{\frac{\beta'}{\alpha'}t} CER(t)\, dt \qquad \textit{Equation 16}$$

Solving Equation 16 numerically using the Trapezoidal rule, the time course for fungal biomass can be calculated.

3 Materials and methods

3.1. Microorganisms and substrates

A. awamori 2B. 361 U2/1 was the strain used in these experiments. Preparation of its spores consisted in purification and subsequent incubation in fermentation flasks, whose solid medium composition consisted of 5% whole wheat flour and 2% agar, both units on w/w and dry basis (db). At the end of the incubation more spores were harvested, suspended in 10% (v/v) glycerol and then stored in 2 ml cryo-vials at -30 °C until needed. These cryo-vials contained 4.05×10^8 spores ml^{-1}, and to be used in these experiments they were thawed in a water bath at 37ºC for 15 min. The suspension was used to inoculate the substrate up to a concentration of 1×10^6 spores g^{-1} of grain solids.

The target substrate in Petri dishes and the bioreactor was a soft variety of Consort wheat grain (Fishers Seeds & Grain Ltd., U.K.). Grains were prepared by pearling the wheat kernels for 5 s in an abrasive mill (model TM05, Satake). Then, the pearled grains were soaked in water for 20 h to attain a desirable

moisture content of 0.8–0.9 g water g^{-1} grain solid. Subsequently, condensed water was eliminated by vacuum filtration and the conditioned grains were autoclave-sterilised at 120 °C for 3h.

3.2 Experimental set up

Results presented in this manuscript were obtained from two different SSF systems: petri dishes and a 1.5 L packed bed bioreactor.

a) Petri dishes: these containers share some similarities with tray reactors and were 10 cm diameter and 1 cm height. The dishes were filled with the inoculated grains, and were placed in an incubation room at 30 °C and allowed to grow statically.

b) Packed bed bioreactor: *A. awamori* was grown in an aerated bioreactor featuring a spray nozzle for water trickling as novelty. The bioreactor was made of a sterilisable glass cylinder having 8 cm diameter and 30 cm height, totalling 1.5 L total volume. Both ends were closed with stainless-steel plates. Two ports in the bottom plate allowed for the introduction of air and the extraction of liquid samples, while venting of exhausts and injection of water proceeded through ports in the top plate. Five temperature probes and four sampling probes were distributed along the bioreactor in a bottom-up sense: thermocouples type K were placed at 5.5, 9.4, 13.8, 18.2 and 21.9 cm. The sampling probes were placed at 6.4, 11.9, 17.4 and 22.9 cm. Temperature was recorded using a Microlab II monitor (Aglicon, UK). Heating was supplied via a jacket made of a silicon tube, wrapped around the bioreactor, recirculating water to a controlled water bath at 30 °C. Oxygen was supplied in the form of filter-sterilised air injected at 1 L min^{-1} through an air distributor fitted to the bioreactor.

Loading involved several tasks. To begin with, wheat grains were sterilised. Subsequently, 800 g thereof having a moisture content of 0.87 g water g^{-1} grain solids were inoculated. This was done by adding 6 ml of spore suspension until attaining a spore concentration of $1x10^6$ spores g^{-1} of grain solids. The bioreactor was sterilized, loaded with these inoculated grains and assembled as previously described by Botella et al. [3].

Gas analysis was performed online. However, it was necessary to reduce the humidity and impurities of exhaust gases, and this was done with a classic silica gel trap fitted with a filter. Then, the cleaned gas was fed directly into an analyser to measure O_2 and CO_2.

3.2 Analytical methods

3.2.1 Moisture in dried solids

This analysis was based on the dry weight of solids from Petri dishes and the bioreactors. Initially, a triplicate of samples was placed in metallic dishes that were previously dried. The metallic dishes were weighed before and after sample addition to give an average of 5 g of wheat grain. Then, the dishes were ready for subsequent sample drying in an oven at 90 °C for 24 hours. Afterwards, the metallic dishes were transferred into a desiccator and rested therein for 15 minutes to cool down. Finally, the dishes were weighed again and equation 17 was employed to calculate moisture in the dried wheat grains. This property is expressed as a mass ratio of water to dried grain solids.

$$m_{(db)} = \frac{w_1 - w_2}{w_2 - w_3} \qquad \textit{Equation 17}$$

where, $m_{(db)}$ is the moisture ratio of wheat grains on dry basis, (g water g^{-1} grain solid)

w_1 is the weight of the dish with the wheat grains before drying, (g)

w_2 is the weight of the dish with the wheat grains after drying, (g)

w_3 is the weight of the dish, (g)

3.2.2 Capacitance in SSF

Capacitance was measured using a Biomass Monitor (Aber Instruments Ltd, UK). The monitor measures capacitance at different frequencies using a probe with four sensors. The probe was submerged inside a beaker with around 20 g of wheat grains and 20 ml of NaCl solution (10 g L^{-1}) which was added to the grains to increase conductivity during the measurements. The low and high operating frequencies chosen were 0.1 and 19.5 MHz respectively. Un-inoculated grains at different moisture contents were used as reference samples (blanks) in order to take into account the capacitance due to moisture.

3.2.3 Metabolic indicators: oxygen and carbon dioxide

Both gases were measured online at the outlet of the bioreactor by a FerMac 368 gas analyser fitted with an infrared detector (Electrolab Ltd, UK).

4 Results and discussion

4.1 Dry weight model results

Figure 1 represents the dry weight measurements obtained during Petri dish fermentations and the estimated fungal growth profile calculated using the dry weight model (Equation 9). Results obtained show a typical growth curve, which was fitted using the logistic equation (Equation 10), with a lag phase during the first 50 h, an exponential growth phase that lasted until 120 h of incubation and a deceleration growth phase. The estimated parameters obtained from the logistic curve (Table 1) were used to re-calculate the total dry weight, obtaining a curve that provides a good approximation of the experimental values except towards the end of the fermentation.

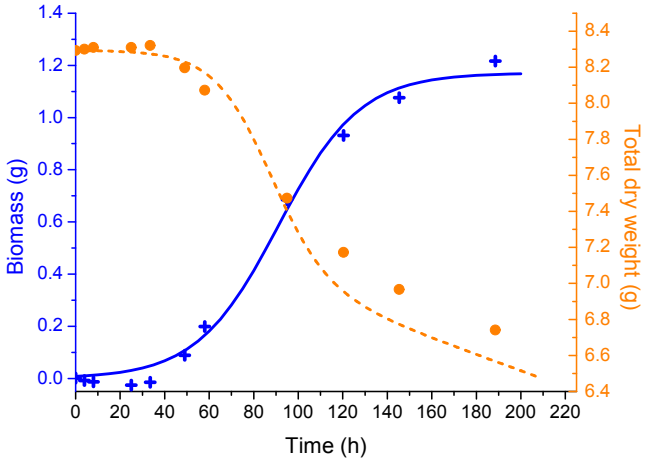


Figure 1 Total dry weight measurements and estimated fungal growth profile calculated using the dry weight model, obtained during fermentations of* A. awamori *on whole wheat grains in Petri dishes.
Experimental total dry weight (●), estimated biomass (+) obtained from Equation 9. The solid line (—) represents the logistic curve (Equation 10) fitted to the biomass data; the dashed line (- - -) represents the total dry weight evolution estimated from Equation 5 using the parameters obtained from the logistic equation.

The good fit of both curves suggests that the approach and assumptions considered during the development of the model were reasonable and the estimated biomass values obtained are probably close to the actual ones. It is also important to point out that the experimental dry weight values represented in Figure 1 came from the measurements of many different Petri dishes, each removed from the incubator at a different time. The fact that the estimated biomass can be fitted with a typical growth curve using individual experiments, gives even more confidence in the model used.

The same approach used for the Petri dish experiments was adopted to obtain the growth profile during fermentation in the 1L-packed bed reactor for the production of the feedstock [38]. Using the same system as that described in the Materials and Methods section, the total dry weight was measured by weighing the reactor every 24 h and measuring the moisture content at incremental heights in the bed. To obtain the dry weight profile, a simple arithmetic operation was performed with the average moisture content, total weight and glucose extracted (for correction as mentioned before). Using Equation 9, the

time course of change in biomass was calculated. Results again show a typical growth curve (Figure 2), which was fitted using the logistic model. As previously explained, the estimated parameters obtained from the logistic model were used to calculate the theoretical change in dry weight. The curve obtained fitted the experimental values of dry weight, supporting again the validity of the model.

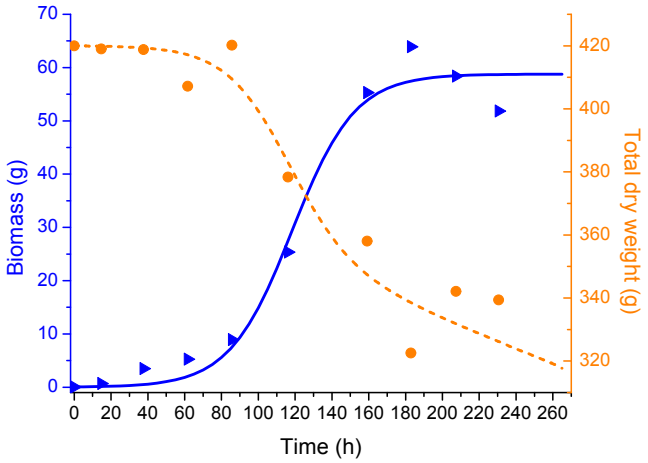


Figure 2 Total dry weight measurements and estimated fungal growth profile calculated using the dry weight model, obtained during the fermentation of A. awamori on whole wheat grains in a packed bed reactor with intermittent trickling of water. Experimental total dry weight (●), estimated biomass (▶) obtained from Equation 9. The solid line (—) represents the logistic curve (Equation 10) fitted to the biomass data; the dashed line (- - -) represents the total dry weight evolution estimated from Equation 5 using the parameters obtained from the logistic equation.

Comparing the biomass concentration values obtained for *A. awamori* in Petri dishes with those obtained in the 1-L packed bed bioreactor, it can be concluded that the growth profiles obtained in both systems were very similar. The maximum biomass concentrations obtained (C_M) were around 0.14 g biomass g^{-1} initial grain solid and the specific growth rates (μ) around 0.06 h^{-1} for both systems (Table 1). However, a longer lag phase was observed in the packed bed reactor system, probably because of the smaller inoculum size and the extraction of nutrients during the trickling of water. Although the correlation coefficients are very good with r^2 higher than 0.96 for both reactor systems, as shown in figures 1 and 2 the total dry weight is slightly deviated from the model predicted with Equation 5 at the late exponential and stationary phases. This could be due to the fact that the model depends on various

yield coefficients which are assumed to be constant, however at late exponential and stationary phases long distance translocation is required to deliver nutrients [39] which could affect those yields coefficients.

Table 1 Comparison of model parameters for the various reactor systems, including maximum biomass concentration (C_M) and specific growth rate (μ) obtained using the logistic equation, correlation coefficients (r^2) of the fitting of the logistic model and dry weight estimation. and Error Sum of Squares (SSE) of the dry weight model estimation.

REACTOR SYSTEM	Trickling cycle	Initial grain solid (g)	Final grain solid (g)	C_0 (g biomass g^{-1} initial grain solid)	C_M (g biomass g^{-1} initial grain solid)	μ (h^{-1})	r^2 (Logistic model)	r^2 (Dry weight estimation)	SSE (Dry weight estimation)
Petri dish	NO	8.3	6.7	0.00012	0.141	0.055	0.993	0.965	0.135
1L reactor	every 6 h	420.0	339.3	0.00008	0.140	0.059	0.982	0.967	849

REACTOR SYSTEM	Mixing event	Trickling cycle	Initial grain solid (g)	Final grain solid (g)	r^2 (Logistic model)	r^2 (Dry weight estimation)	C_0 (g biomass g^{-1} initial grain solid)	C_M (g biomass g^{-1} initial grain solid)	μ (h^{-1})
Petri dish	NO	NO	8.3	6.7	0.993	0.965	0.00012	**0.141**	**0.055**
1L reactor	NO	every 6 h	420.0	339.3	0.982	0.967	0.00008	**0.140**	**0.059**

The use of dry weight in order to identify microbial growth phases has been used previously by Borzani [40]. However, Borzani's approach was slightly different. Instead of solving Equation 9 using experimental data, he fitted the change of dry weight into different equations depending on the shape of the curve. These fits were then used to calculate growth equations with the target of identifying the growth phases without taking into account the biomass values. The model proposed in this paper can, not only be used for identifying growth phases but also to estimate key growth parameters such as the maximum biomass concentration and specific growth rates.

Two further approaches to estimating fungal growth were also explored; one was based on capacitance measurements and the other on CO_2 evolution.

4.2 Capacitance measurements and dry weight model validation

Figure 3 shows the capacitance measurements taken at different incubation periods in petri dish fermentation and compares the results with measured spore concentrations. It can be seen that during the first 50 h of incubation the capacitance values were very low or even negative. These negative values do not have any physical meaning since the measurement of the control without any growth should not have higher values of capacitance than the samples with growth. They could be due to inaccuracies in the measurements using this type of probe at such small cell concentrations showing low capacitance values [41]. This first 50 h, which also corresponded to low spore production, can be considered as the lag phase where the cells are adapting to the new media. From 50 h to 120 h of incubation the capacitance increased. This period corresponds to the exponential phase. Then, the slope of both curves decreased slightly. Patel et al. [42] have found that capacitance measurements during cell death phase is largely depend on the way of the cells are killed and the effect on their cell wall, therefore the use of capacitance as a measurement only of "viable" biomass should be used with caution. However, in the

work presented in this manuscript, without intentional cell disruption, three independent measurements: capacitance, spore concentration and total dry weight decreased after 120 h. These independent measurement confirm that the rising trend of capacitance conferred by the bulk of viable cells decreases while maintaining a rising trend. Probably, this notorious change at 120h is due to the natural tendency of the system to become more disorganized by the increasing effects of non-viable cells whose cell walls are likely to be damaged.

Biomass prediction using capacitance in liquid culture during growth phases has been widely applied in recent years [33]. However, prediction in the decline phase still showed errors in some studies [43-45]. To overcome this limitation, Ehgartner et. al. [45] developed a strategy to control the specific growth rate of *Penicillium chrysogenum*. Their strategy was based on online measurements of viable biomass amounts by means of dielectric spectroscopy throughout the growth and decline phases. [42]

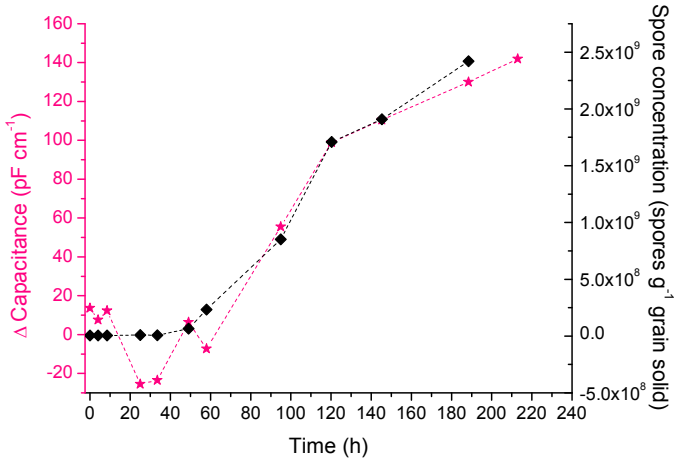


***Figure 3 Capacitance and spore concentration measured during the growth of* A. awamori *in Petri dishes.* Capacitance measurements (★), Spore concentration (◆)**

One of the limitations of capacitance measurements in particulate bioprocessing, as for any indirect method, is that this technique must be calibrated to establish the relationship with biomass, which can only be done in systems allowing direct measurement of the biomass. Liquid culture could be used but there is no guarantee that the biomass will have the same composition nor that the capacitance values would be the same. To solve this problem new approaches have been tried. For instance, culture methods based on artificial medium with overlying membranes where the fungus grows. This allows for biomass withdrawal, mimics solid systems more closely and could be used as a calibration system. However, during particulate bioprocessing using natural substrates, the composition of the substrate

changes during the fermentation and this could also modify capacitance, which would not be considered using an artificial medium. A way of overcoming this problem could be to use the estimated biomass values calculated from the dry weight model as a calibration curve.

Figure 4 compares capacitance measurements taken during petri dish fermentation with the estimated growth profile previously calculated from the dry weight evolution of the same system. Results show that the capacitance measurements could be fitted with the results obtained from the dry weight model, suggesting the potential of this technique to monitor on-line fungal growth. However, this calibration system should be used with caution, since the biomass values were not directly measured and come from a model with different assumptions and limitations.

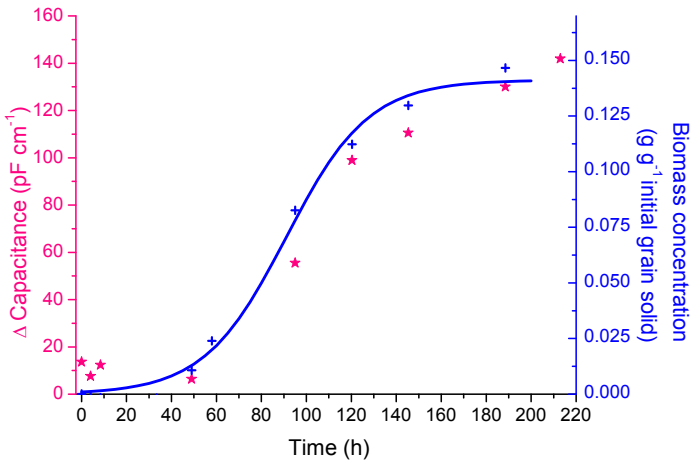


Figure 4 Capacitance and estimated fungal growth profile calculated using the dry weight model during the growth of A. awamori in Petri dishes. Capacitance measurements (★), estimated biomass (+) obtained from dry weight model (Equation 9); the solid line (—) represent the logistic curves (Equation 10) fitted to the biomass data.

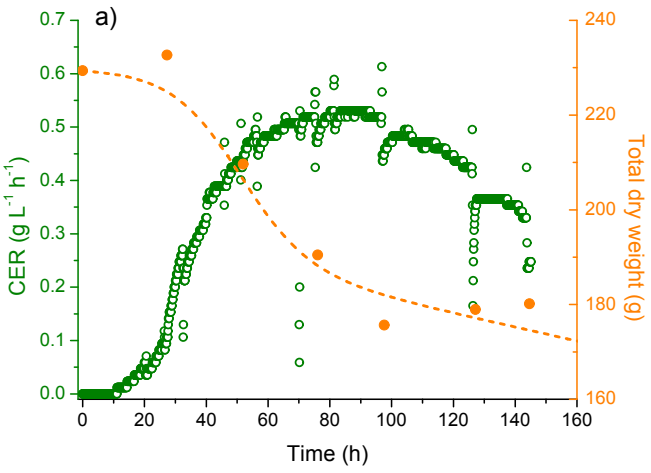
Capacitance was used in several SSF studies to monitor on-line cell growth [41, 46, 47]. The measurements in these works were compared with hyphal length and dry weight losses, concluding that capacitance and hyphal length were closely correlated. The authors of those studies treated the measurement at the beginning of the fermentation as a blank and did not consider changes that the fungus can cause to the media composition during growth. These assumptions may not be reasonable because they do not take into account two important factors. The first is the change in water content,

mainly due to evaporation and metabolic production. A second factor, is the change in the solid matrix due to growth and nutrient consumption. Both factors affect capacitance and should be considered.

To take into account the moisture effect, un-inoculated samples with the same moisture content as those samples at the moment of the measurement were used to give reference values in the experiments reported in this work. However, the second factor is much more difficult to overcome. To be able to have blanks without cells but with the same changes to the solid as would have occurred with growth is virtually impossible because the cells cannot be separated from the solid substrate. It would be necessary to find a way of destroying the microorganism cell walls without affecting the composition of the grains, and without affecting capacitance, and this would be impractical for online measurements. Therefore, although capacitance could be used to follow fungal growth phases, further development studies would be necessary in order to obtain 'absolute' values. A combination of the dry weight model reported in this work and validation using off-line measurement such as hyphal length using the membrane filter technique as reported by Kaminski et. al [41] could be explored in the future.

4.3 Metabolic model and dry weight model validation

The metabolic model was applied to the experimental results obtained from a second fermentation carried out in the 1L-packed bed reactor with intermittent trickling of water. On this occasion, both CO_2 evolution and dry weight were followed during the experiment (Figure 5.a) and both models were tested. Using the dry weight model (Equation 9) as described above, the biomass values were calculated from the metabolic model (Equation 16) and shown in Figure 5.b.



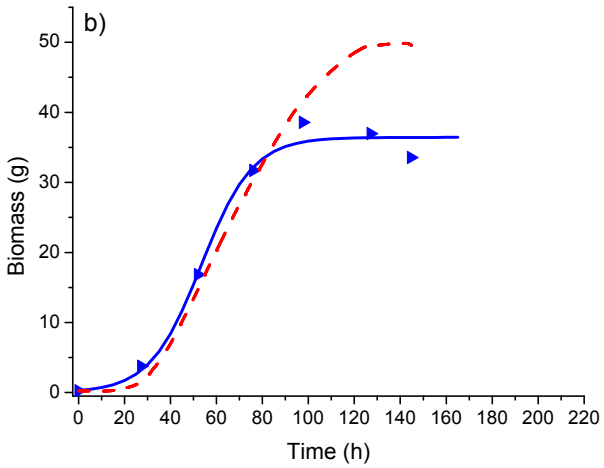


Figure 5 Total dry weight measurements and CO_2 evolution (a); estimated fungal growth profiles (b) calculated using the dry weight model and the metabolic model, obtained during the fermentations of* A. awamori *on whole wheat grains in a 1L packed bed reactor with intermittent trickling of water.

a) CO_2 evolution rate (○), experimental total dry weight (●); The short dashed line (- - -) represents the total dry weight evolution estimated from Equation ~~10~~-5 using the parameters obtained from the logistic equation (SSE=152, r^2=0.996).

b) Estimated biomass obtained from dry weight model using Equation ~~10~~-9 (▶); estimated biomass obtained from metabolic model using Equation ~~10~~-16 (— — —); The solid line (——) represents the logistic curve (Equation ~~10~~-10) fitted to the biomass data.

Cumulative CO_2 and cell dry weight measurements were also recently used by Zolfaghari-Esmaeelabadi and Hejazi [48]. They used these measurements to determine growth kinetic parameters in a semi-mechanistic model describing *Pseudomonas aeruginosa* growth on agar spheres. In their case, they used a model substrate that allowed for studying intra-particle transport phenomena in solid-state fermentation. In our case because the fungal separation is not possible the dry weight model was used instead.

Comparing the estimated biomass from the dry weight model proposed in this paper and the metabolic models, it can be seen, in Figure 5, that both approaches estimate the same biomass profile during the first 80 h of incubation. Towards the end of the fermentation, the metabolic model predicts higher growth than that predicted using the dry weight model. This could be due to a possible overestimation of biomass predicted from the constant yield CO_2 model. Previous authors have identified that the ratio of CO_2 to biomass increases with time [17, 49]. Therefore, it is possible that towards the end of the fermentation the CO_2 is more related to maintenance than to growth. One of the assumptions of both models was that the yield and maintenance coefficients remain constant during fermentation. A change in the coefficients related to the CO_2 means that probably both models slightly overestimate fungal growth. This overestimation is more important in the metabolic model, since parameters α' and β' depend directly on the yield and maintenance coefficient for CO_2. However, the fact that both growth

profiles obtained using two different sets of experimental data are quite similar provides greater confidence in both models.

5 Conclusions

The objective of this work was to provide a reliable measurement of fungal growth during solid state fermentation of *Aspergillus awamori* on wheat grains. During this project, a dry weight model has been proposed, which can be used not only for identifying the various growth phases but also to estimate important growth parameters such as specific growth rate and maximum biomass concentration. By carrying out a mass balance of the total dry weight of the system, a relationship between biomass and the change in dry weight was obtained. Using maintenance and yield coefficients from the literature, estimated values of biomass were obtained for the different systems studied. The biomass profiles were then fitted to logistic models and the parameters calculated were used to re-calculate the change in dry weight. The good fits obtained by comparing estimated with experimental values, suggest that the dry weight model could be used for biomass estimation and the approach and assumptions considered were quite realistic.

Capacitance as a way of measuring biomass was also explored during the project. Although with some limitations, results reported in this work have shown the potential of this online measurement to estimate fungal growth by comparing the results with the dry weight model prediction.

Finally, the dry weight model was compared with a previously proposed metabolic model. The similarity in the prediction of fungal growth using both models validates both approaches. This confirms the potential of the model developed during this project based on dry weight measurement as a simple and low cost tool for estimating fungal growth in SSF.

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