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IMPORTANCE OF DIFFUSSIVE FLUXES ON THE CARBON FATE WITH *Aspergillus niger* SURFACE CULTURES

IMPORTANCIA DE LOS FLUXES DIFUSIVOS EN EL DESTINO DEL CARBONO EN CULTIVOS SUPERFICIALES DE *Aspergillus niger*

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Abstract

This paper presents a simple experimental system to study surface cultures made of test tubes with height, L = 18.5 cm and internal diameter, D = 3.3 cm (A = 8.553 cm^2). In this system, surface cultures of *Aspergillus niger* were supplied with 1.2 g total glucose but with different agar depths, H: 1.2, 2.3, 3.5 and 4.6 cm. Experimental variables were, surface uptake of glucose, biomass production, citric acid production and carbon dioxide as well as the final height of mycelia. The results show a positive linear correlation between S_0 and average glucose flux (J_S) as well as the respiratory rate, Rc. However, the flux of biomass (J_X) and citric acid (J_P) are negatively correlated to substrate initial concentration (S_0). The results suggest that carbon fate in these surface cultures depends on the magnitude of substrate influx to the fungal mat and the substrate uptake seems to be controlled by the rate of diffusion of substrate of the medium to the surface of culture. Such results support the need to understand the importance of J_S, in order to control carbon fate in fungal surface cultures.

Keywords: surface culture, *Aspergillus niger*, uptake glucose, fate of carbon, citric acid.

Resumen

En el presente trabajo se presenta un sistema sencillo para el estudio de cultivos en superficie, el cual consiste en tubos de ensayo con longitud, L= 18.5 cm y diámetro, D=3.3 cm. El sistema experimental fue evaluado con cultivos superficiales de *Aspergillus niger* que contenían 1.2 g de glucosa total pero con diferentes alturas de agar (H = 1.2, 2.3, 3.5 y 4.6 cm). Se midió el consumo de glucosa, y la producción de biomasa, ácido cítrico y bióxido de carbono, así como la altura del micelio. Los resultados muestran una correlación lineal positiva de la concentración inicial de sustrato (S_0) con el flux promedio de glucosa (J_S) y la tasa respiratoria, Rc. Sin embargo, el flux de biomasa (J_X) y ácido cítrico (J_P) mostraron una correlación negativa con S_0. Los resultados indican que el destino del carbono consumido por los cultivos fúngicos superficiales depende de la magnitud del flux de sustrato que se le suministre al cultivo y, a su vez, el consumo del sustrato es controlado por la tasa de difusión del sustrato desde el medio a la superficie del cultivo. Estos resultados sugieren la necesidad de entender la importancia de J_S, en el control del destino del carbono en cultivos fúngicos superficiales.

Palabras clave: cultivos superficiales, *Aspergillus niger*, consumo de glucosa, distribución de carbono, ácido cítrico.

1 Introduction

The carbon fate in fungal surface cultures may have three options: Biomass, CO_2 and other metabolites such as organic acids, enzymes (López-Flores *et al*., 2016) or polyiols (Martínez-Corona *et al*.,2015). Carbon preferential fate for the production of one or another product is important in food spoilage because their physiology seems to be closely linked to Solid-State Fermentation processes (SSF) since in most practical cases, these kind of processes are made of microbial cultures grown on the surface of solid substrates. Nevertheless, the relationship between the diffusive fluxes created by the molds within the solid

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substrate and the fate of carbon is poorly understood. Nopharatana *et al*. (1998) formulated a mathematical model assuming plug-flow grow of the mold above the agar plates where substrate was transported from the agar plate to the uppermost mycelial model by diffusive substrate translocation (Olsson and Jennings, 1991) along the mycelial syncytium. Rahardjo *et al*. (2002) showed that oxygen penetration within a flat growing colony of *A. oryzae* was less than 0.01 cm of mycelial height. But in many cases of food spoilage, mold layers have a depth higher than 0.1 cm. In such cases, most of the mycelium is living in micro aerophilic conditions. Furthermore, Diano *et al*. (2006) reported that oxygen availability is a crucial variable on the fate of carbon substrates consumed by continuous submerged cultures of *A. niger* favoring the production of polyols such as trehalose, erythrol and glycerol with low oxygen tensions within the culture. Ortega-Sánchez *et al*. (2012) have shown that an excess of glucose available for *A. niger* surface cultures diminish the biomass yields and this is consistent with previous comparative work between *A. niger* grown on surface cultures, SSF on small glass beads and submerged cultures in shake flasks (Favela *et al*., 1998).

In this study shows the importance of the effect of average glucose flux (J_S) on the carbon fate (citric acid, biomass or CO_2). The experiments were performed in surface cultures with different agar depth but, maintaining constant amount glucose, VS_0.

2 Materials and methods

2.1 Biological material

Aspergillus niger C28B25 was grown in Erlenmeyer flask of 125 mL with 30 mL of Potato Dextrose Agar (PDA) and incubated at 30° C. Spores were harvested from 5 day cultures with 30 mL of sterile water containing 0.05 % of Tween 80 (Tellez-Jurado *et al*., 2006). The spore suspension was used as inoculum.

2.2 Culture medium and experimental units

The culture medium was Yeast Extract Peptone Dextrose Agar (YEPDA) containing (mg/mL): glucose 20, peptone of casein 5, yeast extract 5 and agar 10. Culture medium was sterilized at 93° C for 15 minutes in cylindrical test tubes (18.5 cm height, 3.3 cm internal diameter, 177 mL volume and surface area 8.553 cm^2); spore concentration was adjusted to 1×10^6 spores mL^{-1} of culture and incubated at 30° C with humidified air flux of 20 mL/min. Four experiments were realized with different agar depth H: 1.1, 2.3, 3.5 and 4.6 cm. All with the same amount of glucose (Table 1). The respiratory activity of a set of experimental duplicates were examined (Hernández-Rodriguez *et al*., 2009), taking automating samples every 90 minutes by a system of measurement of CO_2 and O_2 connected online (Fig. 1).

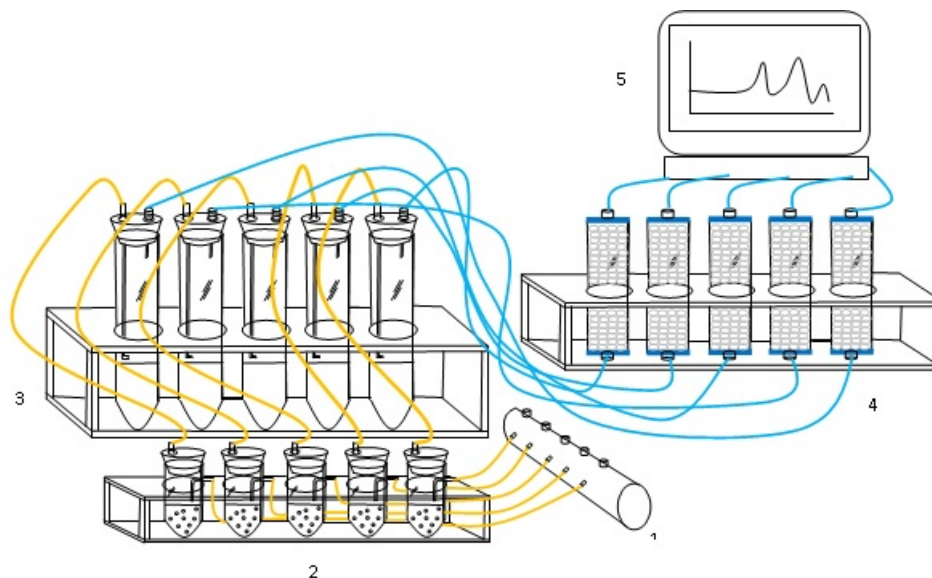

Fig. 1: Experimental system. 1. Air distributor, 2. Humidificator, 3. Superfitial culture system, 4. Dehumidifier (silicates), 5. Quantification system of CO_2 and O_2 (metabolimeter).

Table 1. Experimental design of surface cultures of *Aspergillus niger* on agar cylinders having a constant amount of glucose and different depths and volumes with indicated initial glucose concentrations, S_0.

Trial (No.)	Agar depth, H (cm)	Total glucose amount (g)	S_0^a (mgcm^{-3})	Area/Volume, α (cm^{-1})
1	1.1	1.2	125	0.91
2	2.3	1.2	55	0.43
3	3.5	1.2	40	0.29
4	4.6	1.2	30	0.22

[a] The composition of the culture medium was modified to be proportional to the glucose concentration.

2.3 Determination of glucose uptake and biomass production

In order to quantify the average glucose uptake by *A. niger* C28B25, the culture medium was acidified with 1 mL of a solution of HCl 0.25% (v/v) per mL of medium, then the tubes were heated in a water bath for 15 minutes to solubilize the agar. Once the medium solubilized was filtered through Whatman paper filter No. 41 and the filtrate was used to measure the residual glucose and citric acid produced using the enzyme-LQ® Glucose kit (SPIN REACT) and high performance liquid chromatography (HPLC), respectively (Schlosser *et al*., 1997). On another hand, is important to mention that also the glucose initial in the medium was measured. Material retained on the filter paper was used for biomass produced quantification (prior to constant weight) by dry weight.

2.4 Citric acid quantification

Citric acid quantification was carried out using an HPLC (LC 250 Perkin Elmer®) with a REZEXTM® column (8% cross-linked CALCIUM). Analyses were performed at 60° C using water as the mobile phase adjusting flow at 0.6 mL / min (Gutierrez-Rojas *et al*., 1996). The samples used for citric acid determination were centrifuged at 5000 rpm min-1 for 3 minutes and subsequently filtered through a nylon membrane 0.45 μm.

3 Results

3.1 Effect of initial substrate concentration on the average substrate flux

Figure 2 shows a linear correlation between substrate initial concentration (S_0) and the average flux of substrate, J_S (mgcm^{-2}s^{-1}) across the interfacial area (A = 8.553 cm^2) between the agar and the mycelial mat calculated from data obtained in experiments where the total amount of substrate was fixed (VS_0 = 1.2 g) but the agar depth was changed, H (cm) = 1.1, 2.3, 3.5, 4.6. All tubes had an interior diameter D = 3.3 cm. Average, J_S, was calculated with the equation.

$$J_S = -\frac{\Delta S}{\alpha \Delta t} \tag{1}$$

Where, $\alpha = A/V$, is the specific area of the culture medium. Figure 2 shows a linear correlation (R^2 = 0.99) of the following form

$$J_S = k_J S_0 - J_0 \tag{2}$$

Where, $k_J = 3 \times 10^{-7}$ cms^{-1}, is the kinetic constant of fungal glucose uptake (permeability) and the constant, $J_0 = 3 \times 10^{-7}$ mgcm^{-2}s^{-1}, is a threshold flux of glucose uptake required to start the growth process, that is whenever, $S_0 < 10$ mgcm^{-3}, (10 gL^{-1}) there is not a sustainable growth process of *A. niger*. This agrees with the basic notion that substrate flux follows the Fick laws (Fick, 1855) within a solid reservoir (the agar medium) moved by a sink (fungal glucose uptake) having a minimal maintenance metabolic activity necessary to support fungal growth. Whereas membrane transport system are saturable, it is noteworthy that the linear correlation between J_S and S_0 shown in figure 2, may have deviations for S_0 >125mgcm^{-3}.

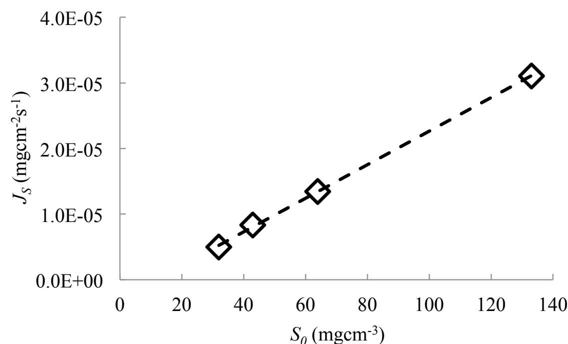

Fig. 2: Positive linear correlation of the average substrate flux, J_S(mgcm^{-2}s^{-1}), with initial substrate concentration, S_0 (mgcm^{-3}), $J_S = 3 \times 10^{-7}$ (S_0 - 10); R^2 = 0.99.

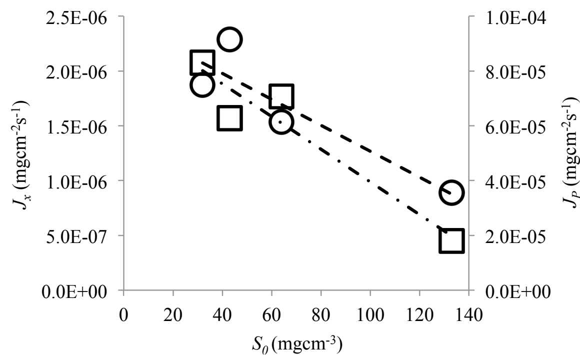

Fig. 3: Negative correlations between the average biomass and citrate rates, J_X (o), J_P (□) expressed as, mgcm^{-2}s^{-1}, and initial substrate concentration, S_0 (mgcm^{-3}). $J_X = 1\times10^{-8}$ (200 - S_0); R^2=0.82; $J_P = 6\times10^{-7}$ (166 - S_0); R^2=0.91.

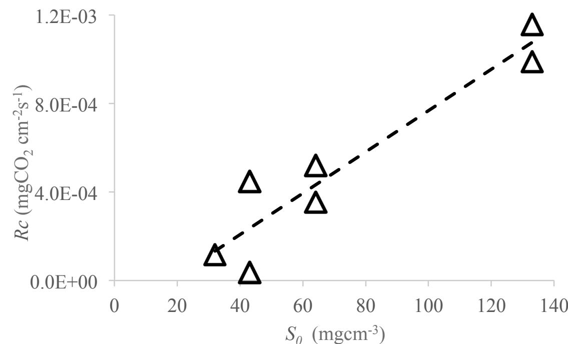

Fig. 4: Positive linear correlation between the respiratory rate, R_C, and initial concentration, S_0. The empirical equation was, $R_C = 9\times10^{-6}$ (S_0 -22); R^2 = 0.89. Each data point corresponds to an independent experiment. Best fit was done using Marquardt Excel algorithm (Solver) adjusting six parameters with more than 80 measurements as indicated in the Appendix.

3.2 *Effect of initial substrate concentration on the rates biomass growth and citrate production*

In a similar fashion as the preceding section, the average rate of surface biomass growth, J_X (mgcm^{-2}s^{-1}) and citrate production, J_P (mgcm^{-2}s^{-1}), were estimated by the following equations and shown in Fig. 3.

$$J_X = \frac{\Delta X}{\alpha \Delta t} \tag{3}$$

$$J_p = \frac{\Delta P}{\alpha \Delta t} \tag{4}$$

Again, J_X and J_P, had linear correlations with S_0 (R^2 = 0.82, 0.91, respectively) but with negative slopes supporting the notion that the corresponding yield coefficients ($Y_{X/}S = -\Delta X/\Delta S$) and ($Y_{P/S} = -\Delta P/\Delta S$) decrease with increasing values of average substrate flux, J_S, as shown by equations (5) and (6)

$$J_x = J_{x0} - aS_0 \tag{5}$$

$$J_P = J_{P0} - bS_0 \tag{6}$$

Where, $J_{X_0} = 2\times10^{-6}$ mgcm^{-2}s^{-1}, and, $J_{P_0} = 1\times10^{-4}$ mgcm^{-2}s^{-1}, are the maximal surface rates of biomass and citric acid production, respectively, and, $a = 1\times10^{-8}$ cms^{-1}, $b = 6\times10^{-7}$ cms^{-1}, are empirical inhibition constants of biomass and citric acid production, respectively. A decreasing correlation of biomass and citric acid production (J_X and J_P) with increasing substrate flux (J_S) is consistent with overflow of metabolic capacity of biosynthesis by increasing inflow of glucose within the cells.

3.3 *Effect of initial substrate concentration on the respiratory rate (R_C)*

The respiratory rate, R_C, is defined in the Appendix as a parameter approximately equal to four times the peak value in a respiratory curve and helps to correlate carbon dioxide production per surface unit with the S_0 that is the driving force of the substrate influx to the fungal mat as shown in Fig. 2. Figure 4 shows a positive linear correlation (R^2 = 0.89) between R_C and S_0 as shown in equation (7)

$$R_C = qS_0 - R_{C0} \tag{7}$$

Where, $R_{C0} = 2\times10^{-4}$ mgcm^{-2}s^{-1}, is threshold value of R_C, $q = 9\times10^{-6}$ (mgCO$_2$/gS)cms^{-1}, is a conversion rate constant of substrate to CO_2. Note that, R_C = 0, when S_0 = 22 mgcm^{-3}. This threshold of S_0 is nearly ten times lower than the threshold value for glucose uptake suggesting that respiratory activity is a more sensitive function of substrate inflow. The opposite sign of the slopes in equations (2) and (7) as compared to the negative value of the slopes in equations (5) and (6) are consistent with partial overspill of substrate through the respiratory pathway (production of CO_2) whenever the biosynthetic pathways (biomass and citrate production) are overflown.

3.4 *Effect of initial substrate concentration on mycelial height and volumetric biomass density*

Fungal organisms grow on top of solid surfaces producing networks (mats) of filamentous structures

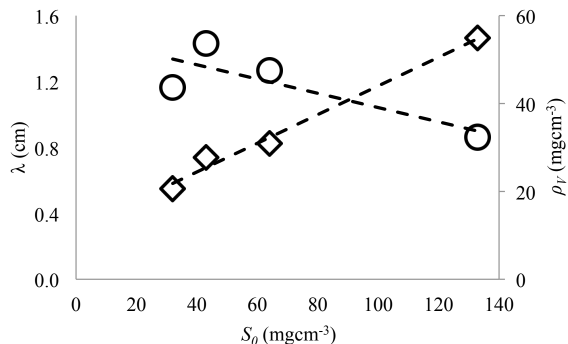

Fig. 5: Linear correlations between mycelial height (λ,◇) or volumetric density, ρ_V (o) vs. initial substrate concentration, S_0, of surface cultures of *A. niger*. The empirical equations were, λ =0.0087(S_0) + 0.3032; R^2 = 0.99; ρ_V = -0.1624(S_0) + 55.3; R^2 = 0.67.

with a height, λ (cm) and average surface density, ρ_A, estimated as the quotient between total biomass, ΔX (mg DW), over the solid surface, A. In turn, volumetric biomass density, ρ_V (mgcm^{-3}), can be estimated with following expression.

$$\rho_V = \frac{\Delta X}{A\lambda} \tag{8}$$

The porosity, ϕ, of the fungal mat can be calculated from the fact that microbial biomass is made of 20% solids and 80% water. This yields the following expression.

$$\phi = 1 - \frac{\rho_V}{0.2} \tag{9}$$

Fig. 5 shows a linear increase of, λ vs. S_0, and a linear decrease one between ρ_V vs. S_0. Those findings are consistent with results shown in Figs. 2 and 3, and the notion that higher substrate fluxes through the fungal mat decrease biosynthesis. That is, fungal mats grow thinner with but taller when diffusional substrate fluxes are increased as a result of higher substrate concentration. Therefore, according to equation (9) and Fig. 4, fungal networks increase their porosity, from ϕ = 0.75 to ϕ = 0.84, with increasing substrate influxes, favoring faster gas exchange rates with the air phase.

3.5 *Effect of initial glucose concentration on the fate of carbon in surface cultures of A. niger*

Table 2 shows the calculated fate of total carbon uptake (100%) as a function of initial glucose concentration in the experimental design shown in Table 1. It should be noted that mass balance was done with the difference between initial and final substrate concentrations as compared to the final amounts of biomass and citric acid, together with the integrated values of carbon dioxide. Therefore there are some differences between the carbon biomass yield given in Table 2 and the estimated average rates of biomass production shown in Fig. 3. However, there is consistency on the carbon fate accumulated in citric acid that decreases with S_0, and the accumulated value of carbon recovered in CO_2 that increases with S_0. According to Fig. 3, biomass rate of production decreases with S_0, but the average carbon assimilation in the biomass remains around 40%. This could be explained by a long pathway of carbon translocation along increasing values of biomass height (λ) shown in Fig. 4 even though the average rate of biomass production is decreasing. Incomplete carbon recovery ($\approx$ 20% to 25%) shown in Table 2 could be due to possible production of volatile compounds, such as ethanol, that were not trapped in a condenser or some other unknown metabolic products. Nevertheless, this mass balance concurs with the idea that citrate metabolic pathways can be overflown in surface cultures of *A. niger* by excessive influx of glucose to the mycelial mat and that carbon dioxide production can work as a partial relief of such overflow.

Average glucose influx, J_S, vs. the fate of carbon uptake (%) in surface cultures of *Aspergillus niger* with indicated initial glucose concentration, S_0.

S_0 (mgcm^{-3})	J_s (mgcm^{-2}s^{-1})	C to Biomass (%)	C to citric acid (%)	C to CO_2 (%)	C recovered (%)
30	5.0×10^{-6}	30	39.8	7	77
40	8.3×10^{-6}	41	24.6	16	81
55	1.4×10^{-5}	36	25.0	21	83
125	3.1×10^{-5}	40	5.8	27	73

Data are average values obtained in two independent replicates of each trial indicated in Table 1.

4 Discussion

Modeling of oxygen and glucose diffusion both in solid particles and fungal bio-layers has been done by Rajagopalan and Modak (1995). They considered the diffusion of glucose from bulk to the biofilm and also diffusion of oxygen from the atmosphere to the interior of the biofilm and assuming first order kinetics for oxygen consumption predicted a maximal biomass density, 20 mgcm^{-3} $< \rho_V <$ 50 mgcm^{-3}. However they did not consider metabolic activities in the anoxic part of the biofilm and assumed this to be "inactive cell mass". Rahardjo *et al*. (2002) found that the penetration depth of oxygen in the biofilm of *Aspergillus oryzae*, was close to 0.006 cm. The model of Nopharatana *et al*. (1998) considered substrate translocation from the bulk to the uppermost region of the biofilm as described by Olsson and Jennings (1991) but none of those authors have considered important metabolic differences between the anoxic and oxygenated regions of the biofilm. For example that glucose can be partially oxidized in the anoxic region and completely oxidized in the oxygenated region of the biofilm. Also, none of those authors have considered the possibility that anoxic metabolism can be channeled to different pathways as a function of balance between local oxygen concentration and biological oxygen demand. For example, ethanol production does not require oxygen whereas citric acid production would require a 1.5 moles of oxygen per mole of glucose and complete oxidation would require 6 moles of oxygen per mole of glucose. Furthermore, anoxic regions of biofilms would are not necessarily be "inactive" because it is known that molds have a maintenance metabolic activity in the absence of oxygen (Diano *et al*., 2006). In fact this the basis for large scale production of citric acid where aerobic respiration is constrained and citric acid is the main product of submerged anoxic fermentations of *A. niger* (Papagianni *et al*., 1998). However it is known that citric acid production in submerged fermentation is quite different to the similar process in SSF processes since the later does not require the use of respiratory inhibitors (Chaudhary *et al*. 1978).

As indicated above, this work supports the general idea that the fate of carbon up-taken by fungal surface cultures depends on the magnitude of the average substrate influx to the fungal mat. The first main result of this work is the observation that the average substrate uptake is controlled by the rate of diffusion of substrate from the solid substrate to the interphase with the fungal biofilm because it is proportional to initial substrate concentration. A second result is the existence of a threshold or minimal substrate influx necessary to support the sustained growth of the fungal culture because average substrate uptake and respiratory rates vanish when initial substrate concentration is below S_0 = 10 mgcm^{-3}. Finally, there is a negative correlation between the average rate of biomass and citrate production and S_0, but there is a positive correlation with respect to the respiratory rate, R_C. Those results support the existence of local gradients below the fungal layer that preclude strong substrate inhibition of substrate uptake and catabolism even though the bulk substrate concentration is hypertonic ($S_0 >$ 100 mgcm^{-3}) but force substrate influx to the fungal layer, perhaps by diffusional substrate and intermediate metabolite translocation to the uppermost aerobic region of the biofilm. This way, the thin aerobic layer can oxidize a mixture of substrate and products. The fact that net citric acid production is inversely proportion to initial substrate concentration can be taken as indirect evidence that molds can use different pathways for the partial oxidation of glucose, especially when the balance between local oxygen mass transfer is negative as compared to biological oxygen demand. Furthermore the observation that apparent mycelial porosity decreases with increasing values of initial substrate concentration can be taken as a modification on the branching mechanism because microbial density will be inversely proportional to branching frequency. For example, dense mycelia will have shorter branches than loose mycelia as shown by Larralde *et al*. (1997). These results can be applied to various fields, such as, modeling food spoilage by molds, optimal design of industrial production of citric acid by solid-state fermentation as suggested by Chaudhary *et al*. (1978) and the use of *Aspergillus niger* for bioremediation of polluted soils (Volke-Sepulveda *et al*., 2003).

Conclusions

Our results suggest that a diffusive over-flow of substrate through the mycelial mat, decreases biomass yields, and citric acid production but increases carbon dioxide production. This result may be interesting as constraints for designing surface cultures oriented to the production of citric acid or biomass related products or alternatively, to use molds to mineralize polluting compounds dispersed within solid materials.

A possible practical application of this work is the

manipulation of metabolism by the adequate design of solid substrates that modify substrate influx into fungal layers, for example, by changing the specific area, the internal porosity and the bulk concentration of the solid support where the soluble substrate is dispersed. Current work of our research group is showing the importance of soluble substrate gradients within solid supports in relation to the physiological manipulation of fungal surface cultures and will be reported elsewhere.

Nomenclature

A	surface area (cm^{-2})
A	empirical inhibition constant of biomass (cms^{-1})
B	empirical inhibition constant of citric acid (cms^{-1})
C	constant ($X_m/X_0 - 1$) (dimensionless)
D	reactor diameter (cm)
H	agar depth of culture medium (cm)
J_0	flux of glucose uptake required to start the growth ($mgcm^{-2}s^{-1}$)
J_P	flux citric acid production ($mgcm^{-2}s^{-1}$)
J_{P0}	maximal flux of biomass production ($mgcm^{-2}s^{-1}$)
J_S	flux substrate across surface area ($mgcm^{-2}s^{-1}$)
J_X	flux biomass across surface area ($mgcm^{-2}s^{-1}$)
J_{X_0}	maximal flux of biomass production ($mgcm^{-2}s^{-1}$)
k_J	permeability (cms^{-1})
L	reactor length (cm)
M	maintenance coefficient ($g_s g_x h^{-1}$)
P	citric acid produced in the culture (mg)
Q	rate of conversion of substrate to CO_2 (gCO_2/gS)cms^{-1}
Rc	respiratory rate ($mgCO_2cm^{-2}s^{-1}$)
Rco	treshold value or Rc ($mgCO_2cm^{-2}s^{-1}$)
R_{max}	maximal respiratory rate ($mgCO_2cm^{-2}s^{-1}$)
R_ε	residual respiratory rate ($mgCO_2cm^{-2}s^{-1}$)
S_0	substrate concentration ($mgcm^{-3}$)
T	time of culture (s)
V	volume of culture medium (cm^3)
X	biomass produced in the culture (mg)
X_0	biomass at t=0 (g)
Xm	maximal biomass of the culture (mg)
$Y_{p/s}$	citric acid yield ($g_p gs^{-1}$)
Y_{x/CO_2}	biomass/CO_2 yield (dimensionless)
$Y_{X/S}$	biomasss yield ($g_x g_{s^{-1}}$)
Greek letters	
μ	specific rate of growth (s^{-1})
A	specific area (cm^{-1})
Γ	maintenance coefficient ($mY_{X/CO_2}/\mu$) (dimensionless)
Λ	biofilm height (cm)
ρ_A	surface density of biomass ($mgcm^{-2}$)
ρ_v	volumetric density of biomass ($mgcm^{-3}$)
ϕ	porosity of fungal networks (dimensionless)

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Appendix

Kinetic model of microbial respiration rates

This model follows closely the mass balance of Lareo *et al*. (2002) with a logistic law for biomass production

$$\frac{dX}{dt} = \mu\left[1 - \frac{X}{X_m}\right]X \tag{A-1}$$

Together with Pirt mass balance for substrate uptake

$$\frac{dCO_2}{dt} = \frac{dX}{Y_{X/CO_2}dt} + m \tag{A-2}$$

Where, X is the biomass amount, with a steady state value, $X = X_m$; μ (1/s), is the exponential growth rate; S is the residual substrate amount; Y_{X/CO_2}, is the biomass yield and, m, the maintenance coefficient.

The solution of the logistic equation is known to be

$$X(t) = \frac{X_m}{1 + (X_m/X_0 - 1)e^{-\mu t}} \tag{A-3}$$

This expression can be simplified using the following substitution, $U(t) = Ce^{-\mu t}$, where, A, is an empirical

parameter related to the initial condition, $C = X_m/X_0 - 1$. Hence, inserting the solution of the logistic equation in the Pirt equation and after some algebraic manipulation the following kinetic expression is obtained

$$R(t) = R_C \left[\frac{\frac{U}{1+U} + \gamma}{1 + U} \right] \quad \text{(A-4)}$$

Where, $R_C = \mu X_m / Y_{X/CO_2}$ (mgCO_2/s) and, |*gamma* = $mY_{X/CO_2}/\mu$, is a dimensionless coefficient related to maintenance metabolism. The respiratory rate, $R(t) = dCO_2/dt$, can be normalized by the surface area of the superficial culture in order to compare it with the substrate, biomass and fluxes. The meaning of R_C is related to the peak of respiratory activity ($R = R_{max}$, when $dCO_2/dt = 0$) and is approximately $R_C = 4R_{max}$, when $0 < \gamma \ll 1$, since it corresponds roughly to the inflection point of the growth curve which is known to be proportional to $1/4\mu X_m$ because the maximal growth rate is obtained when $X = 1/2X_m$. The, value of γ, is important because it helps to explain the residual respiration at the end of the respiratory curve ($R_\varepsilon = \gamma R_C$) when U is negligible. Also, it corrects the peak value using the following expression.

$$R_{max} = \frac{R_C(1+\gamma)}{4(1-\gamma)} \quad \text{(A-5)}$$

When, $\gamma > 1$, it is not possible to find a peak point because maintenance metabolic activity overrides biosynthesis. Therefore, the estimate of parameters, R_C and γ, provide important clues on the general metabolic orientation of a given microbial culture. It should be noted that it is not possible to estimate parameters, Y_{X/CO_2}, and X_m, from respiratory curves because it requires an independent estimate of the yield coefficient. For example, it is necessary to have an independent measurement of biomass and the respiratory rate.