ISSN 1330-9862
(FTB-1655)

review

Why Solid-State Fermentation Seems to be Resistant to Catabolite Repression?

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Received: January 20, 2006
Accepted: March 20, 2006

Summary

A critical review of the phenomenon of resistance to catabolite repression of enzyme synthesis by solid-state fermentation (SSF) has been made. The practical and theoretical importance of such phenomenon is commented, together with the current ideas to explain it. Namely, the possible existence of microscopic gradients within the mass of cell aggregates, or the changes in cell permeability to sugars, which would explain the fact that no catabolite repression is observed in SSF when sugar concentration is as high as 100 g/L, despite the fact that in conventional submerged fermentation (SmF) strong inhibition of inducible enzymes is observed whenever sugar concentration is higher than 10 g/L. Two alternative mathematical models are presented in order to explore the feasibility of those hypotheses, but also to help the planning of future experiments in order to understand the microscopic physiology of SSF. A priori, both hypotheses will explain the phenomenon, but only if the local diffusivity or permeability of sugars in SSF have changed in various orders of magnitude as compared to the observed magnitudes in SmF systems.

Key words: solid-state fermentation, enzyme synthesis, resistance to catabolite repression, mathematical models

Introduction

Most enzymes are manufactured using the conventional technique of submerged fermentation (SmF), where microbial cells are suspended in a large volume of water that is stirred and aerated using mechanical devices. In such systems, chemical composition of the culture medium is nearly spatially homogeneous because the mixing rate is faster than the reaction rate. In those circumstances, it is important to consider the phenomenon of catabolite repression (CR). Such phenomenon is the inhibition of the microbial synthesis of many enzymes that require a chemical signal, called the inducer, and are repressed when there is abundance of a ready fermentable substrate such as glucose, glycerol or other carbon sources (*1*). De Vries and Visser (*2*) commented that the major system responsible for carbon repression in *Aspergillus* is mediated by the carbon catabolite repression protein CreA. CreA is a zinc finger protein which binds to specific sites in the promoters (SYGGRG) of a wide range of target genes. In the presence of easily metabolizable substrates, such as glucose or fructose, CreA inhibits or decreases the expression of the target genes. But there is another alternative technique for enzyme production (*3*) called solid-state fermentation (SSF), where the microbial cells are grown on the solid surface of porous materials (biodegradable or not). In this system, a large fraction of the fermentation mash is occupied by interstitial air and the biomass is in contact with a high level of humidity adsorbed to the mash surface or absorbed into the solid particles supporting the microbial cells (*4*). Ramesh and Lonsane (*5,6*) discovered that the production of alpha-amylase by *Bacillus licheniformis* M27 in submerged fermentation was

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completely inhibited due to catabolite repression in medium containing 1 % glucose. In contrast, the enzyme production in a solid-state fermentation system was 19 550 units/mL in the extract even when the medium contained 15 % glucose. That observation was confirmed by Solís-Pereira *et al.* (*7*) using cultures of *Aspergillus niger.* They reported that the production of endo- (endo-p) and exo-pectinase (exo-p) by SSF was not reduced when glucose, sucrose or galacturonic acid (up to 10 %) were added to a culture medium containing pectin. Moreover, both activities increased when concentrations of carbon sources were also increased. Those original observations using bacterial and fungal cultures were reproduced with a variety of organisms, enzymes, and solid supports as indicated in Table 1 (*8–20*). Such property of SSF systems seemed to be of importance because, according to Díaz-Godínez *et al.* (*10*) and Viniegra-González *et al.* (*21*), it helps to obtain high enzyme yields per gram of biomass, using elevated concentrations of simple sugars (>50 g/L) with relatively low levels of inducers.

Once the resistance to CR by SSF was established, several questions were brought up, as follows:

a) Is the resistance of CR an absolute or a relative property of SSF systems?
b) Do the cells cultured by the SSF use different regulatory system than the cells cultured by SmF? Or do they work in a different microenvironment with the same universal regulatory system?
c) What are the mechanisms involved in this phenomenon of SSF systems?

Those questions are the main task of the present review.

Is the Resistance to CR in SSF an Absolute or Relative Property?

Initial observations in SSF systems (*5–7*) suggested a total absence of CR using cultures of *Bacillus licheniformis* and *Aspergillus niger*, respectively. But, de Souza *et al.* (*15*) and Tlécuitl-Beristain *et al.* (*11*) reported that this property was relative. For example, de Souza *et al.* (*15*) tested the effect of easily metabolizable sugars (glucose, xylose, fructose, maltose, cellobiose and lactose) on xylanase production by *Aspergillus tamarii* using wheat bran, corn cob and sugar cane bagasse as substrate. They found that the addition of different sugars at a concentration of 1 % to sugar cane bagasse or corn cob media caused severe catabolite repression. In contrast, wheat bran systems were resistant to catabolite repression even at high concentrations of glucose (10 %). Also, Tlécuitl-Beristain *et al.* (*11*) studied the effect of high initial concentrations (40–120 g/L) of either glucose or sucrose on the exopolygalacturonase production by *Aspergillus niger* in solid-state fermentation. Polyurethane foam impregnated with media containing 15 g/L of pectin was used as inert support. It was observed that in media containing around 100 g/L of either glucose or sucrose, the organism did present CR, but it did not affect its growth. This latter result was confirmed by Téllez-Jurado *et al.* (*13*). They studied the expression of laccase IV from *Trametes versicolor*, expressed in *Aspergillus niger* using a promoter from amyloglucosidase and grown on polyurethane foam (SSF) with various levels of glucose. They found an increase of the laccase excretion to the culture media by SSF using 50 g/L of glucose as a sole carbon source, but with 100 g/L CR occurred. In shake flasks

Table 1. Examples of published reports on the resistance to catabolite repression (CR) of SSF systems

Organism	Enzyme(s)	Support	Reference
Aspergillus carbonarius	phytase	canola meal	(*8*)
Aspergillus niger	β-fructofuranosidase	sugar cane bagasse	(*9*)
Aspergillus niger	exopectinases	polyurethane foam	(*10*)
Aspergillus niger	exopolygalacturonase	polyurethane foam	(*11*)
Aspergillus niger	pectinase	bagasse pith	(*7*)
Aspergillus niger	pectinesterase and polygalacturonase	sugar cane bagasse	(*12*)
Aspergillus niger	recombinant laccase	polyurethane foam	(*13*)
Aspergillus niger	tannase	polyurethane foam	(*14*)
Aspergillus tamarii	xylanase	wheat bran, corn cob, sugar cane bagasse	(*15*)
Apergillus niger	exopectinase	polyurethane foam	(*10*)
Aspergillus niger	exopectinase	bagasse pith	(*16*)
Aspergillus niger	pectinases	coffee pulp	(*17*)
Bacillus coagulans	α-amylase	wheat bran	(*18*)
Bacillus licheniformis	xylanase	wheat bran	(*19*)
Bacillus sp.	xylanase	wheat bran	(*20*)
Bacillus licheniformis	α-amylase	wheat bran	(*5*)
Bacillus licheniformis	α-amylase	wheat bran	(*6*)

(SmF), laccase activity was negligible for all tested concentrations of glucose (10, 50 and 100 g/L).

Apparently, the use of an absorbent support such as wheat bran or sugar cane bagasse helps the SSF system to be more resistant to CR than the use of less absorbent support, such as polyurethane foam. Also, xylanases seem to be more sensitive to CR than pectinases or amylases.

One possible way to explain those results is the formation of sugar gradients inside the solid support and also in the microbial aggregate, for example, when the support is an absorbent material (agar, wheat bran or sugar cane bagasse) or when it merely adsorbs the liquid broth by capillarity (polyurethane foam). This idea was originally proposed by Georgiou and Shuler (*22*) and confirmed by Mitchell *et al.* (*23*,*24*) and also by Olsson (*25*). Nagel *et al.* (*26*) studied the culture of *Aspergillus oryzae* on membrane-covered wheat-dough slices using H-1-NMR images. Their results indicated the presence of »strong glucose gradients«. For example, glucose concentrations just below the fungal mat remained low due to high glucose uptake rates, but deeper in the matrix glucose accumulated to very high levels. Rajagopalan and Modak (*27*,*28*) and Mitchell *et al.* (*24*) developed diffusion-reaction models to account for those concentration profiles of substrates in SSF systems.

Aguilar and Huitrón (*29*) used a fed-batch SmF system for the production of pectinase by *Aspergillus* sp. in such a way that reducing sugars were limited to less than 4 g/L. They noted that the final activity of pectinase was 4.5 times higher in the fed-batch system than in the conventional non-fed-batch system. Antier *et al.* (*17*) found that the addition of glucose of up to 10 g/L in shake flasks stimulated the synthesis of pectinases by *Aspergillus niger* in the presence of small amounts of pectin, but higher levels of glucose were inhibitory. Those observations are in agreement with the idea that thin layers of cells, growing on a solid support, create a microenvironment where sugar concentration is optimal for growth and enzyme production, *i.e.* between 5 and 10 g/L.

Aforementioned evidence favours the notion that resistance to CR by SSF cultures is a relative property that may depend on the nature of the support. For example, they are more resistant when the support absorbs the substrate. Also, they seem to depend on the sensitivity of each regulatory system.

Do SSF Cultures Use a Different Regulatory System than SmF Cultures?

Maldonado and de Saad (*12*) studied the production of pectinesterase and polygalacturonase by *Aspergillus niger* in submerged and solid-state fermentation systems and found that the membranes of cells from the SSF showed increased levels of C18:1, C16:0 and C18:0 fatty acids. This observation may be taken as an indication of the changes of permeability of fungi grown by SSF technique. Acuña-Argüelles *et al.* (*16*) studied the effect of a depressant of water activity (ethylene glycol) on the production of pectinase by *Aspergillus niger* grown by SSF technique (on sugar cane bagasse). They noted that »the reducing groups accumulated extracellularly suggesting sugar transport limitation as a consequence of a_W depression«.

Oostra *et al.* (*30*) studied cultures of *Rhizopus oligosporus* grown on the surface of solid, nutritionally defined glucose and starch media. Their main result was that the contribution of the aerial hyphae to overall oxygen consumption was negligible. It can be concluded that optimal oxygen transfer in SSF depends on the available interfacial gas-liquid surface area and the thickness of the wet fungal layer. Such depth was measured to be 100 μm, consistent with a diffusion model applied to a biofilm. Furthermore, Rahardjo *et al.* (*31*) worked with a culture of *Aspergillus oryzae* during growth on a wheat-flour model substrate. They found that aerial mycelia are extremely important for the respiration of this fungus. Both results together indicate important differences between fungal species involving different mechanisms of oxygen uptake that can, in turn, modulate the production of enzymes.

The importance of hydrophobins in the regulation of fungal metabolism and differentiation has been studied in basidiomycetes (*32*–*34*). Volke-Sepulveda *et al.* (*35*) found that *Aspergillus niger* grown on a solid support (SSF) had a faster rate of hydrocarbon mineralization than the culture grown in shake flasks (SmF). Similar results were obtained by García-Peña *et al.* (*36*) using a biofilter of toluene inoculated with *Scedosporium apiospermum*. Peñas *et al.* (*34*) commented that it should be kept in mind that this pattern of hydrophobin expression in submerged cultures can differ from solid fermentation processes as metabolism under these two culture conditions also differs.

Iwashita (*37*) reported solid-state specific gene expression of the *glaB* (glucosidase) and *pepA* (protease) genes of *Aspergillus oryzae* and also specific release of enzymes from the cell wall in the production of β-glucosidases in *Aspergillus kawachii*. He explained such phenomena by the presence (or absence) of a specific polysaccharide acting as a secretory signal of the enzymes attached to the cell wall. He also indicated the presence of a specific GC box in the promoter »of the *glaB* promoter region (that) affected solid-state specific gene expression of this gene« (*37*).

An important property of fungal cultures on solid supports is the translocation of material between different mycelial regions. This problem has been studied by Olsson at Lund University. For example, Olsson (*38*) found three distinct patterns of translocation between different parts of the mycelium in surface cultures of 62 different fungi grown on media with opposing gradients of carbon and mineral nutrients. Olsson and Jennings (*39*) supported the mechanism of diffusion for the translocation of materials within the mycelia by measuring the rates of translocation of labeled radioactive phosphate and carbon compounds.

Those observations suggest the need to study the patterns of formation of vegetative aerial hyphae in connection with the induction or repression of specific proteins that, in turn, play a significant role in the adaptation of fungal cultures to SSF and SmF cultures. But, it is worth recalling that resistance to CR by SSF occurs both in bacterial and fungal SSF cultures (see Table 1). Therefore, it is necessary to invoke a more basic and general mechanism in order to explain this phenomenon as a

physiological characteristic of SSF microbial cultures, for example, to postulate a mechanism that does not depend on the formation of aerial or branched structures.

What Are the Mechanisms Involved in the Resistance of SSF Systems to CR?

Proposition of various geometrical models for SSF and SmF

Up to now the experimental evidence supports:

(*i*) Resistance to CR is a general property of bacterial and fungal cells grown as thin layers on the surface of solid supports with aerial interface (Figs. 1 to 4).

(*ii*) Such a property depends on the nature of the solid support. For example, resistance to CR is stronger (tolerates higher levels of sugar without repression) with solid supports having important absorption of liquid broths, such as sugar cane bagasse and wheat bran, and it is weaker with impermeable solid supports such as polyurethane foam, which merely adsorb the liquid broth by capillarity.

(*iii*) As indicated above (*26*), the existence of strong sugar microprofiles within the porous structure of the absorptive solid support and also of oxygen profiles within the thin mycelial mats that obey the conventional diffusion and reaction kinetics has been established by Oostra *et al.* (*30*). Such profiles produce important concentration differences within small distances, typically less than 0.1 mm. In turn, the microbial layers extend on the solid surface for distances in the range of more than 1 mm (*21*).

(*iv*) It is known that microbial cells can be stimulated to produce inducible enzymes in the presence of the adequate inducer if simple sugars are added in such a way that their concentrations remain low (*17*), for example, pectinase production by fed-batch cultures in the presence of pectin and glucose level lower than 10 g/L (*29*).

Viniegra-González *et al.* (*21*) proposed the idea that anisotropic coupling of oxygen and sugar fluxes (different diffusion trajectories) within the thin layer of microbial cells is the main cause for the resistance to CR. They argued that in stirred vats (SmF) the fluxes of oxygen and sugar were isotropic (diffusion along the same spatial coordinate) with respect to biomass concentration, whether the cells were dispersed or aggregated in nearly spherical clumps (Fig. 5). For this reason, the depletion profiles of oxygen and sugar more or less coincided within the accepted distance of 0.1 mm, whereas in SSF system the inherent anisotropy of oxygen and sugar fluxes produced different profiles for each kind of solute (see Figs. 1 to 5). Oxygen was depleted within the critical distance of 0.1 mm (Fig. 5), but sugar may have a much longer trajectory (1 mm) in order to establish quite a different concentration profile within the microbial aggregate (Fig. 5).

The crux of the matter is whether simple diffusion and reaction schemes can produce flat and depressed profiles of sugar concentrations in the interstices of cell aggregates, along the diffusion trajectory of the substrate, but with milder (parabolic-like) oxygen profiles. This way, the whole layer of cells will have an adequate oxygen supply and still low enough sugar concentrations in order to prevent the onset of CR, despite the fact that the average bulk sugar concentration is very high.

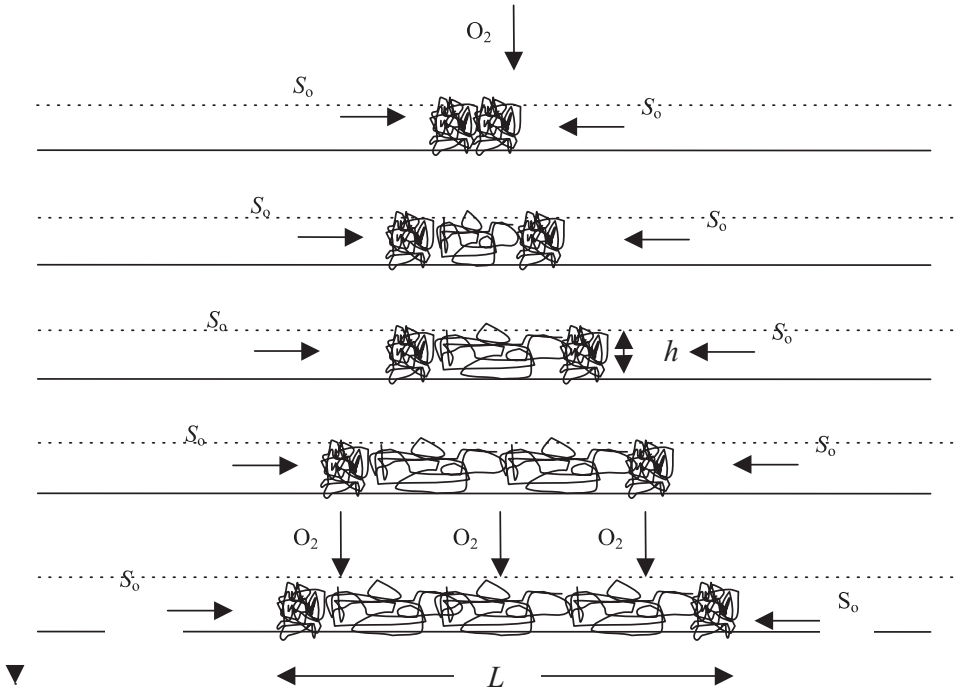

Fig. 1. Diagram of an idealized cell aggregate growing in the horizontal direction on an impermeable solid support, bathed in a thin layer of liquid broth and exposed to the air from above. Biomass layer has a depth of *h* (cm) and a lateral dimension of *L* (cm). This would correspond to a microbial colony growing on a glass or plastic surface inundated with a thin layer of liquid broth

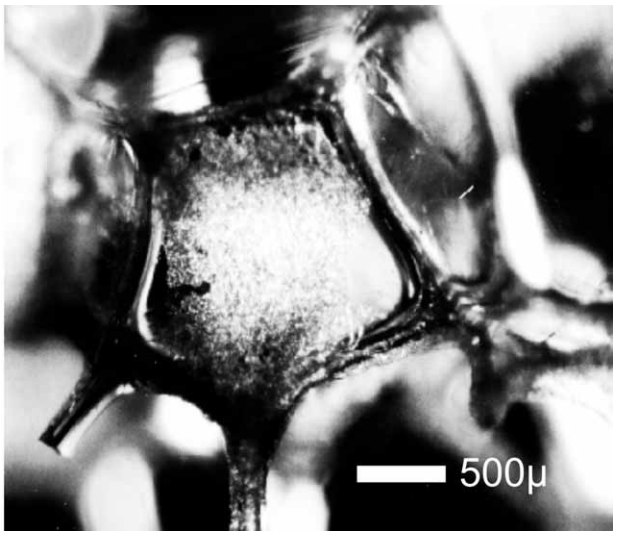

Fig. 2. Micrograph of *Aspergillus niger* C28B25 growing in polyurethane foam under solid-state fermentation conditions after 6 hours of culture (100×). Micrograph from J. S. Romero-Gómez (unpublished results)

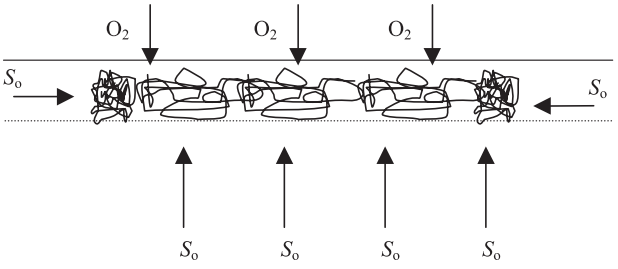

Fig. 3. Diagram of a thin layer of microbial biomass growing on the flat surface of a porous material, such as an agar plate. Substrate diffusion comes from fresh media outside the colony and also from the bottom of the agar plate. Oxygen diffusion comes from the air phase above the microbial colony. The colony is wet because of the capillarity dispersion of the liquid broth

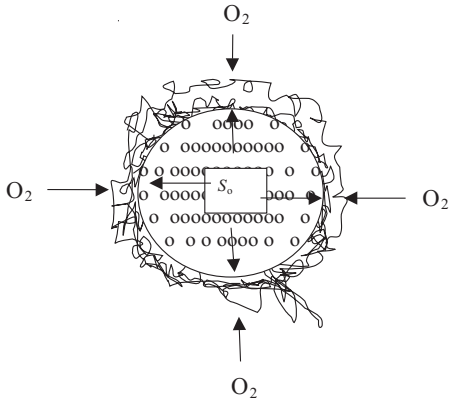

Fig. 4. Diagram of anisotropic fluxes of the substrate, S_o, and oxygen, O_2, to a thin layer of microbial biomass growing on a spherical porous particle imbibed in a liquid broth and exposed to interstitial air. Oxygen diffusion is assumed to be perpendicular to the microbial layer but coming from the outside. Substrate diffusion is assumed to be coming from the inside. Substrate gradients are formed because of the microbial metabolism on the surface of the particle. The biomass increases and substrate decreases with time

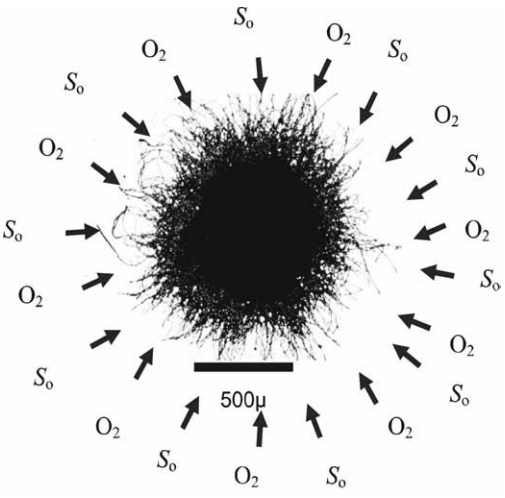

Fig. 5. Diagram of isotropic fluxes of oxygen and substrate to a pellet of *Aspergillus niger,* grown in a shake flask. Micrograph from J. S. Romero-Gómez (unpublished results)

Mathematical models of diffusion and reaction within a microbial layer

Model with homogeneous diffusivity

A fundamental question of the establishment of sugar concentration profiles along the transversal coordinate of microbial layers is the solution of the mass balance equation of the following type:

$$\frac{\partial S}{\partial t} = \frac{\partial}{\partial x}\left[D_S \frac{\partial S}{\partial x}\right] - q_s \rho \frac{S}{K_s + S} \qquad /1/$$

where $S(x)$ is the local concentration at the transversal position x, D_S is the diffusivity of the substrate, q_S is the specific metabolic rate of substrate consumption, ρ is the biomass density, and K_S is the saturation constant of the metabolic activity. An ideal case would be a thin and uniform (rectangular) mycelial mat with depth h and width L, resting on an impermeable solid support and surrounded by a nutrient solution with fixed substrate concentration S (Fig. 1), where $S>K_S$. The normalized version of this model involves the use of the following transformations, $\sigma_c=S/S_o$; $\sigma_{o_2}=S/S_{o_x}$; $\xi=x/L$ or $\zeta=y/h$. At the steady-state ($\partial S/\partial t=0$), Eq. 1 becomes an ordinary differential equation as in Eq. 2:

$$\frac{d^2S}{dx^2} = \frac{q_s \rho}{D_s} \qquad /2/$$

Changing to the normalized coordinates the following differential Eqs. 3 and 4 are obtained:

(*i*) For the steady-state mass balance of the substrate:

$$\frac{d^2\sigma_c}{d\xi^2} = \Phi_s^2 \qquad /3/$$

where $\Phi_S^2=L^2(q_S\rho/D_S S_o)$ is the Thiele modulus for the substrate. The diffusivity of glucose in water, at 20 °C, is taken as $D_S=1.5\cdot10^{-6}$ cm^2/s for glucose;

(*ii*) For the steady-state mass balance of oxygen:

$$\frac{d^2\sigma_{o_2}}{d\varsigma^2} = \Phi_{o_2}^2 \qquad /4/$$

where $\Phi_{o_2}^2 = h^2(qs\rho/Y_{s/o_2}D_{o_2}S_o)$ is the Thiele modulus for the substrate. The coefficient Y_{s/o_2} is the biological oxygen demand (BOD). For example, if the substrate is a hexose, Y_{s/o_2}=1.06 g of O_2/g of substrate. The diffusivity of oxygen in water, at 20 °C, is taken as $D_{o_2}=5\cdot10^{-5}$ cm^2/s.

The solutions of Eqs. 3 and 4 with the above mentioned boundary conditions become parabolic-like profiles calculated by Eqs. 5 and 6, respectively:

$$\sigma_S\xi = \Phi_S^2(\xi-1)\xi+1 \qquad /5/$$

$$\sigma_{o_2}(\varsigma) = \left[\frac{\Phi_{o_2}^2\varsigma}{2}-1\right][\varsigma-1] \qquad /6/$$

Table 2 shows the numerical values used in this model. Fig. 6 indicates that it is possible to choose $D_S^*=1.31\cdot10^{-7}$ cm^2/s for glucose diffusivity in order to make $\sigma_C(½)=0.1$, while $D_{o_2}=5\cdot10^{-5}$ cm^2/s. This way, most of the microbial layer will be oxygenated and the midpoint glucose concentration will be low. However, this model has a drawback. Namely, the glucose profiles are parabolic and most of the microbial layer will have too much glucose ($\sigma_S>0.1$) and perhaps be susceptible to CR. For example, if S_o=100 g/L, most of the cells will be exposed to $S>10$ g/L. Therefore, it is necessary to invoke a more complex model in order to have a flat sugar profile around $\sigma_S=0.1$.

Table 2. Values of parameters of a hypothetical biofilms used in numerical calculations and illustrated in the indicated figures

Parameter	Meaning	Value	Units
D_{o_2}	Diffusivity of oxygen in water	$5.0\cdot10^{-5}$	cm^2/s
D_S	Diffusivity of glucose in water	$1.5\cdot10^{-6}$	cm^2/s
D_S^*	Effective glucose diffusivity necessary in Fig. 6 to make $\sigma(½)=0.1$	$1.3\cdot10^{-7}$	cm^2/s
h	Cell membrane width in Fig. 8	$6\cdot10^{-3}$	cm
H	Biofilm depth in Fig. 7	0.01	cm
K_S	Saturation constant of Monod equation in Fig. 8	0.0025	g/cm^3
L	Biofilm width in Fig. 7	0.10	cm
q_s	Average specific metabolic activity in Fig. 7	2.778	g/g s
S	Substrate concentration		g/cm^3
S_o	Initial bulk substrate concentration in Fig. 7	0.10	g/cm^3
Y_s	Biomass yield in Fig. 8	0.30	g *S*/g *X*
ρ_0	Maximal biomass density (tightly packed biomass)	0.15	g/cm^3
ρ	Average biomass solid density (dry mass) in Fig. 7	0.06	g/cm^3

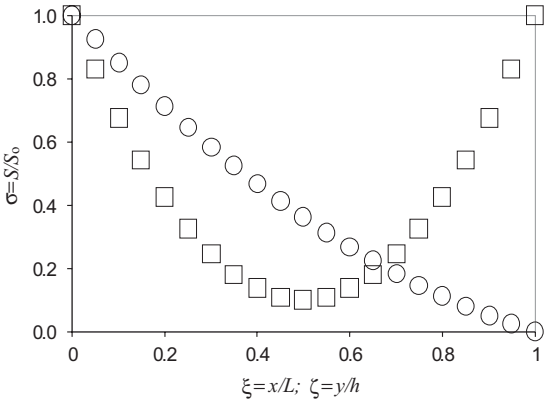

Fig. 6. Estimated concentration profiles of glucose (□) using Eq. 5 and oxygen (○) using Eq. 6 in a hypothetical thin and symmetrical layer of biomass illustrated in Fig. 1. Glucose flux is horizontal ($\xi=x/L$) across a length, L=0.1 cm. Oxygen flux is vertical ($\zeta=y/h$) across a section of depth, h=0.06 cm. Values of parameters are shown in Table 2. Diffusivity of glucose was adjusted to $D_{eff}=1.31\cdot10^{-7}$ cm^2/s in order to make $\sigma_C(0.5)=0.1$. Oxygen diffusivity was assumed to be as in water, $D_{o_2}=5\cdot10^{-5}$ cm^2/s. Notice that the glucose profile is parabolic with a minimum at the midpoint and most of the layer has $\sigma_C>0.1$. Also, 80 % of the microbial layer has positive values of oxygen concentration ($\sigma_{o_2}>0.1$)

Model with heterogeneous diffusivity

The effective diffusivity constant of a solute, within a porous material, such as biomass is estimated by Eq. 7:

$$D_{eff} = D_w\left[\frac{1-\rho/\rho_o}{\tau}\right] \qquad /7/$$

The variable ρ is the solid density of biomass. The value ρ_o is the maximal solid density, for example, when the porosity is zero. Thus, porosity is given by $(1-\rho/\rho_o)$. The coefficient of tortuosity, τ, is the average path length of particles traversing the material within a mesh, as compared to the lineal length of the trajectory. This simple model indicates that dense mycelial mats will have lower effective diffusivity, as compared to loose mycelial mats, and this may produce important differences in the oxygen and substrate profiles of the fungal aggregate. However, this model does not provide a way to estimate changes in tortuosity as a function of biomass density nor explains how porosity and tortuosity can be adjusted to different values of local substrate concentration.

Models from Zhang and Bishop (*40*) have shown that effective diffusivity in bacterial biofilms changes with biomass density. Also, Thurnheer *et al.* (*41*) have shown that bacterial biofilms can excrete polysaccharides that modify the diffusivity of dyed molecules, acting as if the polysaccharide mesh was a molecular sieve. Fan *et al.* (*42*) developed a model where biomass changed the diffusivity as a function of the biomass density. Thus it is justifiable to assume that D_{eff} in cell aggregates can change as a physiological response to different environmental conditions and we can explore the simplest model indicated in Eq. 8, where biomass produces a hypothetical barrier to the diffusion of glucose that is a function of

the glucose concentration itself, for example, following the observations of Thurnheer *et al.* (*41*) and also Torrestiana *et al.* (*43*) that microbial cells excrete polymers that limit the diffusion of sugars.

$$D_{eff}(S) = D_S[1-(S/S_C)^p] \text{ if } S<S_C;\ D_{eff}=D_W/10^4 \qquad /8/$$

In Eq. 8 there is a critical concentration of the substrate, S_C, that measures the sensitivity of the product made from the substrate, *i.e.* a polysaccharide, blocking the diffusion of the substrate itself. The conditional value $D_{eff}=D_W/10^4$ for $S\geq S_C$ prevents the use of negative values for D_{eff}. For example, it is assumed that substrate fluxes through the cell metabolism are diverted at critical values ($S\rightarrow S_C$), proportional to extracellular substrate concentrations, in order to make and excrete polysaccharides that decrease the diffusivity, D_{eff}, of the substrate itself (*44*). The parameter $p>0$ is called the Freundlich exponent in chemical engineering models. It produces different kinds of curvature of the Eq. 8 when plotted *vs.* S. For example, if $p=1$, it is a straight line; for $0<p<1$, it produces a concave curve, and for $p>1$, a convex curve. It is worth recalling that excretion of polymers as a result of cell aggregation is well established by the so called »quorum sensing genes« (*45*) that respond to threshold levels of homoserine lactones, that in turn are a function of biomass density, and indirectly, also a function of the substrate concentration. Davies *et al.* (*45*) suggested that »The involvement of an intercellular signal molecule in the development of *P. aeruginosa* biofilms suggests possible targets to control biofilm growth on catheters, in cystic fibrosis, and in other environments where *P. aeruginosa* biofilms are a persistent problem«. Wisniewski-Dye *et al.* (*46*) reported that »Analysis of *N*-acyl-L-homoserine lactones (AHLs) produced by *Rhizobium leguminosarum* bv. *viciae* indicated that there may be a network of quorum-sensing regulatory systems producing multiple AHLs in this species«. Extension of quorum-sensing signals has been extended to other organisms such as *Candida albicans* (*47*).

On the other hand, chemists have studied the changes in diffusivity of solutes within solutions of various kinds of branched polymers (*48,49*) showing the importance of polymer charge and structure. Walther *et al.* (*50*) developed a simulation model of biofilms of yeast and tried to relate changes in viscosity to the presence of polymers such as agar but they found a decrease in glucose diffusivity of 5 % with respect to water. However, Torrestiana *et al.* (*43*) found a decrease of sucrose diffusivity by two orders of magnitude when xanthan concentration was only 10 g/L as compared to the diffusivity of sucrose in water.

Thus, this model assumes that well oxygenated microbial cell aggregates with depth h are exposed to a critical concentration of the substrate $S>S_C$ and excrete a polysaccharide that decreases the local diffusivity of the substrate. Therefore, the exposure of cells to the excess concentration of the substrate is not the same for all the cells. For example, cells within the cell aggregate will be exposed to a weaker substrate concentration ($S\ll S_C$) than cells near the aggregate boundary exposed to a very strong substrate concentration ($S\geq S_C$). This way, most of the cells will be exposed to the right substrate concentration ($\sigma_S\leq 0.1$) in relation to the local mass transfer of oxygen ($\sigma_{o_2}>0$). On the other hand, it is assumed that the diffusion path for oxygen is short enough ($h\ll L$) and with higher diffusivity than the average diffusivity of the substrate, mainly because in SSF systems the diffusion trajectories for oxygen and substrate are quite different from each other.

In order to test the plausibility of this model a series of simulations were run using the following differential equation obtained by inserting Eq. 8 in Eq. 1 and taking the partial differential derivative of the product between the effective diffusivity, $D_{eff}(S)$, and the substrate gradient, $\partial S/\partial x$, since the diffusivity changes from one point to another (*51*):

$$\frac{\partial S}{\partial t} = \frac{\partial}{\partial x}\left\{D_{eff}(S)\frac{\partial S}{\partial x}\right\} - q_s\rho\frac{S}{K_s+S} \qquad /9/$$

Differentiation of Eqs. 8 and 9 yields Eq. 10:

$$\frac{\partial S}{\partial t} = D_S\left[1-c\left(\frac{S}{S_C}\right)^p\right]\frac{\partial^2 S}{\partial x^2} - D_S\frac{p}{S_c}\left(\frac{S}{S_c}\right)^{p-1}\left(\frac{\partial S}{\partial x}\right)^2 - q_s\rho\frac{S}{K_s+S} \qquad /10/$$

Eq. 10 was normalized using the following definitions, $\alpha=cS_0/S_C$; $\kappa=K_S/S_0$; $\theta=tq_S\rho/S_0$; $\xi=x/L$; $\Phi_S^2=L^2(q_S\rho/D_SS_o)$.

This way Eq. 11 was obtained:

$$\frac{\partial\sigma}{\partial\theta} = \frac{1}{\Phi_S^2}\left\{\left[1-(\alpha\sigma)^p\right]\frac{\partial^2\sigma}{\partial\xi^2} - \left[\alpha p(\alpha\sigma)^{p-1}\right]\left[\frac{\partial\sigma}{\partial\xi}\right]^2\right\} - \frac{\sigma}{\kappa+\sigma} \qquad /11/$$

Parameters Φ_S and κ were estimated from the data shown in Table 2. For the sake of illustration we assumed that $p=1$ or 2, as shown in Figs. 7a and 7b, respectively. In each of those figures, the only free parameter of Eq. 11 was the parameter α. This equation was solved by numerical methods, with the following boundary conditions, $\sigma=1$ ($S=S_o$) for $\xi=0$ and $\xi=1$. Those calculations, shown in Figs. 7a and 7b, indicate that it is feasible to have an adaptive mechanism of microbial aggregates that have strong diffusion barriers toward solutes at the boundaries with the external environment and also a rather homogeneous distribution of substrate within the aggregate. An interesting aspect of this model is the adaptive response of the microbial aggregate to the local substrate concentration regulating its diffusivity. For example if $\alpha\rightarrow 0$, the system becomes insensitive to an excess of substrate concentration (Eq. 11 becomes Eq. 1). Also, for $\alpha\geq 1$, the system is very sensitive to the substrate concentration.

Model based on membrane permeability adaptation

An alternative explanation to the resistance of CR is the reduction of membrane permeability to glucose or other equivalent substrates. In order to explore this possibility it is necessary to consider the mass balance between the transport and the consumption of the substrate, S, in a cylindrical cell with glucose membrane diffusivity, D_M, which can be expressed as follows:

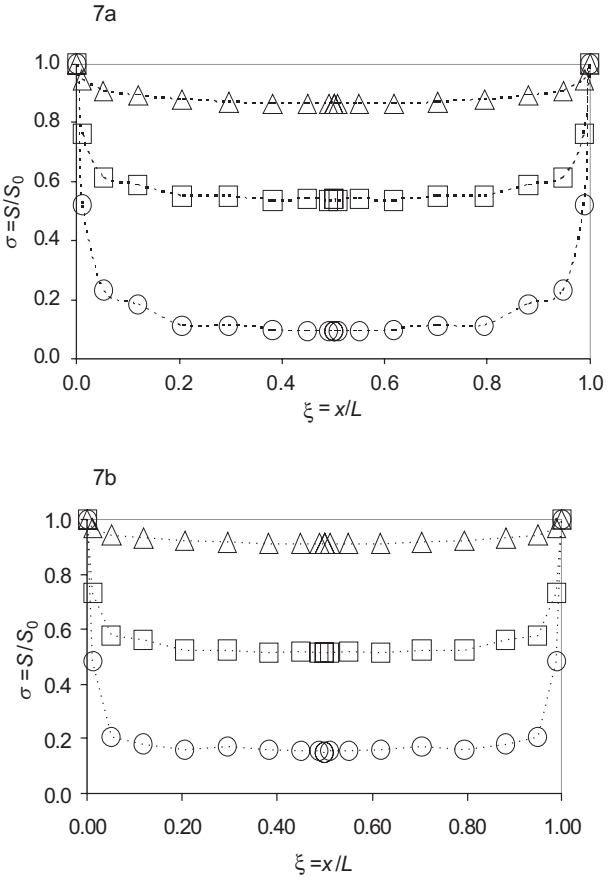

Fig. 7. Numerical solutions of Eq. 11 are shown using orthogonal collocation for a symmetrical system, applying Legendre polynomials with eight interior collocation points. The resulting system of ordinary differential equations was solved with a Runge-Kutta method of the 4th order, checking back the precision with another Runge-Kutta method of the 5th order. In Fig. 7a, p=1, (△)α=1.000; (□) α=1.010; (○) α=1.021. In Fig. 7b, p=2, (△) α=1.000; (□) α=1.050; (○) α=1.2

$$AD_M \left.\frac{\partial S}{\partial r}\right|_{r=R} \approx Vq_S\rho \frac{S}{K_S + S} \qquad /12/$$

In this expression, it is assumed that cellular concentration of the substrate, S, metabolic activity, q_S, and biomass density, ρ, are homogeneous. Furthermore, the gradient of glucose across the membrane with thickness, h, is assumed to be linear and driven by the external concentration of the substrate, S_o.

$$\left.\frac{\partial S}{\partial r}\right|_{r=R} \approx \frac{(S_o - S)}{h} \qquad /13/$$

Inserting Eq. 13 in Eq. 12, assuming cylindrical geometry and solving for D_M, the following expression is obtained:

$$D_M = \left(\frac{Rh}{2}\right)\left(\frac{S}{K_S + S}\right)\frac{q_S\rho}{(S_o - S)} \qquad /14/$$

If we assume that the metabolic system has a limiting step that is working at the midpoint of the saturation in Monod equation, (S=K_S), we can conclude that D_M is nearly proportional to the inverse of S_o:

$$D_M \approx \left(\frac{Rh}{4}\right)\frac{q_S\rho}{S_o} \qquad /15/$$

Data from Larralde-Corona *et al.* (*52*) show that R and q_S=$\mu/Y_{x/s}$ decrease with increasing the values of S_o. Fig. 8 shows the estimated values of D_M, using Larralde-Corona *et al.* (*52*) data. They follow a definite inverse correlation with S_o. The estimated permeability, P=$D_M h$ (cm/s), is in the range of 10^{-20} cm/s<P<10^{-18} cm/s, and is various orders of magnitude lower than the permeability of glucose in erythrocytes.

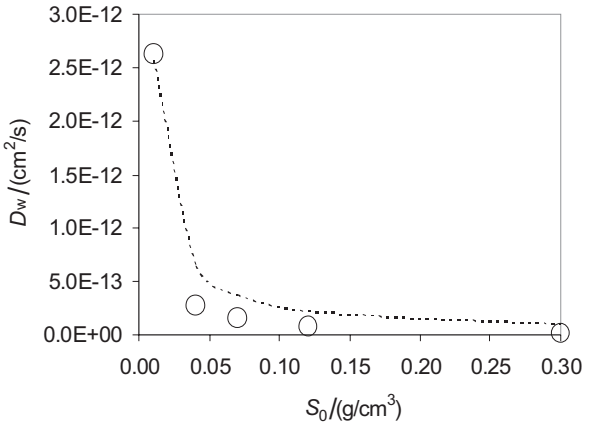

Fig. 8. Estimated values of glucose diffusivity across a cellular membrane, D_M, with various external concentrations of the substrate, S_o. The calculations were done using Eq. 14 with the assumptions indicated in the text and data taken from Larralde-Corona *et al.* (*52*). The interrupted line corresponds to the empirical equation Y=10^{-14} (0.514/X) cm^2/s

This alternative model does not require the excretion of polysaccharides to block the diffusion of substrates within the biofilm, but requires an explanation why the membrane permeability is regulated in such a way in SSF systems and is not in submerged fermentation. One possible explanation is cell to cell communication through a network of quorum sensing genes discussed above. But, pellet mycelial aggregates (Fig. 5) observed in submerged fermentation are conspicuously sensitive to CR. Therefore, a new property related to the differential fluxes of oxygen and substrate ought to be invoked to account for the peculiar resistance of SSF to CR.

Conclusions

The main conclusion of this review is the need to search in a systematic way for the physiological mechanisms that microbial aggregates may have in order to regulate the diffusion of substrates within the biofilm or across the cell membranes in SSF systems. Here, a set of alternative theoretical models is presented where (*i*) diffusivity is assumed to be homogeneous within the biofilm, (*ii*) it is assumed to change as response to an excess

concentration of substrate or (*iii*) it is assumed homogeneous within the biofilm, but with changing the permeability of the cell membranes. The resistance to CR is assumed to depend on the regulation of diffusivity of the substrate by one of the two later models, or perhaps by a combination of both. This kind of mechanisms can be shared by different kinds of microbial aggregates exposed to oxygen and substrate diffusion, especially if they have different diffusion trajectories, the diffusion path for oxygen being much shorter than the diffusion path for the substrate. One possible way to explore this interesting phenomenon is the use of microchemical analysis of microbial aggregates in such a way that microheterogeneity of chemical composition, porosity, tortuosity and diffusivity can be detected and structured models of solid-state fermentation can be supported by experiment. The models presented here indicate the plausibility of this kind of reasoning. Future experimental work will be the acid test for it.

Acknowledgement

The authors thank encouraging discussions and suggestions from Professors Mariano Gutiérrez-Rojas, Felipe López-Isunza and Enrique Galindo-Fentanes. Calculations were done by a graduate student Inés Reyes-Ocampo. Research was supported by a special project of Universidad Autónoma Metropolitana on solid-state fermentation.

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