



THE EFFECT OF THE RATIO BETWEEN SUBSTRATE CONCENTRATION AND SPECIFIC AREA OF THE SUPPORT ON THE BIOMASS YIELD OF FUNGAL SURFACE CULTURES

EFEECTO DE LA PROPORCIÓN ENTRE LA CONCENTRACIÓN DEL SUSTRATO Y EL ÁREA ESPECÍFICA DEL SOPORTE SOBRE EL RENDIMIENTO DE CULTIVOS FÚNGICOS SUPERFICIALES

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Abstract

A parameter, $\sigma_0 = S_0\alpha^{-1}$ (gcm^{-2}), is defined. Where, S_0 , (gcm^{-3}) is the initial substrate concentration and α is the specific area of the solid support. This parameter helps comparing the biomass yields, $Y_{X/S}$, of fungal superficial cultures either with a fixed S_0 value and variable α or with fixed α and variable S_0 . *A. niger* cultures followed the logistic equation with maximal surface density ρ_{AM} (gcm^{-2}). Final average thickness, h , was measured by image analysis. The values of h , followed a saturation function of σ_0 ($R^2 = 0.965$) with extrapolated value $h_{MAX} \approx 0.4$ cm. But the volumetric density $\rho_V = \rho_{AM}h^{-1}$ was nearly constant $\rho_V \approx 0.046 \pm 0.005$ (gcm^{-3}). The regression $Y_{X/S}^{-1} = Y_0^{-1} + \sigma_0\epsilon^{-1}$ ($R^2 = 0.95$) in the range, $5.9 \text{ mgcm}^{-2} < \sigma_0 < 62.8 \text{ mgcm}^{-2}$ indicated, $Y_0 = 0.5$, as the maximal biomass yield, and $\epsilon = \rho_V h_{MAX} \approx 0.02 \text{ gcm}^{-2}$, as a measurement of the biosynthetic efficiency. This approach could be useful for the normalization and comparison of screening tests of fungal surface cultures with a variety of solid supports and culture conditions. For example, cultures with different S_0 values but, similar yield coefficient $Y_{X/S}$.

Keywords: fungal surface cultures, mass balance, specific area, biomass yield, *Aspergillus niger*.

Resumen

Se define, $\sigma_0 = S_0\alpha^{-1}$ (gcm^{-2}). Donde, S_0 , (gcm^{-3}) es la concentración inicial del sustrato y α , (cm^{-1}) es el área específica del soporte sólido. Dicho parámetro ayuda a comparar los rendimientos de la biomasa, $Y_{X/S}$, de cultivos fúngicos superficiales, sea con un nivel fijo S_0 , y diversos valores de α , o con un valor fijo de α , y diversos valores de S_0 . Las curvas de crecimiento fueron seguidas por la ecuación logística con una densidad superficial máxima ρ_{AM} (gcm^{-2}). Su espesor final, h , se midió por análisis de imágenes que siguieron una función de saturación en σ_0 ($R^2 = 0.965$) con un valor extrapolado $h_{MAX} \approx 0.4$ cm. Pero, la densidad volumétrica, $\rho_V = \rho_{AM}h^{-1}$ fue casi constante $\rho_V \approx 0.046 \pm 0.005$ (gcm^{-3}). La regresión $Y_{X/S}^{-1} = Y_0^{-1} + \sigma_0\epsilon^{-1}$ ($R^2 = 0.95$) en el intervalo, $5.9 \text{ mgcm}^{-2} < \sigma_0 < 62.8 \text{ mgcm}^{-2}$, indicó, $Y_0 = 0.5$, y $\epsilon = \rho_V h_{MAX} \approx 0.02 \text{ gcm}^{-2}$, ambos como medida de la eficiencia bio-sintética. Este enfoque puede ser útil para normalizar y comparar ensayos de selección de cepas fúngicas en diversos tipos de soportes y condiciones de cultivo. Por ejemplo, diferentes valores de S_0 , pero similares valores de $Y_{X/S}$.

Palabras clave: cultivos fúngicos superficiales, balance de materia, área específica, rendimiento de la biomasa, *Aspergillus niger*.

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1 Introduction

Fungal surface cultures on agar plates have been used for more than a century as laboratory models of the growth of filamentous fungi, and, could be seen as simplified models of Solid State Fermentations (SSF). For example, without the problems of inter particle diffusion barriers that affect SSF systems. However, cultures of *Aspergillus niger* grown on packed beds of small Amberlite beads imbibed with sugar broths showed higher biomass yields than similar cultures on agar plates (Favela-Torres *et al.*, 1998) when the comparison was made on terms of the initial substrate concentration. But, an important difference between those systems was the specific area, α (Area/Volume, ratio), of the solid support, with a value $\alpha \approx 2 \text{ cm}^{-1}$ for agar plates and $\alpha \approx 100 \text{ cm}^{-1}$ for small Amberlite beads, estimated from data published by Auria *et al.* (1990). Furthermore, Rahardjo *et al.* (2005) found that the yield of α -amylase and total oxygen consumption, per gram of initial substrate, of cultures of *A. oryzae* grown on round flat cakes of wheat grains, was proportional to the specific area of those cakes. However, to the best of our knowledge, there are no quantitative accounts about the combined effect of substrate concentration and specific area of the solid support on the biomass yield of fungal surface cultures.

Thus, this work is related to the effect of the ratio¹ $\sigma_0 = S_0\alpha^{-1}$, on biomass yield ($Y_{X/S}$) where S_0 (gcm^{-3}) is the bulk substrate concentration, and α (cm^{-1}) is the specific area of the solid support. This definition helps to explore the effects of changing the specific area, α , on $Y_{X/S}$ having a fixed value of S_0 , as compared to the effect of changing S_0 , with a fixed α value. The purpose of this study is to show that σ_0 is an important parameter affecting fungal biomass yield. This goal is achieved by showing that surface *A. niger* cultures follow a linear regression model between the measured values of $Y_{X/S}^{-1}$ (g of substrate consumed/g biomass produced) with respect to σ_0 . Also, the correlation between final superficial biomass production ρ_{AM} (gcm^{-2}) and the measured thickness of the fungal layer, h , tries to test whether the estimated volumetric density, calculated as, $\rho_V = \rho_{AM} \cdot h^{-1}$, is constant for different values of σ_0 . Such results would be helpful to define the constraints of *A. niger* layers with different thicknesses as a result of using different σ_0 values. All those results seem useful for the rational design of fungal surface cultures that are

¹The ratio $\sigma_0 = S_0\alpha^{-1}$ can be defined as the initial amount of substrate, $Q = S_0V$, within a solid support with volume, V , divided by the area, A , of the surface culture of the same solid support. This comes from the usual definition $\alpha = A/V$.

commonly utilized in mass screening procedures for strain selection.

2 Materials and methods

2.1 Microorganism

The *Aspergillus niger* strain C28eco3-13 used in this study was previously reported by Téllez-Jurado *et al.* (2006) and is a laccase transformant derived from a wild strain studied by Antier *et al.* (1993). It belongs to the UAM (Universidad Autónoma Metropolitana) fungal collection. Such strain was propagated in Potato Dextrose Agar (PDA) at 30°C and the spores were preserved in vials with silica gel. For the present experiments, a few grains of silica gel were spread onto the surface of 25 mL of PDA medium within 9 cm diameter Petri dishes, followed by incubation at 30°C until black spores were formed (usually at 5 days). The resulting spores were harvested by pouring 100 mL of a sterile Tween-80 0.5% (v/v) solution into the dish and gently stirring the agar surface with a magnetic bar for 5 min. The resulting spore suspension was poured into a sterile Erlenmeyer flask. Spore concentrations were measured with a Neubauer chamber.

2.2 Medium composition

The culture medium was similar to one reported by Kafert (1977), but the only carbon source was glucose labeled as S_0 (gL^{-1}). The salt composition was proportional to $S_0 = 100$: (gL^{-1}): NaNO_3 (15), KCl (5.2), KH_2PO_4 (8.1), K_2HPO_4 (10.4), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (5.2), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.052), EDTA (0.5), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.22), H_3BO_3 (0.11), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0.05), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.016), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.016), $(\text{NH}_4)_6 \text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ (0.11). The, C/ N, ratio was maintained at a constant level (16 gC/ gN). The initial pH value of the medium was adjusted to 6.5 and 1.5% agar was used in all experiments.

2.3 Experimental units

The experimental units were 9 cm Petri dishes ($A = 63.62 \text{ cm}^2$) with different volumes of culture medium (see Table 1). Inoculation was performed with 1 mL containing 10^5 spores and scattered evenly over the surface (lawn pattern). Dishes were stored in a stove at 30°C for different periods of time.

Table 1. Specific growth rates (μ), average volumetric density ($\rho_V = \rho_{AM}h^{-1}$) and degree of glucose utilization (f) of *Aspergillus niger* grown on agar plates with indicated σ_0 , S_0 and α , values.

Exp. No.	σ_0 (gcm ⁻²)	S_0 (gcm ⁻³)	α (cm ⁻¹)	μ (h ⁻¹)	ρ_V (mgcm ⁻³)	f (%)
1	0.0059	0.0125	2.12	0.107±0.002 ^a	0.039	97±1.6
2	0.0118	0.0250	2.12	0.125±0.031 ^a	0.049	98±0.2
3	0.0157	0.1000	6.36	0.098±0.009 ^a	0.054	98±0.9
4	0.0236	0.0500	2.12	0.082±0.003 ^a	0.051	93±0.8
5	0.0394	0.1000	2.54	0.127±0.007 ^a	0.047	98±0.9
6	0.0472	0.1000	2.12	0.090±0.041 ^a	0.043	99±0.5
7	0.0629	0.1000	1.59	0.102±0.008 ^a	0.044	88±7.9
Average				0.104±0.017	0.046±0.005	96±3.8

Specific growth rates (μ) were estimated from equation (1), the values of ρ_{AM} , h and f , were measured as it is indicated in Materials and Methods (see also Fig. 1). Rows with the same superscript do not differ significantly (Tukey comparison test, $p < 0.05$). All experiments in Petri dishes with surface area $A = 63.617 \text{ cm}^2$. The value of specific area, $\alpha = A/V$ ratio.

2.4 Experimental design

The experiments were performed with different values of σ_0 , obtained in two different ways: a) Changing S_0 (gcm⁻³) = 0.0125, 0.025, 0.050 and 0.100, with a fixed agar volume $V = 30 \text{ mL}$ ($\alpha = 2.12, \text{ cm}^{-1}$) or b) changing V (mL) = 10, 25 and 40, ($\alpha = 6.36, 2.54, 1.59, \text{ cm}^{-1}$) with a fixed $S_0 = 0.100, \text{ gcm}^{-3}$. This experimental design is shown in Table 1 numbering the rows with increasing values of $\sigma_0 = S_0\alpha^{-1}$.

2.5 Analysis

The dry weight of the biomass was measured after melting the agar medium. The biomass, together with the agar, was introduced into a baker containing 200 mL of acidified water with HCl (pH 3), which was placed in a microwave oven (Panasonic NV-950B) at level 9 for 2 minutes. The resulting liquid was then filtered through a tared Millipore filter membrane (nominal pore size 0.45 μm), washing through the filter with distilled water and placing it at 60°C until a constant weight was obtained. The total amounts of biomass, X , per agar plate were determined by the dry weight difference, and were expressed as average surface densities ρ_A (gcm⁻²). Growth curves were obtained using triplicate values of ρ_A at eight regular intervals over approximately 96 hours. The experimental values were followed by the logistic equation (1) and the asymptotic values (for $t \rightarrow \infty$) were calculated as, ρ_{AM} .

$$\rho_A(t) = \frac{\rho_{AM}}{1 + Ce^{-\mu t}} \quad (1)$$

where, $C = (\rho_{AM} - \rho_0)/\rho_0$ is an integration constant and the parameters ρ_{AM} , ρ_0 , and μ can be estimated from the least value of the sum of squared residuals between the experimental data and the calculated data points. Calculations were performed with the program Solver (Marquardt algorithm) of an Excel spreadsheet.

Residual glucose present in the filtrate was measured by the DNS method (Miller, 1959). The thicknesses, h (cm), of fungal layers were measured at the end of each run ($\rho_A \approx \rho_{AM}$). Vertical random sections of such layers were made with a conventional scalpel. Every section was observed under a microscope (OLYMPUS KP-D51/D50) adapted with a camera (OLYMPUSKP-D51/D50). Illumination source was the halogen bulb of the microscope (6V/30W) with intensity 4 in a scale from 0 to 7. Measurements were performed using commercial image analysis software (Image J, USA); the images were displayed as a gray images having 512x512 pixels, the thickness of interest was selected manually and measured with a predetermined software function (analyze and measure). For each experiment, 100 independent measurements were made in three separate Petri dishes.

2.6 Statistical analysis

Analysis of variance and multiple average Tukey's comparison tests were performed, with a level of statistical significance $p < 0.05$. The Minitab 15.0 (Pennsylvania, USA) statistical software was used.

3 Results

Table 1 shows that the average degree of substrate depletion was $f = 0.96 \pm 0.04$ and the average specific growth rate was, $\mu = 0.10 \pm 0.02 \text{ h}^{-1}$, both without statistical significance ($p > 0.05$) with respect to changes in σ_0 . Hence biomass production was measured when practically all the substrate was utilized by the fungal mat and with growth rates comparatively similar in all the experiments. The yield coefficient, $Y_{X/S}$, was calculated from the mass balance ($Y_{X/S} V f S_0 = A \rho_{AM}$) as follows:

$$Y_{X/S} = \frac{\alpha \rho_{AM}}{f S_0} \quad (2)$$

It is worth noticing that all quantities on the second term of Eq. (2) are experimental values measured as indicated above. If $\rho_V = \rho_{AM} h^{-1}$, is the average volumetric density of the mycelium, h , is the thickness of the mycelium mat and recalling the definition $\sigma_0 = S_0 \alpha^{-1}$, the following relationship follows:

$$Y_{X/S} = \frac{\rho_V h}{f \sigma_0} \quad (3)$$

Figure 1 shows the trends of experimental values of, h and ρ_{AM} , vs. σ_0 . Both dispersions of experimental data are quite similar to each other. The dispersion of thickness, h , was followed very closely ($R^2 = 0.965$) by the hyperbolic Eq. (4)

$$h = \frac{h_{max} \sigma_0}{K_h + \sigma_0} \quad (4)$$

where, $h_{max} = 0.386 \text{ cm}$, is the extrapolated value for very large σ_0 and $K_h = 0.0431 \text{ gcm}^{-2}$ is the σ_0 value when $h = h_{max}/2$. Table 1 shows that the estimated average of quotient $\rho_{AM} h^{-1}$ was $\rho_V = 0.048 \pm 0.004 \text{ gcm}^{-3}$ with low correlation with respect to σ_0 ($R^2 = 0.046$). Also, the dispersion of experimental values of ρ_{AM} can be followed by Eq. (4) multiplied by a fixed value of, $\rho_V = 0.049 \text{ gcm}^{-3}$ (see Fig. 1). This supports the model where the final value, ρ_V , is approximately constant for all experiments and the main source of biomass increase, with respect to σ_0 , is related to the thickness of the fungal mat. The published discrepancies with this model will be discussed in the following section.

Substitution of Eq. (4) into Eq. (3) leads, after some rearrangement and assuming that $f \approx 1$, to

$$\frac{1}{Y_{X/S}} = \frac{1}{Y_0} + \frac{\sigma_0}{\varepsilon} \quad (5)$$

where, the physiological interpretation of parameters, Y_0 (maximal yield for small σ_0) and $\varepsilon = \rho_V h_{max}$ (maximal biomass surface density for large σ_0), are derived in the Appendix A and commented in the Discussion. The calculated values, corresponding to Fig. 2 were, $Y_0 = 0.50$; $\varepsilon = 0.0149 \text{ gcm}^{-2}$; $R^2 = 0.950$. Therefore, there is consistency between, the saturation model given by Eq. (4) and the empirical biomass yield equation (5), supporting a simple model where *A. niger* grows with approximately constant volumetric density, ρ_V , having a maximal thickness, h_{max} . It is worth noticing that the maximal yield $Y_0 \approx 0.5$ with glucose, is limited by the thermodynamic efficiency of biosynthesis (Heijnen and Roels, 1981).

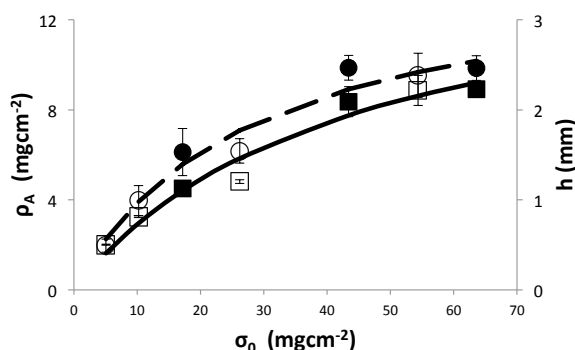


Fig. 1. Correlations between surface density (ρ_{AM} , circles) and bio-film thickness (h , squares) in relation to surface availability (σ_0) of *Aspergillus niger* grown as lawn culture in 9 cm Petri dishes. Open symbols correspond to $S_0 = (\text{gcm}^{-3}) 0.0125, 0.025, 0.050, 0.100$ and $\alpha = 2.12 \text{ cm}^{-1}$. Closed symbols correspond to $S_0 = 0.100 \text{ gcm}^{-3}$ and $\alpha (\text{cm}^{-1}) = 1.59, 2.54$ and 6.36 . Continuous curve was drawn as the least square fit of the saturation equation (4) of h as a function of σ_0 . Interrupted line was drawn as the product of values estimated by equation (4) multiplied by $\rho_V = 0.049 \text{ gcm}^{-3}$.

4 Discussion

As indicated at the Introduction, Rahardjo et al. (2005) studied the effect of changing the value of α of flat rounded cakes made of wheat grains, on the pattern of total oxygen consumption and α -amylase production by *R. oligosporus*. They found a linear correlation of such variables in the range from $\alpha = 1 \text{ cm}^{-1}$ to $\alpha = 12.9 \text{ cm}^{-1}$. This is indirect evidence that $Y_{X/S}$ is proportional to α , as indicated by the mass balance in equation (2) because *R. oligosporus*, should hydrolyze starch in order to grow on wheat grains

and most of oxygen consumption is also linked to the growth process. Present results show in a more precise way how, α and S_0 affect $Y_{X/S}$. This can be done combining eqs. (4) and (5) to become Eq. (6)

$$Y_{X/S} = \frac{Y_0 \varepsilon}{\varepsilon + Y_0 \sigma_0} \quad (6)$$

From Eq. (6) it can be derived that for very low σ_0 ($Y_0 \sigma_0 \ll \varepsilon$), biomass yield will be maximal ($Y_{X/S} \rightarrow Y_0$). But for large σ_0 ($Y_0 \sigma_0 \gg \varepsilon$), biomass yield will be a decreasing function of σ_0 ($Y_{X/S} \approx \varepsilon \sigma_0^{-1}$). Appendix A shows the final mass balance of substrate uptake based on the rates of biosynthesis (μ) and maintenance (m). The combination of such balance with the logistic equation of growth, produces two yield factors, a maximal biomass yield, Y_0 , and another term proportional to the ratio, m/μ . This ratio seems to be an indication of how much substrate was used by non-productive metabolism. For example, when the ratio, m/μ , is small, the yield coefficient is Y_0 . This helps to interpret the physiological meaning of ε as the productivity of the surface culture that is large when the ratio, m/μ is small. The parameter ε has the dimensions of biomass surface density since, according to Appendix A, $\varepsilon = \rho_V h_{MAX}$, is the maximal biomass production on the surface of the solid support.

Our calculations yielded, $\varepsilon = 0.033 \text{ gcm}^{-2}$ from biomass yield data published by Favela-Torres *et al.* (1998), obtained on agar plates with a wild strain of *A. niger*. This result shows that our mutant strain was two times less productive ($\varepsilon = 0.015 \text{ gcm}^{-2}$) when cultured on agar plates than the wild strain studied by them. However, the maximal yield values were quite similar ($Y_0 \approx 0.5$) for both sets of data. Again, our calculations from previous data (Favela-Torres *et al.*, 1998) obtained in columns packed with small beads of Amberlite, we obtained, $\varepsilon = 0.009 \text{ gcm}^{-2}$. This is a smaller value than the aforementioned values of ε obtained on agar plates. It should be noted that the range of initial substrate concentration, S_0 , was quite similar in all those experiments ($0.01 \text{ gcm}^{-3} < S_0 < 0.45 \text{ gcm}^{-3}$) but the values for σ_0 of Amberlite columns were quite different because of large difference in the specific area, since the specific area of agar plates is small ($\alpha \approx 1 \text{ cm}^{-1}$) as compared to the large value of Amberlite beads ($\alpha \approx 100 \text{ cm}^{-1}$) with diameter close to 0.06 cm. Such comparisons help to conclude that, a) The maximum yield, Y_0 , of *A. niger*, seems to concur with the usual biomass yield of aerobic microbial cultures when using glucose as the main source or carbon (Heijnen and Roels, 1981) and, b). The value of ε depends both on the fungal

strain (wild type vs. mutant) and the culture medium (Petri dish vs. Amberlite beads). The importance of such comparison is that yield values should not be compared in terms of bulk substrate concentrations, S_0 , but on the basis of parameter $\sigma_0 = S_0 \alpha^{-1}$. In other words, to compare the yield of different fungal strains, grown on different kinds of solid supports, it is necessary to normalize such comparisons with similar σ_0 values. Otherwise the importance of ε will not be appreciated.

The constancy of the volumetric density, ρ_V , of *Aspergillus niger* found in this work is somewhat different to the reports of Nopharatana *et al.* (2003) and Camacho-Díaz *et al.* (2010) with surface cultures of *Rhizopus oligosporus*. They found a decreasing function of volumetric density, ρ_V , on the thickness, h , in lawn cultures (Nopharatana *et al.*, 2003) or on the radius of circular colonies (Camacho-Díaz *et al.*, 2010). In the present work the variation of ρ_V was found to be small for different values of h in the range, $0.05 \text{ cm} < h < 0.22 \text{ cm}$. One explanation for those differences could be a sharper upper boundary in *Aspergillus niger* as compared to a loose defined boundary of the fungal mat of *Rhizopus oligosporus*.

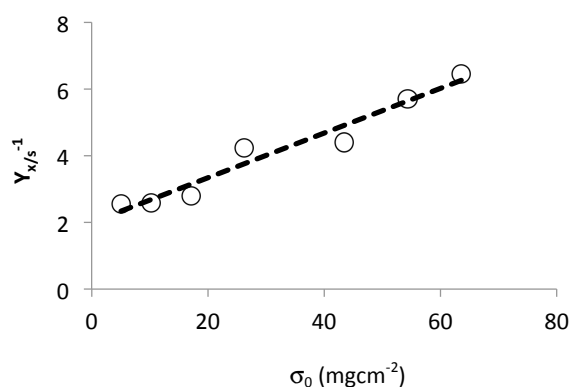


Fig. 2. Correlation between $Y_{X/S}^{-1}$ (estimated as $\sigma_0 \rho_A^{-1}$) vs. σ_0 . Data points indicated as (O). The linear equation, indicated as interrupted line, was defined as, $Y_{X/S}^{-1} = Y_0^{-1} + \sigma_0 \varepsilon^{-1}$. Estimated parameters: $Y_0 = 0.50$, $\varepsilon = 0.0149 \text{ gcm}^{-2}$, $R^2 = 0.950$.

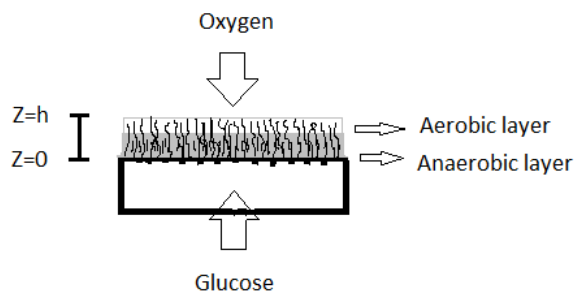


Fig. 3. Diagram of a fungal layer growing on top of an agar plate. Oxygen diffuses downwards (from the air into the mycelium) and glucose moves upwards (from the agar to the top of the fungal layer). Oxygen penetration depth is smaller than the total depth, h , of the mycelium. Axial coordinate, z , is taken from the air interface ($z = 0$) to the agar surface ($z = h$).

This point requires further analysis. For example, it seems worth looking in future work, at biomass density distributions of different fungal species grown on solid surfaces.

Hill (1929) and Pirt (1966, 1967) proposed the concept of oxygen penetration depth, here labeled as h_C , for different kinds of biomass surface cultures. Measurements of h_C done by Rahardjo *et al.* (2002) with microelectrodes inserted in the fungal mat of *Aspergillus oryzae*, reported $h_C = 0.0082$ cm, and $h_C = 0.0060$ cm for *Rhizopus oligosporus*, reported by Oostra *et al.* (2001). Those results indicate that biomass with depth higher than h_C is deprived from oxygen. Oxygen limitation of *Aspergillus niger* was found related to polyol production and reduction of $Y_{X/S}$ to values close to 0.25 (Diano *et al.*, 2006). This phenomenon has been studied in solid-state fermentations by Gutiérrez-Rojas *et al.* (1995) and Ruitjer *et al.* (2004). Also, Olsson and Jennings (1991) described the phenomenon of substrate translocation in fungal cultures. Hence, thick layers of *A. niger*, seem to be made of an uppermost and thin aerobic layer ($h < h_C$) with $Y_{X/S} \approx 0.5$ and a deep anaerobic layer ($h > h_C$) with lower $Y_{X/S}$, producing secondary metabolites and trans-locating the excess substrate from the agar plate to the top aerobic layer (Fig. 3). The optimal value $\sigma_0 = 0.001$ gcm^{-2} can be estimated using Eq. (3) when, $f \approx 1$; $H \approx 0.01$ cm; $Y_{X/S} = 0.5$, and $\rho_V = 0.05$ gcm^{-3} . For example, most recipes for microbial cultures recommend $S_0 = 0.01$ gcm^{-3} , and the corresponding optimal specific area would be $\alpha = 10$ cm^{-1} . That is, an agar plate of height 0.1 cm (see Appendix B). This means that cultures using thicker agar plates could

have a mixture of aerobic and anaerobic metabolism.

Appendix B provides a way to estimate the optimal characteristic length λ for different shapes of solid support, plates ($\lambda = H$), cylinders ($\lambda = R$) or spheres ($\lambda = R$). Such calculations help to choose the geometry and size of particles supporting the growth of thin fungal layers having maximal biomass yield for a given substrate concentration, S_0 , and physiological conditions (Y_0 , h_C , and ρ_V values). This result could be important for the design of screening procedures requiring a specific kind of metabolism. In example, the use of very thin agar plates for aerobic metabolism and thick plates for different mixtures of aerobic and anaerobic metabolism. Furthermore, hypertonic syrups ($S_0 \approx 0.1$ gcm^{-3}), require small solid particles with α values higher than 100 cm^{-1} in order to maximize biomass yields. For example, spherical particles with diameter smaller than $d = 0.06$ cm. Therefore, the proper selection of σ_0 seems to be an important parameter to orient biomass yield (and fungal metabolism) of surface cultures.

Conclusions

Mass balance of surface fungal cultures is a direct way to study how the biomass yield, $Y_{X/S}$, is affected by the combined effect of specific area, α (cm^{-1}), of the support and the bulk initial substrate concentration, S_0 (gcm^{-3}), using the composite parameter, $\sigma_0 = S_0\alpha^{-1}$. Measurements of the final surface density, ρ_{AM} (gcm^{-2}) and the final thickness, h (cm), with changes of S_0 or α , independent from each other, led to the conclusion that the estimated volumetric density, $\rho_V = \rho_{AM}h^{-1}$ was nearly constant for all studied values of σ_0 whereas the thickness, h , was hyperbolic on σ_0 . This supports that fungal mats of *Aspergillus niger* grow mainly at the expense of the thickness, h , which is bounded to a maximal value, h_{max} . Linear correlation between $Y_{X/S}^{-1}$ and σ_0 , indicated that fungal surface cultures have a maximal biomass yield, $Y_{X/S} = Y_0$ when the parameter, σ_0 , is very low. This linear correlation has a slope, $1/\varepsilon$, where, ε is the maximal productivity of the fungal culture. Maximal biomass yield is obtained with fungal layers thinner than the reported oxygen penetration depth ($h_C \approx 0.01$ cm). Conversely, when σ_0 is large (large amounts of substrate available per unit area) fungal mats will be thicker than the oxygen penetration depth. That is, thicker than 0.01 cm and according the experimental results of Oostra *et al.* (2001) and Rahardjo *et al.* (2002) implying that the cells below

oxygen penetration depth will be anoxic. The fact that biomass reaches a maximal density ($\rho_V < 0.05 \text{ g cm}^{-3}$) could be taken as an indication of a constraint between the porosity of the fungal mat, higher than 75%, and the rate of oxygen consumption. Namely, for lower porosities (higher densities), oxygen transfer rate will be lower than oxygen demand. A practical consequence of this analysis is the importance of the combined parameter $\sigma_0 = S_0\alpha^{-1}$ affecting the biomass yield, $Y_{X/S}$, in a reproducible way. This parameter seems important for the optimization of strains, under normalized conditions, using fungal surface cultures.

Nomenclature

A	area of the agar plate (cm^2)
A_S	surface area of the solid support (cm^2)
C	oxygen concentration (mg cm^{-3})
D	diffusion coefficient ($\text{cm}^2 \text{ s}^{-1}$)
H	height of the agar plate (cm)
h	thickness of the fungal layer (cm)
h_{max}	maximal thickness of the fungal layer (cm) for very large σ_0
h_C	oxygen penetration depth in the fungal layer (cm)
K	saturation constant, σ_0 value to reach $h_{max}/2$ (g cm^{-2})
L	length of cylindrical particles (cm)
M	$= Y_0 S_0 / \rho_V$ relative biomass yield (dimensionless)
m	maintenance coefficient ($\text{gS gX}^{-1} \text{ h}^{-1}$)
Q_0	$= S_0 V_S$ initial amount of substrate within the solid support (g)
q_s	specific metabolic rate ($\text{gO}_2 \text{ gX}^{-1} \text{ s}^{-1}$)
R	radius of spherical or cylindrical particles (cm)
S_0	initial substrate concentration (g cm^{-3})
t	time (s or h)
V_S	support volume (cm^3)
V_X	volume occupied by the fungal layer (cm^3)
X	total amount of biomass (g)
Y_0	maximal biomass yield (gX gS^{-1})
$Y_{X/S}$	biomass yield (gX gS^{-1})
z	depth within the fungal layer (cm)

Greek letters

α	$= A/V$ specific area of the solid support (cm^{-1})
ε	$= \rho_V h_{max}$ efficiency factor (g cm^{-2})
Λ	relative thickness (h_C/L , dimensionless)
λ	characteristic length of solid supports (cm)
μ	specific growth rate (h^{-1})

ρ_A	biomass surface density (g cm^{-2})
ρ_{AM}	maximal biomass surface density (g cm^{-2})
ρ_V	dry mass volumetric biomass density (g cm^{-3})
ρ_0	initial biomass surface density (g cm^{-2})
σ_0	quotient S_0/α (g cm^{-2})

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Appendix A

Mathematical model of mold growth on solid surfaces

It is assumed that a mycelial fungus grows over a solid surface with area, A , producing quantity, X (grams in dry basis) according to the logistic equation, as follows

$$\frac{dX}{dt} = \mu \left[1 - \frac{X}{X_M} \right] X \quad (\text{A.1})$$

Where, μ (h^{-1}), is the initial rate of growth when, $0 < X \ll X_M$, and X_M is the maximal biomass quantity to be produced on that solid surface. The limiting substrate, i.e. the carbon source, is distributed evenly within the solid support with volume, V , at initial concentration, S_0 . Hence the initial amount of substrate is $Q_0 = S_0V$. The rate of substrate uptake is given by the following expression.

$$- \frac{dQ}{dt} = \frac{1}{Y_0} \frac{dX}{dt} + mX \quad (\text{A.2})$$

where, Y_0 , is the maximal yield (g of X per g of S) and m (g $S/\text{g}X \cdot \text{h}$), is the maintenance coefficient.

Combining Eqs. (A.1) and (A.2) and after integration, the following expression is obtained

$$(Q_0 - Q) = \frac{X - X_0}{Y_0} + \frac{\mu X_M}{m} \ln \left[\frac{1 - X_0/X_M}{1 - X/X_M} \right] \quad (\text{A.3})$$

After some rearrangement, assuming that, $X_0 \ll X < X_M$, and defining, $\phi = X/X_M$, it is possible to estimate the decrease of the substrate, $-\Delta Q = Q_0 - Q$, as follows

$$-\Delta Q = \frac{X}{Y_0} + \frac{mX_M}{\mu} \ln \left[\frac{1}{1 - \phi} \right] \quad (\text{A.4})$$

After a very long period of time, $\phi \rightarrow 1$, $X \rightarrow X_M$, and $Q \rightarrow 0$

$$Q_0 \approx \frac{\phi X_M}{Y_0} + \frac{mX_M}{\mu} \ln \left[\frac{1}{1 - \phi} \right] \quad (\text{A.5})$$

Assuming, $\phi = 0.99996$, an approximate estimate of $Y_{X/S}^{-1} = Q_0/X_M$, is obtained

$$\frac{1}{Y_{X/S}} \approx \frac{1}{Y_0} + \frac{m}{\mu} \ln \left[\frac{1}{1 - 0.99996} \right] = \frac{1}{Y_0} + 10 \frac{m}{\mu} \quad (\text{A.6})$$

Equation (A6) shows that the inverse of the apparent yield, $Y_{X/S}^{-1}$, increases with the ratio, m/μ , which is an indication of how much substrate was used to make other products different from biomass. When the ratio, m/μ is very small, the yield is maximal ($Y_{X/S} \rightarrow Y_0$)

The experimental evidence shows that the inverse yield is proportional to σ_0 , according to Eq. (A7) that can be compared to Eq. (A6)

$$\frac{1}{Y_{X/S}} = \frac{1}{Y_0} + \frac{\sigma_0}{\varepsilon} \quad (\text{A.7})$$

Therefore, $1/\varepsilon$, is an index of the efficiency of biomass production because it is proportional to the ratio, m/μ . Furthermore, if the volumetric biomass density, ρ_V , is almost constant, the total amount of biomass over the surface is given by

$$X = A\rho_{AM} = A\rho_V h \quad (\text{A.8})$$

Where, h , is the average thickness of the fungal layer distributed with final average surface density, ρ_{AM} . Again, experimental evidence shows that Eq. (4) holds, and for the sake of clarity is repeated as

$$h = \frac{h_{max}\sigma_0}{K + \sigma_0} \quad (\text{A.9})$$

When $\sigma_0 \gg K$, the thickness reaches its maximal value $h \approx h_{max}$, and from Eq. (3) and assuming

complete substrate utilization ($f \approx 1$), Eq. (A.10) is obtained

$$\frac{1}{Y_{X/S}} \approx \frac{\sigma_0}{\rho_V h_{max}} \quad (\text{A.10})$$

From Eq. (A.7) for large values of σ_0 , Eq. (A.11) is obtained

$$\frac{1}{Y_{X/S}} \approx \frac{\sigma_0}{\varepsilon} \quad (\text{A.11})$$

Hence, comparing eqs. (A10) and (A11), the following identification is obtained, $\varepsilon = \rho_V h_{MAX}$. Therefore, the parameter ε is the maximal amount of biomass per unit area. For example, a mutant strain that is diverting most of the substrate to collateral products will be less productive than a wild type that has evolved to be highly productive with little diversion of the substrate in collateral products. Also, a column of packed bed particles could have more hindrance for the growth of the fungal layer than an open surface of a Petri dish. This is analyzed in the Discussion.

Appendix B

Optimal size of supports for solid state fermentation

Fungal cultures growing on the surface of solid support will have optimal dimensions when the initial amount of the substrate, Q_0 , is just enough to support a fungal layer that is fully oxygenated. In order to calculate the size of the solid support it is necessary to make an analysis of mass transfer of oxygen through the fungal mat. This should consider the fact that there are two inter-phases, a) A lower inter-phase with the solid support where the nutritive liquor is embedded and b) An upper inter-phase with air from which oxygen diffuses in a passive way following the Fick's law as follows. The mass resulting mass balance is given in (B1) and is illustrated in Fig. 3.

$$\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial z^2} - q_s \rho_V \quad (\text{B.1})$$

Where, $z = 0$, corresponds to the aerial inter-phase, and $z = h$, corresponds to the inter-phase with the solid substrate. The steady-state solution of Eq. (B1) yields the following equation

$$\frac{\partial C}{\partial t} = 0 \rightarrow C = C_0 \left[1 - \frac{z}{h_C} \right]^2 \quad (\text{B.2})$$

This equation is consistent with the boundary condition, $C = C_0$, for $z = 0$, and also with $C \approx 0$,

for $z = h_C$, as indicated by Hill (1929) and Pirt (1966, 1967). The value of $h_C = 0.006$ cm has been reported by Oostra *et al.* (2001) for *Rhizopus oligosporus*, and $h_C = 0.0082$ cm by Rahardjo *et al.* (2002) for *Aspergillus oryzae*. Thus, if $h > h_C$, oxygen will be practically absent (anaerobic layer) as suggested in Fig. 3.

If ρ_V is the average volumetric density, the following mass balance holds

$$\rho_V V_X = Y_{X/S} S_0 V_S \quad (\text{B.3})$$

Where, V_X is the volume of the fungal layer and V_S is the volume of the solid support. An important restriction on fungal growth is to assume that the amount of initial substrate, $Q_0 = S_0 V_S$, is just enough to support the growth of an aerobic fungal layer with uniform thickness h_C and $Y_{X/S} = Y_0$. Therefore, the optimal ratio, V_X/V_S , can be derived from Eq. (B.3) yielding

$$\frac{V_X}{V_S} = \frac{Y_0 S_0}{\rho_V} \quad (\text{B.4})$$

Restrictions for a flat solid support of fungal cultures

Assuming that the solid support is a slab (agar plate), with area, A , and height H , ($V_S = AH$) and recalling that the final surface fungal density is $\rho_{AM} = \rho_V h_C$, the following relation holds

$$\frac{h_C}{H} = \frac{Y_0 S_0}{\rho_V} \quad (\text{B.5})$$

Defining, $\Lambda = h_C/H$ and $M = Y_0 S_0/\rho_V$, the following equation is obtained

$$\Lambda = M \quad (\text{B.6})$$

This simple equation indicates that the optimal height, H_{opt} , of the solid support is proportional to the product of the oxygen penetration depth, h_C , times the ratio between the average biomass density over the optimal yield as shown by

$$H_{opt} = h_C \left[\frac{\rho_V}{Y_0 S_0} \right] \quad (\text{B.7})$$

For example, for a fungal culture, with $h_C = 0.01$ cm, supplied with initial glucose concentration, $S_0 = 0.01$ gScm⁻³, maximal yield, $Y_0 = 0.5$ gX/gS, and biomass density, $\rho_V = 0.05$ gXcm⁻³, the optimal height of the agar plate will be $H_{opt} = 0.1$ cm. For cultures with $H > H_{opt}$, the apparent yield will be lower than Y_0 because part of the biomass will be anaerobic. This

calculation can have implications for the design of culture media with different values of biomass yields, but with the same initial substrate concentrations, by choosing different heights of the agar plate.

Restrictions for solid supports of fungal cultures with cylindrical and spherical geometries

The fungal layer can be considered as a shell with external radius, $R + h_C$, and with internal radius, R , length, L , and volume, $V_X = (4\pi/3)[(R + h_C)^2 - R^2]L$. Also, the solid support has a volume $V_S = (4\pi/3)R^2L$. Hence, the mass balance Eq. (B.3) leads to

$$\rho_C [(R + h_C)^2 - R^2] = Y_{X/S} S_0 R^3 \quad (\text{B.8})$$

Solving for, $\Lambda = h_C/R$, as a function of M , the following equation is obtained.

$$\Lambda = (M + 1)^{1/2} - 1 \quad (\text{B.9})$$

In a similar fashion, the mass balance of Eq. (B.3) yields Eq. (B.8) for solid support particles with spherical geometry.

$$\Lambda = (M + 1)^{1/3} - 1 \quad (\text{B.10})$$

The corresponding optimal radius, R_{opt} , for cylindrical or spherical particles are given by eqs. (B.9) and (B.10), respectively

$$R_{opt} = \frac{h_C}{(M + 1)^{1/2} - 1} \quad (\text{B.11})$$

$$R_{opt} = \frac{h_C}{(M + 1)^{1/3} - 1} \quad (\text{B.12})$$

For example, for a fungal culture grown on the surface of small spherical particles, with $h_C = 0.01$ cm, supplied with initial glucose concentration, $S_0 = 0.1$ gScm⁻³, maximal yield, $Y_0 = 0.5$ gX/gS, and biomass density, $\rho_V = 0.05$ gXcm⁻³, the optimal particle radius will be $R_{opt} = 0.038$ cm. This calculation indicates that fungal cultures with hypertonic syrups can have maximal biomass yield if they grow on small solid particles embedded with the hypertonic broth. It is worth noticing that reported values of oxygen penetration depths ($h_C < 0.01$ cm) and measured biomass density $\rho_V = 0.05$ gcm⁻³, give an approximate superficial density, $\rho_{AM} = 0.005$ gcm⁻², which is lower than the parameter $\varepsilon = 0.009$ gcm⁻² discussed in Appendix A and calculated from data published by Favela-Torres *et al.* (1998).