Full Length Research Paper

Assessment of *Aspergillus niger* biofilm growth kinetics in minibioreactors by carbon dioxide evolution

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Aspergillus niger ATCC 10864 was grown on bubble minicolumns aerated with pure oxygen either as biofilm or free-mycelium submerged systems. Growth kinetics was followed by estimating biomass from released carbon dioxide using a titration method and mathematical models. Submerged cultures showed increased endogenous respiration rates (0.0298 vs. 0.0004 g biomass/g biomass.h) possibly related to stress condition since this type of growth is not the natural way of life of *A. niger*. On the other hand, *A. niger* biofilm growth followed a logistic model having higher maximal specific growth rate than submerged cultures (0.061 vs. 0.045 h^{-1}). This may be related to better mass transfer processes due to a channeled mycelial microstructure. Biofilm growth can be easily and reliable assessed by evaluating the CO_2 released during the fermentation in minibioreactors.

Key words: *Aspergillus niger*, biofilm, mathematical modeling, endogenous respiration, Cryo-SEM.

INTRODUCTION

Aspergillus niger is currently one of the microbial species of main biotechnological importance because it produces about 40 different commercial enzymes with different applications in the food industry, beverages, textiles, agriculture, pulp and paper and others. It is also widely used in the production of organic acids such as citric, gluconic, fumaric and oxalic acids (Ward et al., 2006).

Moreover, the knowledge gained in its various fermentation processes, is being used in the production of recombinant proteins (Gyamerah et al., 2002; Wang et al., 2003). Additionally, many of its enzymes and citric acid have been deemed GRAS by the FDA, which has enhanced its use for food processing applications (Schuster et al., 2002).

Industrial enzymes are mainly produced by submerged fermentation, although, alternatively, solid-state fermentation has been used on a smaller scale. In this sense, there are many reports on the analysis and comparison of these two systems in the production of enzymes in the laboratory (Aguilar et al., 2001; Díaz-Godínez et al., 2001; George et al., 1997; Hölker et al., 2004; Maldonado and Strasser de Saad, 1998; Papagianni et al., 1999, 2002; Romero-Gómez et al., 2000; Viniegra-González et al., 2003).

Although, solid-state fermentation has advantages related to higher productivity and concentration of enzymes, lower catabolic repression, among others, difficulties in the scale-up limit their ability to replace industrially submerged fermentation. However, the role of physiological and genetic properties of microorganisms growing on surfaces could explain the advantages of solid-state fermentation on submerged fermentation (Hölker et al., 2004). In this aspect, a new fermentation category, named surface adhesion fermentation (SAF), was proposed based on the importance of the cell adhesion process in the physiological characteristics and differential gene expression of attached microorganisms, particularly filamentous fungi, which at low free liquid the system behaves as solid-state fermentation and at high free liquid the system will give a biofilm fermentation (Gutiérrez-Correa and Villena, 2003). The latter process may use the infrastructure and operating characteristics of submerged fermentation, as well as opening opportunities for the design of new type of bioreactors (Villena and Gutiérrez-Correa, 2006).

The growth of filamentous fungi adhered to surfaces has been commonly regarded as a type of immobilization, which is an engineering approach to achieve high cell densities and continuous-flow culture systems.

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However, this approach does not take into account that cell adhesion is a natural biological process that leads to significant morphological and physiological changes that include the differential expression of genes (Gamarra et al., 2010; Villena and Gutiérrez-Correa, 2007; Villena et al., 2009a, b, 2010). Only recently, filamentous fungi are also being considered capable of biofilm formation as do bacteria and yeasts (Gutiérrez-Correa and Villena, 2003; Harding et al., 2009; Wang and Chen, 2009). In that sense, this type of culture can lead to improved metabolite production systems. Also, different types of bioreactors are beginning to be used for biofilm fermentation by filamentous fungi (Cheng et al., 2010).

The use of small bioreactors during the early stages of biofilm research allows mathematical modeling and the results are more useful in later stages of the production process development (Villena and Gutiérrez-Correa, 2006). Due to the attached growth of biofilms, quantification of biomass in a bioreactor requires non-disruptive techniques that allow time-course experiments. The indirect estimation of growth by CO_2 evolution is commonly used in solid-state fermentation (Auria and Revah, 1994) where it is difficult to directly quantify biomass, and is particularly useful for heterogeneous systems (Koutinas et al., 2003). Unlike these systems where the indirect measurement of biomass may not reflect real growth kinetics because indirect measures are difficult to interpret, the use of CO_2 as an indicator of growth in both submerged and biofilm cultures where it is possible to directly quantify biomass, is a simpler alternative compared to other noninvasive methods such as image analysis. In this study, a growth kinetics assessment of *A. niger* biofilms was carried out by using a low cost and reliable method for estimating fungal biomass.

MATERIALS AND METHODS

Fungal strain and culture medium

In this study, *A. niger* ATCC 10864 was used. The strain was maintained on potato dextrose agar (PDA) slants. For the inoculum, spores were washed from 5-day PDA plates, using 10 mL of 0.1% (v/v) Tween 80 to a final concentration of 1 x 10^6 spores per mL. The growth medium composition (Duff, 1988) per liter was: KH_2PO_4, 2 g; $CaCl_2.2H_2O$, 0.3 g; $MgSO_4.7H_2O$, 0.3 g; urea, 0.3 g; NH_2SO_4, 1.4 g, Tween 80, 2 ml; $FeSO_4.7H_2O$, 5 mg; $MnSO_4.2H_2O$, 1.6 mg; $ZnSO_4.7H_2O$, 1.4 mg; $CoCl_2.6H_2O$, 2 mg; and lactose, 10 g. The initial pH of culture medium was 5.5.

Minibioreactors

Bubble minicolumns made of 15 x 3 cm glass tubes were used as bioreactors, as previously reported (Villena and Gutiérrez-Correa, 2006). However, each reactor was modified to connect the spout gas tube to a collecting glass vial containing 25 mL of 1.5 N NaOH solution, as shown in Figure 1 (A, C-E). For biofilm cultures, each reactor contained a 3.6 x 1.5 cm piece of polyester cloth attached to a stainless steel spiral frame placed on the central shaft tube. Pure

oxygen at 3.6 L/h was sparged through the liquid culture from a 30-G syringe needle connected to the bottom end of the shaft tube.

Minibioreactor cultivation

Minibioreactors have become relevant in small-scale experiments, and the bubble minicolumns have shown the same behavior as laboratory reactors, in contrast to shaken flasks. These types of minireactors are particularly useful for systems with moderate oxygen demands (Kumar et al., 2004).

Biofilm and free mycelium cultures were performed in minibioreactors containing 40 mL of growth medium. Each minibioreactor was inoculated with a 3% (v/v) spore suspension (1 x 10^6 spores/mL). For biofilm cultures, each minibioreactor containing 40 mL of sterile distilled water was inoculated as aforementioned and incubated at 175 rpm for 30 min at room temperature. Then, it was washed two times to remove the unbound spores and the wash water was replaced by 40 mL of growth medium to start the fermentation. To avoid foaming, Sigma antifoam 289 was used. The inoculated minibioreactors were connected to the oxygen supply and incubated in a controlled water bath at 28°C (Figure 1B). Four replicates were used for each sampling point.

Biomass was measured by determining the dry weight of each sample. For submerged fermentation cultures, the total content of each minibioreactor was vacuum filtered using pre-weighed filter paper (Whatman No. 1), which was then dried at 80°C to constant weight. Biofilm cultures support with biofilms were removed, washed three times and dried at 80°C to constant weight; the remaining detached biomass was filtered in the same way for submerged fermentation cultures.

Determination of endogenous respiration

Endogenous respiration rate was determined using 72 h growth cultures grown on medium containing 2% glycerol as the sole carbon source. The cultures were washed repeatedly with sterile 100 mM phosphate buffer, pH 6.5, and incubated at 175 rpm for 2 h at 28°C to exhaustion of nutrients in the mycelium. From this, biofilm or mycelium samples were transferred to flasks containing 20 mL 100 mM phosphate buffer, pH 6.5, and incubated without agitation at 28°C for 14 h to determine the amount of CO_2 produced as shown in Figure 2. Four replicates were used for each sampling point.

The CO_2 produced was quantified as described by Sugama and Okasaki (1979). The CO_2 was collected in flasks containing 25 mL of 1.5 N NaOH. For titration, the content of each flask was transferred to a 250 mL flask and 10 mL of 10% $BaCl_2$ solution were added. The mixture was titrated to pH 9 with 1.5 N HCl. The amount of CO_2 (mg) was calculated from this titer (a ml) and that (b mL) of an equal volume used by a blank NaOH solution using the following relationship:

$$CO_2 \,(mg) = (b - a) \times Normality \times weight \; equivalent \qquad (1)$$

Cryo-scanning electron microscopy (cryo-SEM)

Either biofilm or mycelium samples were immersed in 10% glycerol for 2 h at 4°C. Then, they were thoroughly washed with 0.05 M phosphate buffer (pH 7.4) and frozen by plunging them into liquid nitrogen. Surface water was removed by sublimation at -65°C for 10 min and specimens were sputter-coated with gold (Villena and Gutiérrez-Correa, 2007). Samples were then examined at -80°C in a LEO 1420 PV variable pressure scanning electron microscope (LEO Electron Microscopy, Cambridge, U.K.).
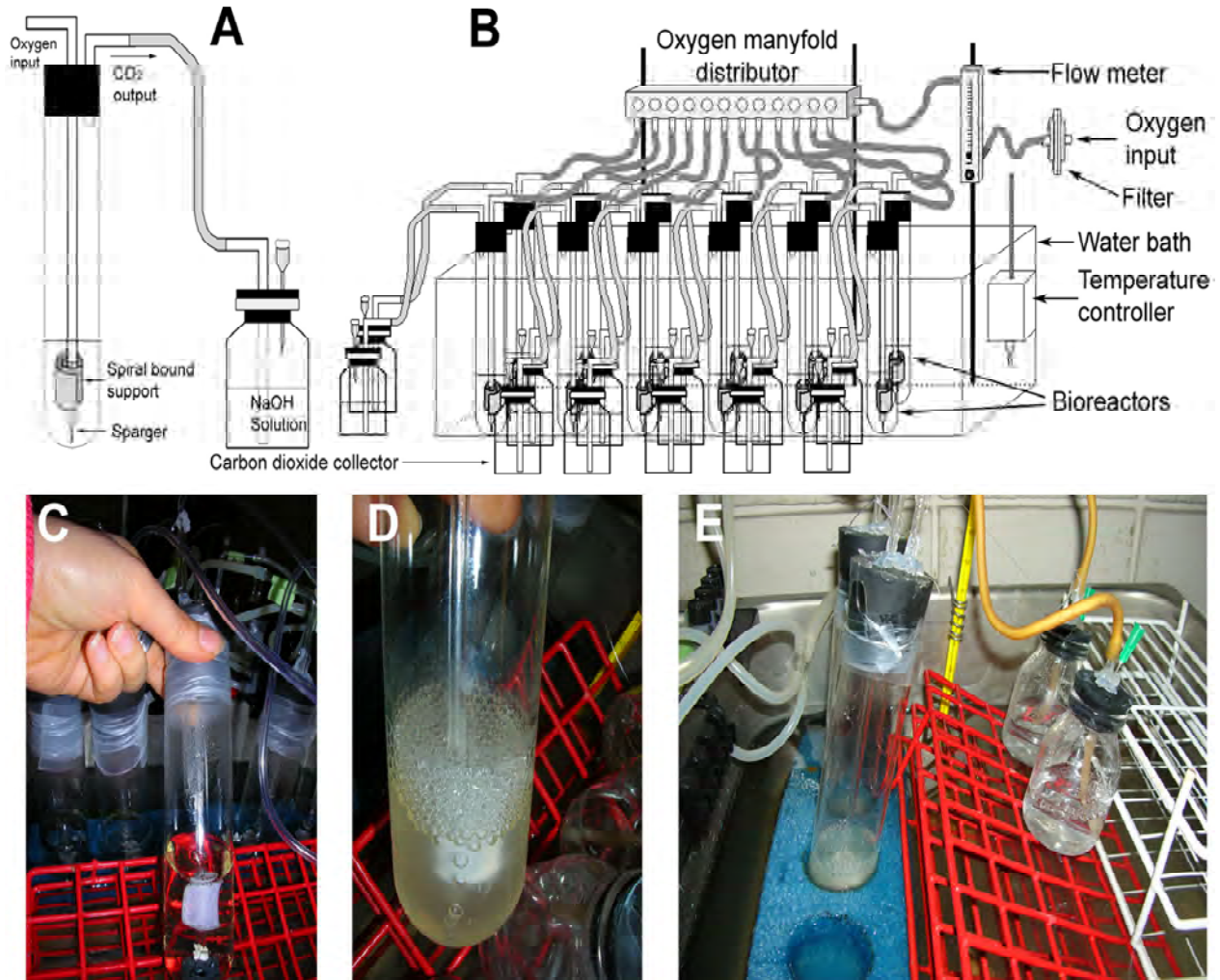
Figure 1. Minibioreactor experimental set. Diagram of minibioreactor of bubble column type showing connection to CO_2 collector vial (A). Diagram of a battery of minibioreactors (B). Close-up photographs of minibioreactors (C-E).

Mathematical models

Kinetic models contribute to the basic knowledge needed in the design and optimization of fermentation processes. Currently, available models are useful to describe and predict growth, consumption and product formation, among others, allowing the analysis and validation of models in certain conditions. On the other hand, work on a small scale, as a starting point for process optimization, is gaining increased research interest (Schäpper et al., 2009). Small-scale work has the advantage of parallelization, repetition and control of variables at reduced costs. Since microbial growth is associated with the consumption of oxygen and nutrients and the production of heat, water vapor, carbon dioxide or other products, the Pirt model can be successfully used to predict growth from CO_2 levels produced during fermentation.

Balance equations were used for biomass and lactose consumption to describe the fermentation process in both systems. Also, a model was obtained to predict fungal growth from accumulated CO_2 and its usefulness as an indirect and noninvasive method in predicting growth was evaluated.

Biomass production was estimated as x (g/L) by the logistic equation (Liu et al., 2003; Wachenheim et al., 2003; Weiss and Ollis, 1980):

$$\frac{dx}{dt} = \mu_m\left[1 - \frac{x}{x_m}\right] \qquad (2)$$

Where, μ_m is the maximum specific growth rate (h^{-1}) and x_m is the maximal biomass concentration reached when $dx/dt = 0$ for $x > 0$. The solution of Equation (2) when x_0 is the initial condition at $t = 0$, is:

$$x(t) = \frac{x_m}{1 + \left(\dfrac{x_m - x_0}{x_0}\right)e^{-\mu_m t}} \qquad (3)$$

The rate of CO_2 production can be described with linear-growth model of Pirt as (Lareo et al., 2006):
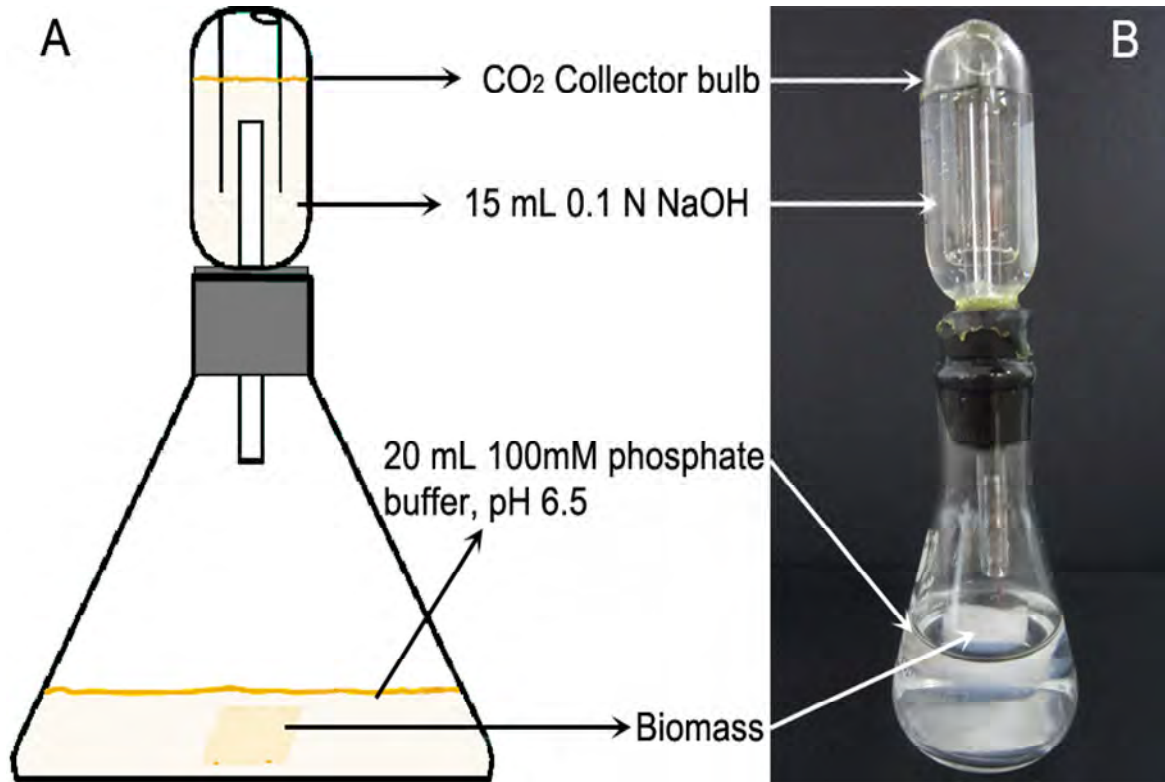
Figure 2. Diagram (A) and photograph (B) of the device used for measuring endogenous respiration.

$$\frac{dCO_2}{dt} = \frac{1}{Y_{x/CO_2}}\frac{dx}{dt} + m_{CO_2} x \qquad (4)$$

Replacing Equations (2) and (3) into Equation (4) the following expression is obtained:

$$\frac{dCO_2}{dt} = \left\{ \frac{m}{Y_{x/CO_2}}\left[1 - \frac{1}{\left(\frac{x_m}{x_0}-1\right)e^{-mt}+1} \right] + m_{CO_2} \right\}\left[\frac{x_m}{\left(\frac{x_m}{x_0}-1\right)e^{-mt}+1} \right] \qquad (5)$$

Solving Equation (5) as a function of t, Equation (6) is as follows:

$$CO_2(t) = CO_{2(0)} + x_m \left\{ \frac{1}{Y_{x/CO_2}\left(\frac{x_m}{x_0}-1\right)e^{-mt}+1} - \frac{1}{Y_{x/CO_2}\frac{x_m}{x_0}} + \frac{m_{CO_2}}{m}.\ln\left[\frac{\left(\frac{x_m}{x_0}-1\right)e^{-mt}+1}{\frac{x_m}{x_0}e^{-mt}} \right] \right\} \qquad (6)$$

Where, $CO_2(t)$ is the actual cumulated CO_2 (g/L), $CO_{2(0)}$ the initial CO_2 concentration (g/L), Y_{x/CO_2} the yield coefficient of biomass on CO_2 ($g_{biomass}/g_{CO2}$), and m_{CO_2} the maintenance coefficient on CO_2 ($g_{CO2}/g_{biomass}$.h). By using Equation (5) with the experimental data the parameters $Y_{x/CO2}$ and m_{CO2} could be estimated.

On the other hand, Equation (4) can be integrated as a function of t to obtain:

$$x(t) = e^{-m_{CO_2}Y_{x/CO_2}t} \left[x_0 + \int_0^t Y_{x/CO_2} e^{m_{CO_2}Y_{x/CO_2}t} \frac{dCO_2}{dt} \right] \quad (7)$$

This integral cannot be solved analytically but by numerical methods. The solution of Equation (7) gives:

$$x(t_i) = \frac{1}{-} e^{\left(--t_i\right)} \left[\sum_{i=0}^n \frac{h}{2}\left(y(t_i) + y(t_{i+1})\right) + x_0 \right] (8)$$

Where,

$$y(t_i) = e^{\left(-t_i\right)} \frac{dCO_2}{dt} \quad (9)$$

$$= \frac{1}{Y_{x/CO_2}} \quad (10)$$

$$= m_{CO_2} \quad (11)$$

Therefore, by using Equation (8) it is possible to estimate the values of $x(t)$ from the experimental determination of dCO_2/dt knowing the values of Y_{x/CO_2} and m_{CO_2}.

The quantification of endogenous respiration is an alternative way to evaluate the provision of energy for maintaining cell viability. The maintenance energy comes from the degradation or oxidation of the biomass when the limiting substrate has been completely consumed. Under these conditions, cell growth is negligible and the balance of biomass can be expressed as:

$$\frac{dx}{dt} = -k_e x \quad (12)$$

Where, k_e is the coefficient of endogenous respiration ($g_{biomass}/g_{biomass}.h$). The second term in this equation can be considered as the rate of cell death. Assuming k_e as a constant, Equation (12) can be solved as a function of t:

$$x(t) = x_{t_1} e^{-k_e(t-t_1)} \quad (13)$$

Where, t_1 is the time at which the substrate is fully consumed and x_{t1} is the biomass at time t_1. Therefore, the balance equation for CO_2 production can be simplified to:

$$\frac{dCO_2}{dt} = m_{CO_2} x \quad (14)$$

Replacing Equation (13) into Equation (14) and solving as function of time:

$$CO_2(t) = CO_2(t_1) + \frac{m_{CO_2}x_{t_1}}{k_e} - \frac{m_{CO_2}x_{t_1}e^{-k_e(t-t_1)}}{k_e}$$

(15)

By using Equation (15) with the experimental data, k_e could be estimated.

Statistical analysis

Data were analyzed by statistical analysis system (Version 8.1) software (SAS Institute, Inc., Cary, NC, USA). Model parameters were estimated using the model OLS procedure with Gauss method of SAS software, which minimizes the experimental and estimated data.

RESULTS

A. niger ATCC 10864 grown on bubble minicolumns aerated with pure oxygen either as biofilm or free-mycelium submerged systems. As freely suspended mycelium, *A. niger* grew producing compact and fluffy pellets. This could be due to higher shear stress attained by oxygen bubbling in minibioreactors. On the other hand, attached growth on the polyester cloth gave a well developed biofilm with neither freely floating mycelium nor sloughing. Morphology of mycelium in submerged and biofilm cultures is depicted in Figure 3. Superficial cryo-SEM views of biofilm mycelium and pellet are clearly different. Biofilm mycelium showed an orderly distribution of hyphae with both surface and inner channeled structure (Figure 3A), while pellet mycelium showed highly intertwined superficial hyphae and a densely packed deep mycelium (Figure 3B).

Figure 4 shows the growth kinetics of both biofilm and submerged cultures. In both cases, there was a trend of logistic growth with a longer lag phase for the submerged culture (Figure 4A, B). Model Equation (4) was used to describe the accumulation of CO_2, as a product of cellular respiration primarily intended to produce energy for biomass production. The correlation coefficients ($R^2 > 0.9$) confirmed a close relationship between the amounts of CO_2 produced and cell growth, especially for biofilm cultures, as shown in Figure 4C and D. In both systems, during the first hours of growth, production of CO_2 was coincident with the growth and in biofilm cultures both curves showed the same trend over time (Figure 4A). However, in submerged culture (Figure 4B) at 72 h,
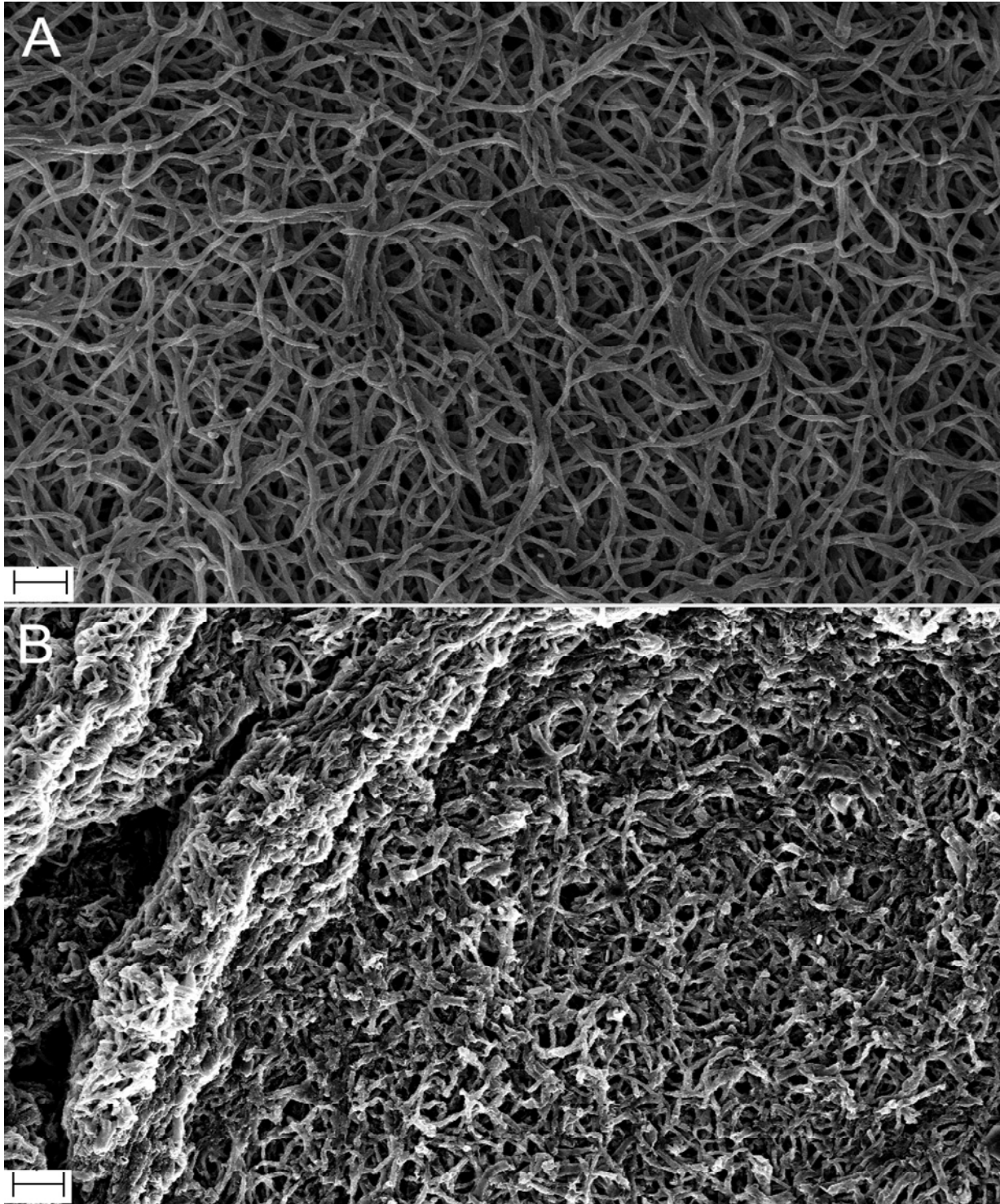
Figure 3. Cryo-scanning electron microscope photographs of *Aspergillus niger* biofilm (A) and pellet from submerged fermentation (B). Bars represent 20 µm.

growth there was an inflection in the growth curve deviating from the production of CO_2 curve. This is also reflected in lower coefficients of correlation ($R^2 = 0.94$) compared to that obtained in biofilm cultures.

Figure 5 shows the biomass kinetics adjustment based on the CO_2 produced, according to Equation 7, which gave the model Equation (16) for biofilm cultures and Equation (17) for submerged cultures.

$$x(t) = e^{-0-0188\ t} \cdot \left[0.0463 + \int_0^t 0.9912\ e^{0.0188\ t} \cdot \frac{dCO_2}{dt} \right] \quad (16)$$

$$x(t) = e^{-0.0011\ t} \cdot \left[0.0338 + \int_0^t 0.3388\ e^{0.0011\ t} \cdot \frac{dCO_2}{dt} \right] \quad (17)$$

The data fit the model satisfactorily, with correlation coefficients above 0.9. Again, the experimental behavior of biofilms was closest to the predictions of the model (Figure 5A) compared to that obtained in submerged culture (Figure 5B). Parameters estimated with model equations are depicted in Table 1. Values of $Y_{x/CO2}$ and m_{CO2} are remarkably different between the two culture systems, showing that in biofilms much CO_2 is produced almost directly by cell growth while in submerged culture only a fraction of CO_2 comes directly from the growth. Furthermore, the endogenous respiration coefficient was higher in submerged culture. In biofilms, the estimated value showed high variability so it cannot be sure it is different from zero, although, experimental data indicate a minimum value.

DISCUSSION

Fermentation is a very complex process, and it is often very difficult to obtain a complete picture of what is actually going on in a particular fermentation. A model is that which describes relationships between principal state variables and explains quantitatively the behavior of a system. The model can provide useful suggestions for the analysis, design and operation of a fermenter. Mathematical modeling is an essential tool for optimizing bioprocesses. Not only can models guide the design and operation of bioreactors but they can also provide insights into how the various phenomena within the fermentation system combine to control overall process performance (Mitchell et al., 2003). Fermentation models are normally divided into two classes: structured models where intracellular metabolic pathways are considered and unstructured models where the biomass is described by one variable. Structured model seems complicated for normal use. Unstructured models are much easier to use, and have proven to accurately describe much fermentation.

Biofilm fermentation may be an alternative to solid state fermentation since it combines the advantages of the latter system and the use of the submerged fermentation technology (Villena and Gutiérrez-Correa, 2006, 2007; Gamarra et al., 2010). Unfortunately, there are very few reports on enzyme production by *A. niger* biofilms because filamentous fungal biofilms are just beginning to be accepted as another type of fermentation systems (Harding et al., 2009). Therefore, both simple and complex kinetic analyses are needed to understand and utilize *A. niger* biofilms as industrial production systems. To implement a fermentation process based on filamentous fungal biofilms, the determination of biomass concentration throughout fermentation is essential. In order to accomplish this, the problem caused by the attached growth on solid supports should be overcome. Since the use of a direct analytical method such as the dry weight method is impractical, the use of an indirect estimation method is the only alternative. Non disruptive methods like CO_2 evolution may be useful to estimate cell growth in biofilm systems by applying a mathematical model that correlates fungal growth with CO_2 evolution (Koutinas et al., 2003). However, the estimation of biomass by CO_2 requires that the $Y_{x/CO2}$ and m_{CO2} values are known, which may vary significantly according to growing conditions (Koutinas et al., 2003).

The estimated values of m_{CO2} and $Y_{x/CO2}$ for biofilm cultures are in the range of those reported for other fungal species while those in submerged cultures are significantly lower (Gelmi et al., 2002; Lareo et al., 2006). Low m_{CO2} and $Y_{x/CO2}$ values may be associated with oxygen limitation conditions but because the aeration conditions for both systems were the same, differences in oxygen transfer could be related to morphologies developed in each case. As seen in Figure 3, morphologies of biofilm and pellets formed in submerged fermentation are different being biofilms characterized by a channeled structure that allows better mass transfer processes as was previously shown (Villena and Gutiérrez-Correa, 2006, 2007; Villena et al., 2010). Also, assuming that CO_2 produced comes mainly from the oxidation of the carbon source (Lareo et al., 2006), and this is not a limiting nutrient, the value of $Y_{x/CO2}$ for submerged cultures suggests transfer limitations directing the provision of energy for maintenance from endogenous respiration since higher k_e values were found in this type of culture (Table 1). Moreover, biofilm cultures attained higher maximal specific growth rates (μ_m = 0.061 h^{-1}) than submerged cultures (μ_m = 0.045 h^{-1})
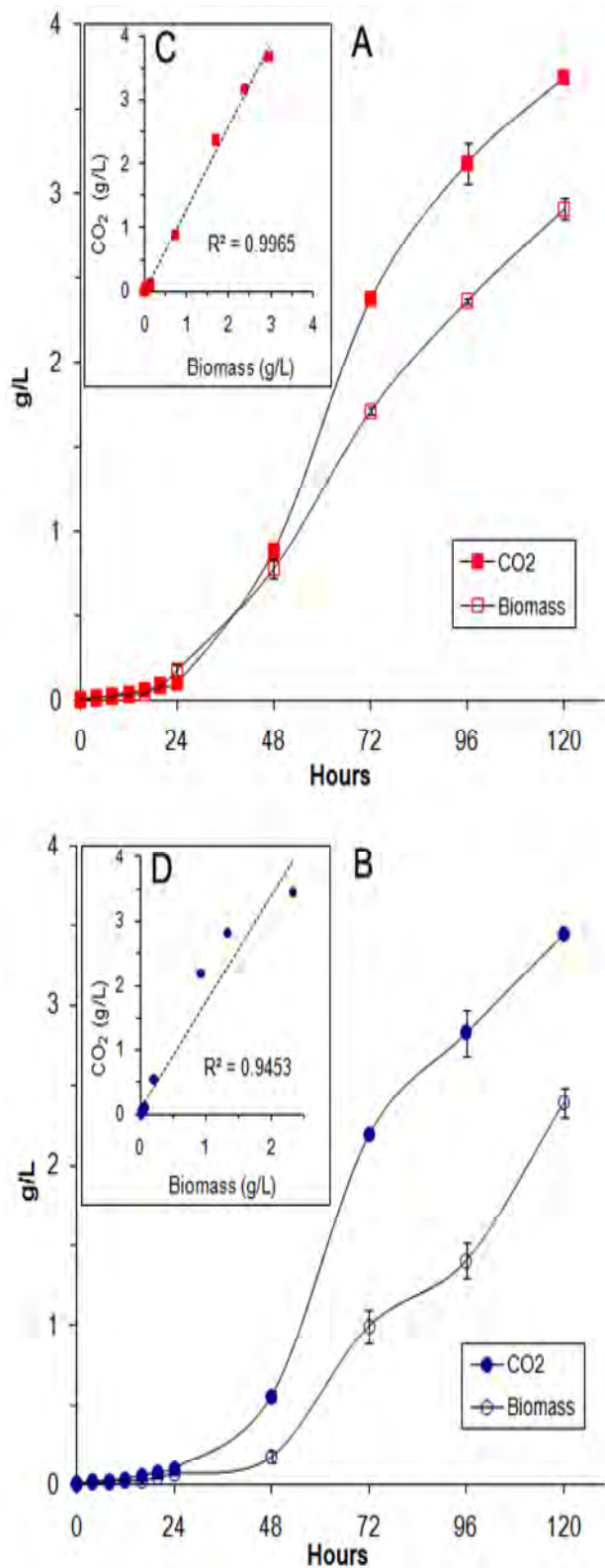
Figure 4. Biomass and carbon dioxide evolution kinetics of *Aspergillus niger* biofilm (A) and submerged (B) fermentations. Inserts show correlations between carbon dioxide evolution and biomass in biofilm (C) and submerged (B) fermentations. Error bars on the graph represent four replicates.
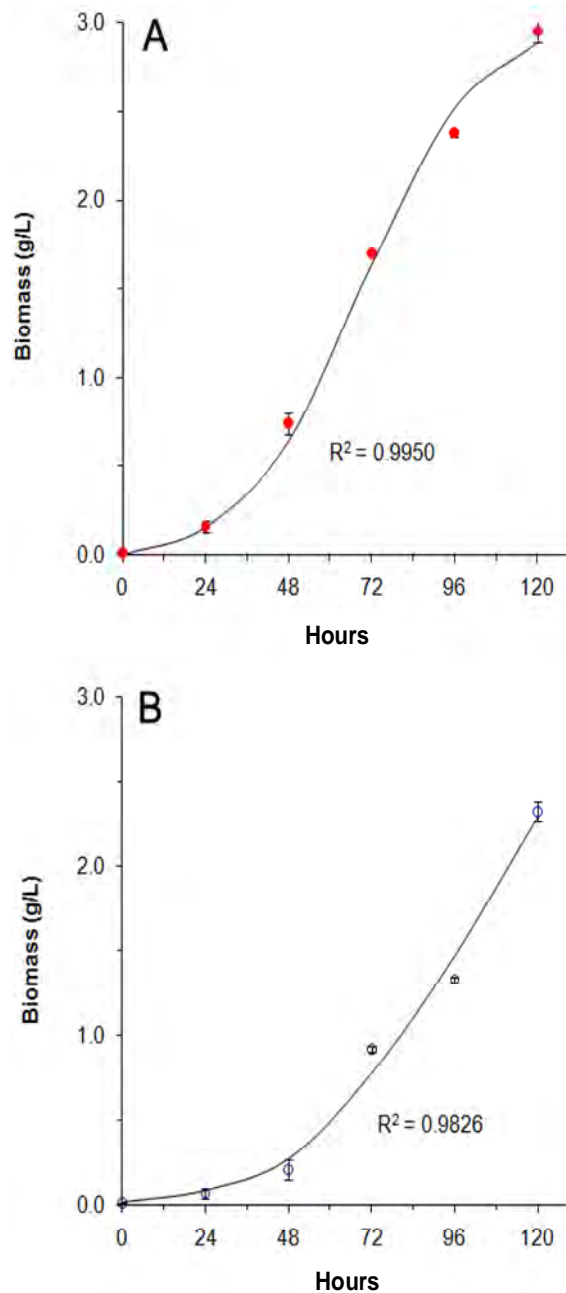

Figure 5. Biomass kinetics of *Aspergillus niger* biofilm (A) and submerged (B) fermentations based on carbon dioxide actual data (symbols). Solid lines were performed using the best fit equation models (16) and (17), respectively. Error bars on the graphs represent four replicates.

(Table 1). This may be related to a stress condition of submerged cultures since this type of growth is not the natural way of life of *A. niger* as shown by the presence of some stress-related proteins in its intracellular proteome (Villena et al., 2009b).

In conclusion, *A. niger* biofilm growth follows a logistic model having higher maximal specific growth rate than

Table 1. Estimated growth kinetic parameters of *Aspergillus niger* biofilm and submerged fermentations in minibioreactors according to model equations.

Estimated parameter	Biofilm fermentation	Submerged fermentation
x_o (g/L)	0.045 ± 0.013	0.035 ± 0.016
x_m (g/L)	2.99 ± 0.11	3.26 ± 0.64
μ_{max} (h^{-1})	0.061 ± 0.005	0.045 ± 0.007
$1/Y_{X/CO2}$ (g/g)	1.009 ± 0.107	2.950 ± 0.922
m_{CO2} (g CO_2/g biomass. h)	0.0198 ± 0.0020	0.0034 ± 0.0026
k_{eCO2} (g biomass/g biomass.h)	0.0004 ± 0.0001	0.0298 ± 0.0068
$k_{eCO2}{}^{calc}$ (g biomass/g biomass.h)	0.0279	0.0514

Initial biomass concentration (x_0), maximal biomass concentration (x_m), maximal specific growth rate (μ_{max}), yield coefficient of biomass based on CO_2 ($Y_{x/CO2}$) and maintenance coefficient on CO_2 (m_{CO2}) were estimated by Equation (6). Endogenous respiration coefficient (k_{eCO2}) was estimated by Equation (15); $k_{eCO2}{}^{calc}$ is the endogenous respiration coefficient calculated from experimental data.
The Model OLS procedure with Gauss method of SAS softwarewas used to estimate parameters.

submerged cultures. This may be related to better mass transfer processes due to a channeled mycelial microstructure. Biofilm growth can be easily and reliable assessed by evaluating the CO_2 released during the fermentation in bioreactors. The method hereby described may be improved and facilitated by using either an automatic titration system or a dedicated gas chromatographer.

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