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Advantages of fungal enzyme production in solid state over liquid fermentation systems

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Abstract

The present paper attempts to explain why enzyme production in solid-state fermentation (SSF) is higher than in submerged fermentation (SmF). Recent work done in our laboratory [Biotechnol. Lett. 22 (2000) 1255; J. Ind. Microbiol. Biotechnol. 26 (5) (2001) 271; J. Ind. Microbiol. Biotechnol. 26 (5) (2001) 296] related to the production of invertase, pectinases and tannases, by *Aspergillus niger* grown by SSF and SmF is reviewed. To do such a comparative study, logistic and Luedeking–Piret equations are used in order to estimate the values of the following coefficients: maximal specific growth rate (μ_M), maximal biomass level (X_M), enzyme/biomass yield ($Y_{P/X}$) and secondary rate of production, or breakdown (k). It is shown that enzyme productivity is proportional to group, $\mu_M Y_{P/X} X_M$, corrected by a function of $\nu = k/Y_{P/X}\mu_M$. In all three cases of enzyme production studied, productivity using a SSF system was higher than in SmF. Studies with invertase resulted in higher values of $\mu_M X_M$. Studies with pectinases resulted in higher values of $Y_{P/X} X_M$. Studies with tannases resulted in higher $Y_{P/X}$ and less negative values of k. Finally, a reaction–diffusion model is presented to try to explain such differences based on micrographic measurements of mycelial aggregates for each kind of fermentation system.
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Keywords: Solid state fermentation; Filamentous fungi; Submerged culture; Enzyme production; Proteolysis

1. Introduction

Enzyme production is a growing field of biotechnology. Annual world sales figures are close to a billion dollars [1] with increasing number of patents and research articles related to this field. Most enzyme manufacturers produce enzymes using submerged fermentation (SmF) techniques with enzyme titers in the range of grams per liter [2]. Such levels are a prerequisite if specific compounds are to be considered as commodities because product recovery costs are inversely proportional to concentration in a fermentation broth [3]. There is, however, a significant interest in using solid-state fermentation (SSF) techniques to produce a wide variety of enzymes, mainly from mold origin, as indicated by the growing number of research papers in the literature [4] and the marketing and development by a small but visible number of fermentation industries [5]. Among the advantages for SSF processes it is often cited that enzyme titers are higher than in SmF, when comparing the same strain and fermentation broth [6]. However, there is a scarcity of papers regarding physiological studies comparing SSF and SmF when trying to explain why microorganisms produce higher titers in the first kind of process as compared to the second. This lack of information makes difficult any assessment regarding the value of one process versus the other, thereby hindering the fundamental approach to process optimization and design for SSF technique in areas such as strain improvement, solid substrate engineering and process control.

In this paper an attempt is made to develop a general approach to compare productivity of three fungal enzymes (invertase, pectinase, tannase) using SSF and SmF techniques To do such a comparison, logistic and Luedeking–Piret equations are used in order to estimate the values of the following coefficients: maximal specific growth rate (μ_M), maximal biomass level (X_M), enzyme/biomass yield ($Y_{P/X}$) and secondary rate of production or breakdown (k).

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2. Theory

Biomass production is followed up by the Velhurst-Pearl logistic equation [7], originally developed for population growth.

$$\frac{dX}{dt} = \mu_M \left[1 - \frac{X}{X_M}\right] X \tag{1}$$

where X is biomass density (g per l, per cm^2 or per kg), μ_M the maximum specific growth rate (h^{-1}) and X_M the equilibrium level of X for which, $dX/dt = 0$ for $X > 0$. Solution to Eq. (1) can be written as follows

$$X(t) = \frac{X_M}{1 - ((X_M - X_0)/X_0)\, e^{-\mu_M t}} \tag{2}$$

where X_0 is the initial condition for X. Eq. (2) is useful to fit experimental data by Eq. (1), finding the least value of the sum of squared errors as a function of parameters, X_0, X_M and μ_M. Eq. (1) assumes that microbial cultures are practically saturated by substrate level and consequently, the specific growth rate is not a function of substrate concentration. If that were the case, then $\mu_M = f(S)$, and the Monod equation would read as follows:

$$\mu_M = \frac{\mu_M^* S}{S + K_S + (S^2/K_I)} \tag{3}$$

where K_S is the saturation constant of μ_M and K_I the inhibition constant by S. Substrate consumption can be modeled using a two-term expression proposed by Pirt [8] as follows:

$$-\frac{dS}{dt} = \frac{1}{Y_{X/S}}\frac{dX}{dt} + mX \tag{4}$$

where S is the substrate concentration (g per l, per cm^2 or per kg), $Y_{X/S}$ the biomass yield coefficient (g X/g S) and m the maintenance coefficient (g S/g X h). Solution of Eq. (4) can be obtained as a function of X as follows:

$$S(t) = S_0 - \frac{X - X_0}{Y_{X/S}} - \frac{mX_M}{\mu_M} \ln\left[\frac{X_M - X_0}{X_M - X}\right] \tag{5a}$$

where S_0 is the initial condition for substrate level, S. Eq. (5a) helps to test the importance of the maintenance coefficient, m, because a state plot of $S(t)$ vs. $X(t)$ will yield a straight line with slope, $1/Y_{X/S}$, whenever m is negligible. Otherwise, a logarithmic correction will appear with coefficient, mX_M/μ_M. Defining the increase of substrate consumption, $\sigma = (S_0 - S)$ and $\xi = X/X_M$, Eq. (5a) can be rewritten as follows:

$$\sigma = S_0 - S(t) = \alpha(\xi - \xi_0) + \beta \ln\left[\frac{1 - \xi_0}{1 - \xi}\right] \tag{5b}$$

where $\alpha = X_M/Y_{X/S} \leq S_0$ is the amount of substrate consumed when $X \to X_M$ and $S \to 0$ and $t \to \infty$. The equality holds when $\beta = mX_M/\mu_M$ is negligible. In all other cases when $X_M < Y_{X/S}S_0$ Eq. (5b) is useful for comparing overall growth physiology of different microbial cultures. For example, when $\beta \approx 0$, the plot X_M vs. S_0 will be a straight line. If β is positive and increases with, S_0, such a plot will be convex, i.e., resulting in a saturation curve with decreasing values of, X_M, when S_0 increases (Fig. 2).

Kinetics of product formation can be modeled using the Luedeking and Piret [9] equation as follows:

$$\frac{dP}{dt} = Y_{P/X}\frac{dX}{dt} + kX \tag{6}$$

where P is the product concentration, $Y_{P/X}$ the product yield in terms of biomass (units of product per unit of biomass) and k the secondary coefficient of product formation or destruction. Eq. (6) is similar to Eq. (4), but here the coefficient k can be negative, zero, or positive, since product formation or destruction is not necessarily related to growth. Again it is possible to solve Eq. (6) as a function of biomass

$$P(t) = P_0 + Y_{P/X}(X - X_0) + \frac{kX_M}{\mu_M} \ln\left[\frac{X_M - X_0}{X_M - X}\right] \tag{7}$$

Defining $\lambda = (P - P_0)$, Eq. (7) can be rewritten as follows:

$$\lambda = Y_{P/X} X_M \left\{(\xi - \xi_0) + \nu \ln\left[\frac{1 - \xi_0}{1 - \xi}\right]\right\} \tag{8}$$

where $\nu = k/Y_{P/X}\mu_M$ is the ratio between the rate of secondary formation, k (or destruction) of P as related to the maximal rate of product formation, $Y_{P/X}\mu_M$. The variable, λ represents the increase of product formation, i.e., enzyme titers. For, $-1\ \nu < 0$, the plot (λ vs. ξ) is convex with a definite peak value of λ, showing the presence of product breakdown during the fermentation period. For $\nu = 0$, the plot is a straight line, showing that P is only associated with the growth process. For $\nu > 0$, the plot is concave, showing the formation of P during the stationary phase of the culture process. Thus, plots derived from Eq. (8), help in comparing the shape of production curves and to diagnose the relative importance of ν, for a variety of microbial cultures (see Fig. 4).

2.1. Basic model for enzyme productivity

Enzyme productivity for fermentation systems can be expressed in different ways. In this paper it is chosen to define productivity, Γ, in terms of the liquid content within the overall culture medium. For example, if the porosity and the liquid content of a given SSF are known, productivity, in terms of reaction volume, can be corrected by corresponding proportional factors. Also, if enzymes are excreted to the medium and leached out at the end of the fermentation, final productivity can be estimated by taking into account the dilution factor. However, in all cases, the initial figure, related to microbial physiology is productivity defined as follows:

$$\Gamma_{obs} = \text{maximum of} \left[\frac{P}{t}\right] \tag{9}$$

That is, for a given fermentation curve, Γ_{obs}, will be the maximum of the ratio between the product level (enzyme titer) per liquid broth volume, P, added to the system and

divided by the fermentation time, t. In most cases, Γ_{obs}, will be evaluated at the peak of enzyme production, but, as will be shown below this is not always the case because of the time factor involved and the asymptotic nature of end fermentation points. Fermentation time, t, can be estimated from Eq. (2) as follows:

$$t = \frac{1}{\mu_M} \ln\left[\frac{(1-\xi_0)\xi}{\xi_0(1-\xi)}\right] \tag{10}$$

Combining Eqs. (7), (9) and (10), using enzyme titers per unit volume, and neglecting the value of P_0, the following relation is obtained

$$\Gamma_{cal} \equiv \mu_M Y_{P/X} X_M \left[\frac{(\xi-\xi_0)+\nu\ln[(1-\xi_0)/(1-\xi)]}{\ln[(1-\xi_0)\xi/(1-\xi)\xi_0]}\right] \tag{11}$$

The latter expression of Γ_{cal} is quite useful because it helps to demonstrate that productivity depends on four main physiological and interacting factors: (a) the maximal specific growth rate, μ_M; (b) the yield factor defined as enzyme per biomass unit, $Y_{P/X}$; (c) the ratio between the rates of product breakdown and synthesis, ν and (d) the dimensionless variable of growth, ξ.

Therefore, Eq. (11) helps to compare enzyme production by SSF and SmF techniques by asking which one of the aforementioned coefficients is the major factor explaining higher productivity for one of those techniques. In particular, the term $\Gamma_{ref} = \mu_M Y_{P/X} X_M$, named as reference productivity, helps to identify the major physiological factors involved in productivity of a given experimental system, corrected by a function of ξ and the coefficient ν.

3. Results and discussion

3.1. Better productivity because of higher biomass production

Romero-Gómez et al. [10] published data showing that three separate strains of *A. niger* produced higher titers of invertase and had higher observed productivity, Γ_{obs}, when cultured by SSF technique as compared to SmF technique. Fig. 1 shows the plot of measured biomass levels of *A. niger* (strain C28B25), X, at different incubations times, t, and for various levels of initial sucrose, S_0 (g l^{-1}) = 6.25, 12.50, 25.00, 50.00, 100.00. By fitting those values by using the logistic equation it was possible to estimate the asymptotic values, X_M, as shown in Fig. 2 which indicates a remarkable difference between SmF system with a hyperbolic saturation curve ($R^2 = 0.9980$) having maximal value X_M = 14.6 g l^{-1} and initial slope close to $Y_{X/S} = 0.3$. This is in contrast with the SSF system where a linear relationship ($R^2 = 0.9916$) between X_M and S_0 and a slope $Y_{X/S}$ = 0.35 is observed. Fig. 3 shows the effect of S_0 over the specific growth rate estimated by the logistic equation (μ_M). The trend for SmF and SSF systems was followed up by the Monod equation with substrate inhibition although the

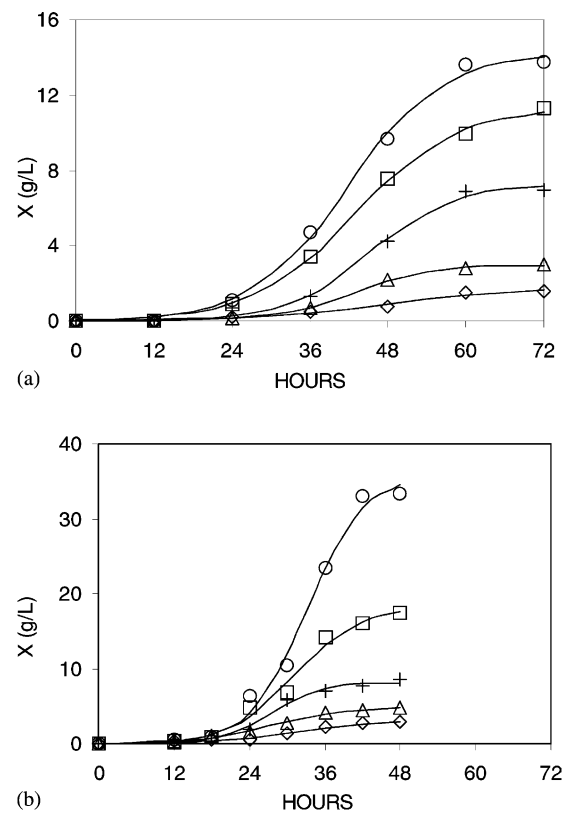

Fig. 1. Growth curves of *A. niger* C28B25 cultured in shake flasks (a = SmF) or on shallow beds of polyurethane foam (b = SSF) with various initial levels of sucrose, S_0 (g l^{-1}): (◇) 6.25, (△) 12.50, (+) 25.00, (□) 50.00, (○) 100.00.

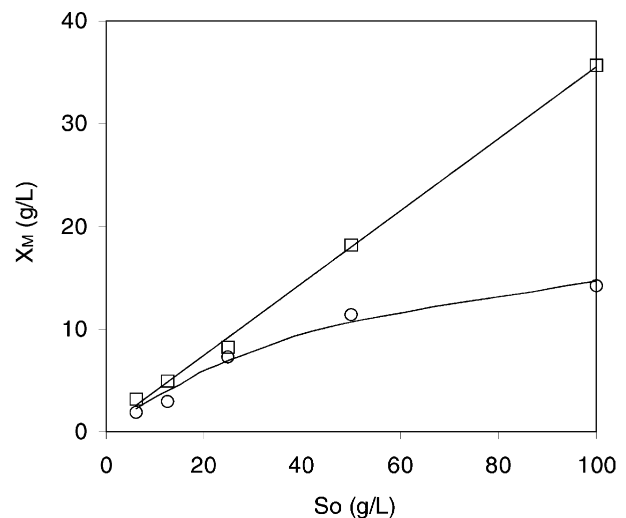

Fig. 2. Correlation between the calculated maximal value of biomass, X_M (g l^{-1}), of *A. niger* C28B25 from data shown in Fig. 1, and the initial sucrose level, S_0 (g l^{-1}), for SmF (○) and SSF (□) systems. Lines correspond to a linear function for SSF ($y = 0.33519x + 0.338$, $R^2 = 0.9980$) or a quadratic function for SmF ($y = -0.0012x^2 + 0.2627x + 0.8234$, $R^2 = 0.9983$).

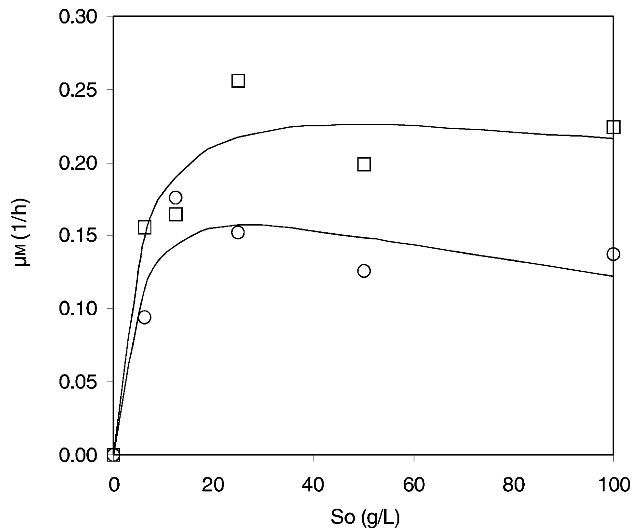

Fig. 3. Correlation between the calculated values of specific growth rates, μ_M, of *A. niger* C28B25, from data shown in Fig. 1 and the initial level of sucrose, S_0 (g l^{-1}), for SmF (◯) and SSF (☐) systems. Lines correspond to the best fit by Monod equation with substrate inhibition (see text). Parameters were for SmF, $\mu_M^* = 0.22\,1\,h^{-1}$, $K_S = 5.7\,g\,l^{-1}$, $K_I = 130\,g\,l^{-1}$, and for SSF, $\mu_M^* = 0.27\,1\,h^{-1}$, $K_S = 5.2\,g\,l^{-1}$, $K_I = 460\,g\,l^{-1}$.

correlation coefficients were not very high ($R^2 = 0.64$ and 0.75, respectively). Nevertheless, it is worth noticing that K_I value for SSF was significantly higher than for SmF. Altogether, results shown in Figs. 2 and 3, support the notion that *A. niger* grew more efficiently in SSF than in SmF system when the initial level of sucrose was high.

Fig. 4a shows the effect of initial sucrose level (S_0) on the titers of invertase (P) as a function of the relative degree of advancement of the fermentation ($\xi = X/X_M$). The shapes of such curves (Fig. 4a) for SmF system were convex ($\nu < 0$) for all values of S_0, except for, $S_0 = 100\,g\,l^{-1}$ ($\nu > 0$). This indicated that excess sucrose helps to prevent the breakdown of invertase. Also, the maximal values of P obtained for each value of S_0 increased with S_0. Comparison of similar curves for SSF (Fig. 4b) showed the opposite effect ($\nu > 0$ for $S_0 = 12.5$ and $25.0\,g\,l^{-1}$ and $\nu < 0$ otherwise) suggesting different physiological conditions for *A. niger* grown in both systems. However, the most remarkable differences were the very high levels of P obtained by SSF system (ca. five times higher) as compared to SmF system. Fig. 5 shows the trend of estimated enzyme yields, $Y_{P/X}$, obtained by fitting experimental data by the model of Luedeking and Piret and the logistic equation. The trend for SmF was a decreasing function of S_0, whereas it was the opposite for SSF system. Again, for $S_0 = 100\,g\,l^{-1}$, $Y_{P/X} = 335\,U\,g^{-1}$ for SSF system and only $Y_{P/X} = 63\,U\,g^{-1}$ for SmF system, supporting the notion of a more efficient biosynthesis of *A. niger* when grown using high levels of S_0 and SSF system, as compared to SmF system. This confirms that excess sucrose helps prevent the breakdown of invertase in SSF system.

Fig. 6 shows the effect of sucrose concentration, S_0, on the productivity, Γ_{obs}, of invertase by strain C28B25 of *A. niger*

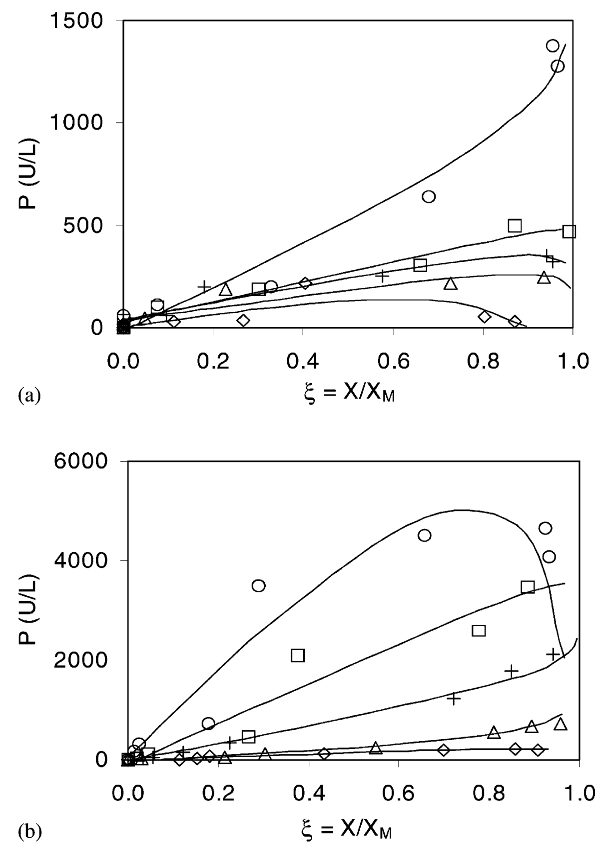

Fig. 4. Production trends of invertase titers, P (U l^{-1}) by *A. niger* C28B25 grown on SmF (a) and SSF (b) reaching various levels of relative biomass production, $\xi = X/X_M$, and with different levels of initial sucrose, S_0 (g l^{-1}): (◇) 6.25, (△) 12.50, (+) 25.00, (☐) 50.00, (◯) 100.00. Lines correspond to calculated values by the Luedeking and Piret and logistic equations (see text).

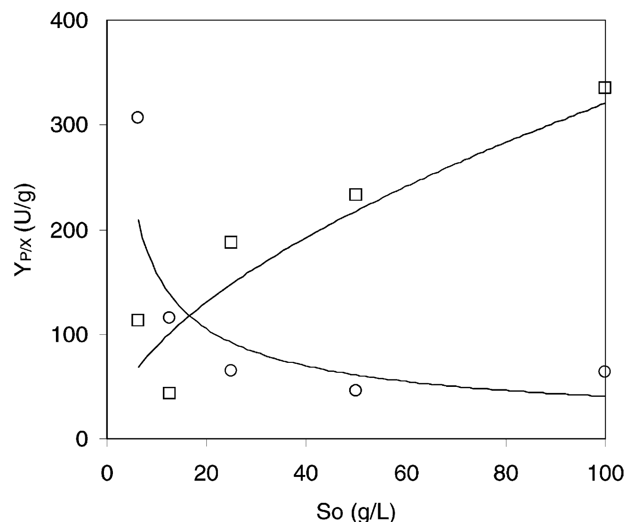

Fig. 5. Invertase yield production by *A. niger* C28B25 on SSF (☐) and SmF (◯) at different initial sucrose concentrations.

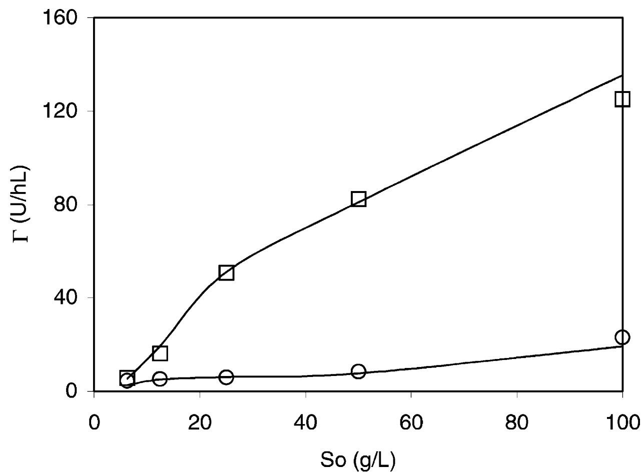

Fig. 6. Comparison of productivities, Γ (U (l h)$^{-1}$), of invertase by *A. niger* C28B25 grown by SmF (○) and SSF (□) techniques as a function of the initial substrate level, S_0. Lines correspond to calculated values according to Luedeking and Piret and logistic equations using data presented in Figs. 1–5. Open symbols correspond to maximal values of quotient, *P*/*t*, taken from experimental data.

grown in shake flasks (SmF) or polyurethane foam (SSF). Increasing levels of S_0 produced higher levels of Γ_{obs} in both systems but the effect was much stronger for SSF having, Γ_{obs}, value nearly five times higher than for SmF when $S_0 = 100\,g\,l^{-1}$. Experimental values of Γ_{obs} were followed closely by the values calculated by Eq. (11) showing the usefulness of such a kinetic model.

These results are a bit surprising because they show that SSF technique can have very effective biomass production without effective mixing (tray reactors) when biological oxygen demand is very high. Biomass production by SmF can be improved by changing from shake flasks as in the experiments reported by Romero-Gómez et al. [10] to stirred vats supplied with pure oxygen and very effective mixing. However, this requires expensive equipment and high-energy expenditures. Apparently in SSF process mold cultures are grown in such a way that is possible to have very good biosynthetic efficiency with very high levels of oxygen demand, without the need for important energy expenditures. The consequences of such an observation will be discussed at the end of this paper.

3.2. Better productivity because of higher biomass production and lower protein breakdown

Díaz-Godínez et al. [11] have shown that *A. niger* produces much higher titers and have higher productivity in SSF system than in SmF system. Fig. 7 shows the profiles of substrate consumption *S*(*t*) and biomass production *X*(*t*) as a function of time. Comparison between Fig. 7a and c (SmF and SSF with $15\,g\,l^{-1}$ of pectin) and Table 1, show similar results in the rates, $\mu_M = 0.22$ and $0.25\,h^{-1}$, and biomass production, $X_M = 4.2$, $4.0\,g\,l^{-1}$, respectively. However, Fig. 7b and d, and Table 1, show a remarkable difference when $50\,g\,l^{-1}$ of sucrose were added to $5\,g\,l^{-1}$ of pectin ($\mu_M = 0.20\,h^{-1}$ vs. $0.47\,h^{-1}$; $X_M = 10.9\,g\,l^{-1}$ vs. $24.2\,g\,l^{-1}$). Those results are consistent with higher values of biomass yield ($Y_{X/S} = 0.37$ vs. 0.26) for pectin without sucrose, and lower biomass yields in SmF vs. SSF when sucrose was added ($Y_{X/S} = 0.19$ vs. 0.38) indicating that

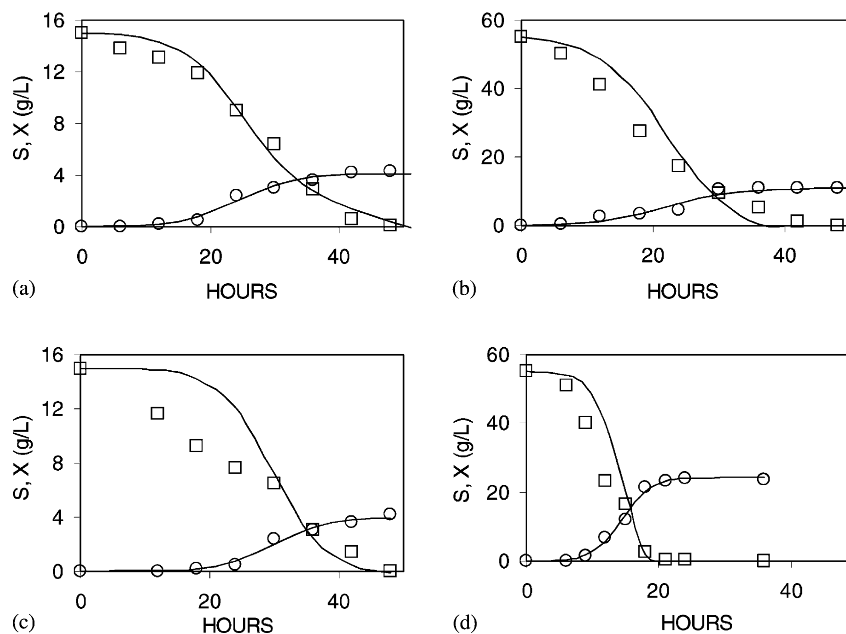

Fig. 7. Evolution of substrate, *S*(*t*), shown as (□) and biomass *X*(*t*), shown as (○), in $g\,l^{-1}$, from cultures of *A. niger* C28B25. Continuous lines correspond to the fit of experimental data by numerical solutions to Pirt and logistic equations, respectively, as discussed in the text: (a) SmF with $15\,g\,l^{-1}$ pectin; (b) SmF with $5\,g\,l^{-1}$ pectin and $50\,g\,l^{-1}$ sucrose; (c) SSF with $15\,g\,l^{-1}$ pectin; (d) SSF with $5\,g\,l^{-1}$ pectin and $50\,g\,l^{-1}$ sucrose (see Table 1).

Table 1
Comparison of growth, metabolic and enzyme production parameters of *A. niger* cultured by shake flasks (SmF) and solid substrate techniques (SSF) using pectin ($15\,g\,l^{-1}$) or pectin ($5\,g\,l^{-1}$) plus sucrose ($50\,g\,l^{-1}$)[a]

	Units	SmF	SSF	SmF	SSF
S_0	$g\,l^{-1}$	15.00	15.00	55.00	55.00
X_M	$g\,l^{-1}$	4.15	3.99	10.93	24.19
X_0	$g\,l^{-1}$	0.02	0.01	0.14	0.03
μ_M	h^{-1}	0.22	0.25	0.20	0.47
$Y_{X/S}$	$g\,X/g\,S$	0.37	0.26	0.19	0.38
m	$g\,S/g\,X\,h$	0.02	0	0	0
$Y_{P/X}$	$U/g\,X$	696.00	131.00	0	204.00
k	$U/g\,X\,h$	−19.00	−5.00	1.00	−1.00

[a] Data from [11].

substrate was consumed and biomass was produced faster and more efficiently in SSF than in SmF when the level of BOD was rather high ($55\,g\,l^{-1}$). Fig. 8a and b shows pectinase titers, P ($U\,l^{-1}$) correlated to the relative biomass level, ξ, indicating that with low levels of BOD ($15\,g\,l^{-1}$ of pectinase) both in SmF and SSF such correlations are convex with a maximum when $3\,g\,l^{-1} < X < 5\,g\,l^{-1}$ and maximum, P, inferior to $2000\,U\,l^{-1}$. In fact, pectinase production is higher in SmF than in SSF. But with $50\,g\,l^{-1}$ of sucrose (Fig. 8c and d) there is a marked inhibition of pectinase production for SmF system but a definite activation in SSF. In such a case the agreement between the kinetic model and experimental data is rather poor, perhaps because of the appearance of two pectinase peaks during the fermentation run, but maximal pectinase titers were higher than $4000\,U\,l^{-1}$.

Fig. 9 shows the time correlation of enzyme titers, P (circles) and the ratio, P/t (triangles) for SmF (Fig. 9a and c) and SSF (Fig. 9b and d) systems without and with sucrose supplementation, respectively. The inhibition of pectinase production by an excess of sucrose in SmF is obvious ($P_{max} \leq 400\,U\,l^{-1}$ with sucrose vs. $P_{max} \geq 1600\,U\,l^{-1}$ without it) as compared with the opposite effect in SSF ($P_{max} \approx 5000\,U\,l^{-1}$ with sucrose vs. $P_{max} \leq 400\,U\,l^{-1}$ without it). This result is in line with previous work done by Solís-Pereira et al. [12] showing the absence of catabolite repression for the production of pectinases by SSF. The ratio, P/t, is a measure of the productivity of enzyme in the liquid phase. For each experiment it is possible to estimate the maximal value of such a ratio by using a parabolic fit of experimental data. Such value is defined as Γ_{obs} in Eq. (11). Table 2 shows that the highest value of $\Gamma_{obs} = 283\,U\,(h\,l)^{-1}$ was found in the SSF supplemented with $50\,g\,l^{-1}$ of sucrose. That result has practical significance because productivity is a major parameter for industrial production of enzymes and deserves further analysis. In Table 2, combined parameters from Table 1 are used to try and explain differences in Γ_{obs}. The parameter $\Gamma_{ref} = \mu_M Y_{P/X} X_M$, helps to point out that the major contributing factor to higher productivity in SSF with sucrose is the higher level of $X_M \approx Y_{X/S} S_0$ together with the apparent resistance to catabolite repression. Large differences between Γ_{ref} and Γ_{obs} may be explained by the effect of fermentation time and also the effect of enzyme decay ($\nu = k/\mu_M Y_{P/X} < 0$) as suggested in the discussion of kinetic models presented above. Comparison of Γ_{cal}, estimated by the calculated value of the enzyme titre, $P(t)$, using the equation of Luedeking and Piret divided by the fermentation time, is in close agreement with values of Γ_{obs}. Initial slope of such correlations correspond to the coefficient, $Y_{P/X}$. The trend of $P(X)$ for $X > X_M/2$ is used to estimate the dimensionless parameter, ν, as shown in Tables 1 and 2. Table 2 shows that, ν, gave negative values for all cases presented but was close to zero when SSF was supplemented with sucrose. Fig. 10 shows the proteolytic

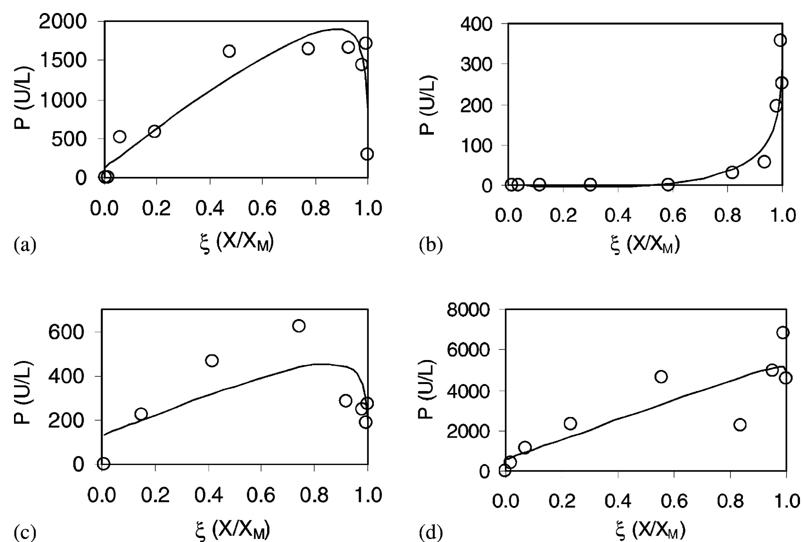

Fig. 8. Correlation between enzyme titres P ($U\,l^{-1}$) produced by *A. niger* C28B25 and ξ. Experimental data are shown as open circles. Solid lines correspond to the fit of experimental data by Luedeking and Piret model and logistic equation (see text). (a) SmF with $15\,g\,l^{-1}$ pectin; (b) SmF with $5\,g\,l^{-1}$ pectin and $50\,g\,l^{-1}$ sucrose; (c) SSF with $15\,g\,l^{-1}$ pectin; (d) SSF with $5\,g\,l^{-1}$ pectin and $50\,g\,l^{-1}$ sucrose (see Table 1).

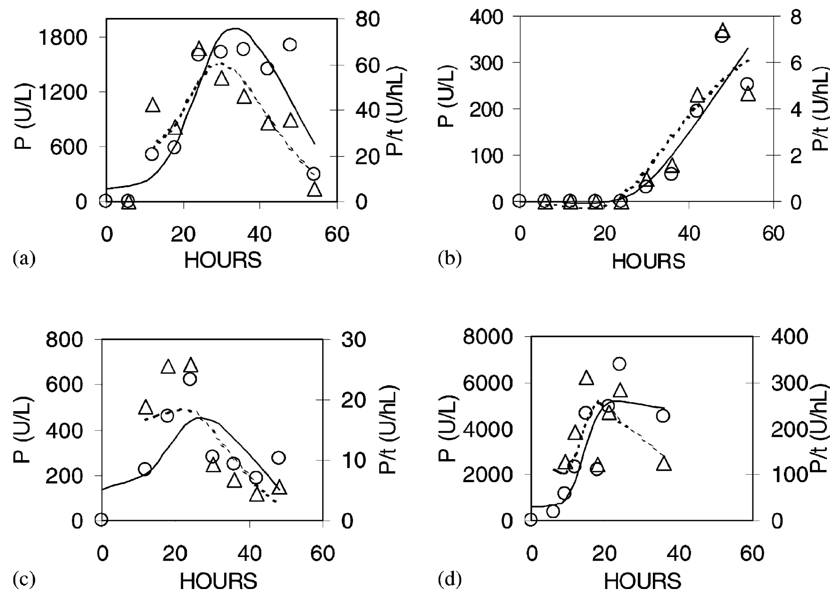

Fig. 9. Correlation between pectinase titres P ($\mathrm{U\,l^{-1}}$) shown as open circles and solid line produced by *A. niger* C28B25 and the quotient P/t ($\mathrm{U\,(l\,h)^{-1}}$) shown as triangles and dotted lines versus time (h). Experimental data are shown as triangles. Interrupted lines correspond to the fit of experimental data by Luedeking and Piret model and logistic equation (see text). (a) SmF with $15\,\mathrm{g\,l^{-1}}$ pectin; (b) SmF with $5\,\mathrm{g\,l^{-1}}$ pectin and $50\,\mathrm{g\,l^{-1}}$ sucrose; (c) SSF with $15\,\mathrm{g\,l^{-1}}$ pectin; (d) SSF with $5\,\mathrm{g\,l^{-1}}$ pectin and $50\,\mathrm{g\,l^{-1}}$ sucrose.

Table 2
Combined parameters of biomass and pectinase production by *A. niger* in SmF and SSF systems (see Table 1)

	Units	SmF	SSF	SmF	SSF
$Y_{X/S}S_0$	$\mathrm{g\,l^{-1}}$	5.55	3.90	10.45	20.90
$q_S = \mu_M/Y_{X/S}$	$\mathrm{g}\,S/\mathrm{g}\,X\,\mathrm{h}$	0.59	0.96	1.05	1.24
$q_P = \mu_M Y_{P/X}$	$\mathrm{U/g}\,X\,\mathrm{h}$	153.00	33.00	0	96.00
$v = k/Y_{X/S}S_0$	Dimensionless	−0.12	−0.15	nd	−0.01
$\Gamma_{ref} = \mu_M Y_{P/X} X_M$	$\mathrm{U\,(l\,h)^{-1}}$	635.00	131.00	0	2319.00
Γ_{obs} [a]	$\mathrm{U\,(l\,h)^{-1}}$	46.00	9.00	5.00	283.00
Γ_{cal} [b]	$\mathrm{U\,(l\,h)^{-1}}$	48.00	10.00	5.00	192.00

[a] Estimated by a quadratic fit to experimental values.
[b] Estimated from the model of Luedeking and Piret (see text).

titers in the fermentation broth indicating that the lowest level of proteolytic activity was precisely for SSF with sucrose (Fig. 10d). It is worth recalling that in this system production of proteases was a secondary and undesirable outcome of pectinase production because it decreases the net productivity. Therefore, the best result was found precisely in this latter case (SSF with sucrose). Other workers have studied the production of proteases by SSF technique as a major product, to be used by organisms in order to break down proteins as a main source of nitrogen [13]. This latter case should not be confused with the unintended production of proteases, perhaps as a result of physiological stress. In summary, SSF system achieved higher productivity for pectinase when sucrose was added in large quantities, mainly because of three factors: higher levels of biomass production, apparent resistance to catabolite repression and reduced breakdown of pectinases by contaminant proteases.

Again, it is necessary to point out that resistance to sugar inhibition of inducible enzymes such as pectinases in SSF system as compared to high sensitivity in SmF system requires a mechanistic explanation discussed at the end of this paper.

3.3. Better productivity because of higher enzyme yield and lower proteolysis

Aguilar et al. [16] studied tannase production by *A. niger* grown on finely ground samples of PUF (density of $113\,\mathrm{g\,l^{-1}}$) with the purpose of measuring enzyme productivity by SSF under conditions where growth was limited by steric hindrances [14,15]. This work was different to previous reports by Romero-Gómez et al. [10] and Díaz-Godínez et al. [11] who used regular commercial samples of PUF (density of $15\,\mathrm{g\,l^{-1}}$). Aguilar et al. [16] compared SSF production of tannase grown in ground PUF to SmF production in shake flasks (Fig. 11). Tannase titers were found to be much higher in SSF ($14{,}000\,\mathrm{U\,l^{-1}}$) than in SmF ($2800\,\mathrm{U\,l^{-1}}$) despite the fact that maximal biomass

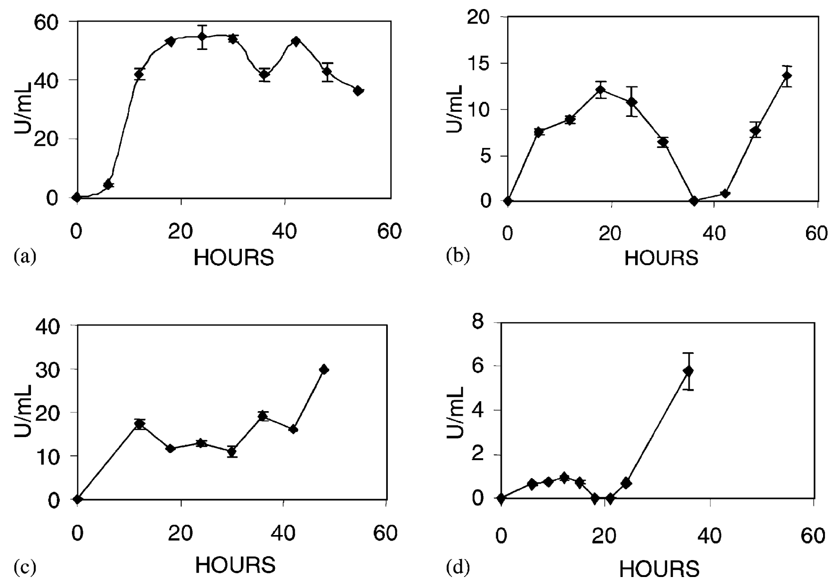

Fig. 10. Correlation between protease titres (U/ml) produced by *A. niger* and time (h): (a) SmF with $15\,g\,l^{-1}$ pectin; (b) SmF with $5\,g\,l^{-1}$ pectin and $50\,g\,l^{-1}$ sucrose; (c) SSF with $15\,g\,l^{-1}$ pectin, (d) SSF with $5\,g\,l^{-1}$ pectin and $50\,g\,l^{-1}$ sucrose.

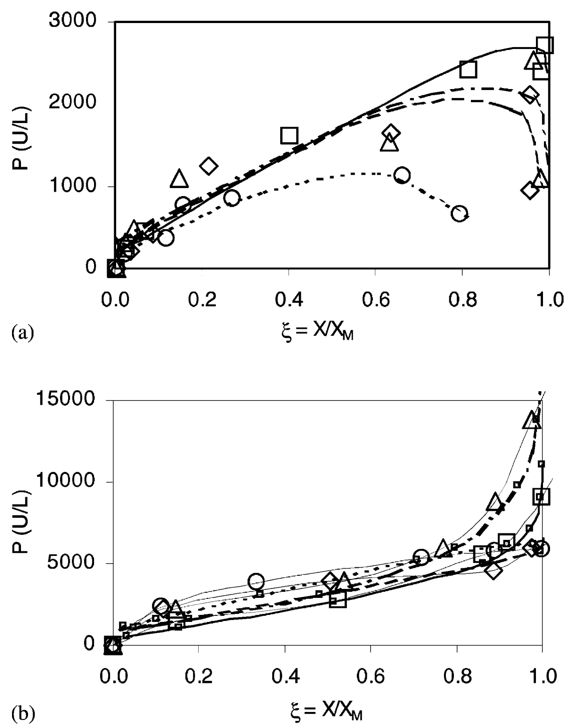

Fig. 11. Correlation between tannase titers, E ($U\,l^{-1}$), and relative biomass level, $\xi = X/X_M$, produced by *A. niger* Aa20 cultured by SmF (a) and SSF (b) with the following initial levels of tannic acid, S_0 ($g\,l^{-1}$): (○) 12.5, (◇) 25.0, (□) 50.0, (△) 100.0. Symbols correspond to experimental data and lines to the best fit by Luedeking and Piret model together with the logistic equation (see text).

levels (Table 3) were much lower in SSF ($4.5\,g\,l^{-1}$) than in SmF ($11.5\,g\,l^{-1}$). Obviously, the enzyme yield, $Y_{P/X}$, was much higher in SSF ($870\,U\,g^{-1}$) than in SmF ($461\,U\,g^{-1}$). Enzyme productivities (Table 3) were also higher in SSF ($\Gamma_{cal} = 313\,U\,(l\,h)^{-1}$) than in SmF ($\Gamma_{cal} = 57\,U\,(l\,h)^{-1}$). Addition of increasing amounts of glucose to the culture medium with a fixed amount of tannic acid as inducer ($25\,g\,l^{-1}$) decreased the values of $Y_{P/X}$, both in SSF and SmF cultures (Table 3). Furthermore, the shape of state plots, $P(t)$ vs. $X(t)$, showed a definite peak for SmF ($\nu < 0$) and concave form for SSF ($\nu > 0$) indicating a strong decay of enzyme titers in SmF during the fermentation as compared to a steady increase in SSF without apparent decay. Protease activity in the liquid broth was found to be eight times higher in SmF than in SSF. Moreover, the addition of protease inhibitors to SmF, at peak time, reverted the decay of enzyme titers without any significant effect on biomass production.

These results show that it is possible to obtain higher enzyme productivity Γ, in SSF, as compared to SmF, even when growth is restricted by physical hindrances (excessive packing density) if the mold happens to have a higher enzyme yield factor, $Y_{P/X}$, and a lower rate of decay ($\nu \geq 0$).

3.4. Microscopic and geometrical comparison of SSF and SmF cultures

Cultures of *A. niger* developed by SmF technique usually produce quasi-spherical aggregates called pellets as indicated in Fig. 12. Such aggregates have a diameter in the millimeter range (i.e. $3000\mu M$) with two well defined zones, an outer layer made of loose mycelium which dyes lightly with methylene blue and has an approximate thickness in the range of 10^{-3} cm (i.e. $h_P = 200\mu M$) and a central core, made of tightly woven mycelium which dyes strongly with methylene blue.

The fine structure of PUF is made of a honeycomb of impermeable polymer pillars having approximately $300\mu M$

Table 3
Comparison of parameters of tannase production by *A. niger* Aa20 grown by SmF (shake flasks) and SSF (PUF packed bed reactor)

S_0 (g l^{-1})	X_M (g l^{-1})	$Y_{X/S}$ (g X/g S)[a]	μ_M (h^{-1})	$Y_{P/X}$ (U g^{-1})	k (U/g X h)	ν	Γ_{ref} (U (l h)$^{-1}$)	Γ_{obs} (U (l h)$^{-1}$)	Γ_{cal} (U (l h)$^{-1}$)
SmF									
12.5	5.04	0.40	0.13	947	−53.23	−0.42	639	32	29
25.0	8.12	0.32	0.23	468	−19.54	−0.19	860	53	61
50.0	19.85	0.40	0.25	167	−2.45	0.06	823	71	77
100.0	13.01	0.13	0.29	261	−10.40	−0.14	970	63	61
Mean	11.50	0.31	0.22	461	−21.4	−0.19	823	55	57
Standard deviation	6.46	0.13	0.06	348	22.34	0.17	138	17	20
SSF									
12.5	3.83	0.31	0.19	1588	−15.07	−0.05	1231	178	157
25	4.04	0.16	0.26	1079	10.53	0.04	1125	141	118
50	4.58	0.09	0.22	616	52.74	0.39	619	236	219
100	5.55	0.06	0.18	98	95.00	5.32	99	381	353
Mean	4.50	0.15	0.21	870	35.80	0.50	769	297	313
Standard deviation	0.77	0.11	0.03	676	48.37	0.86	520	61	55

[a] Estimated as $Y_{X/S} = X_M/S_0$. Data from [16].

of thickness and forming polyhedral cells with a diameter close to 1000μM (Fig. 12a). Added liquid broth (ca. 20 cm^3 per g of PUF) is distributed evenly by capillarity in a thin layer having a thickness $h_W = 60 \pm 5\mu$M. When inoculated with *A. niger* spores, a population of mycelial aggregates is formed which grows inside the water layer and spreads out as mycelial slabs having an average thickness of $h_X = 300 \pm 50\mu$M. Average polymer density of light PUF is close to 15 g l^{-1}. Using a simple geometrical model where volume is equal to the surface by the thickness of the layer, it is possible to estimate an area to volume ratio of a completely dispersed liquid broth as follows:

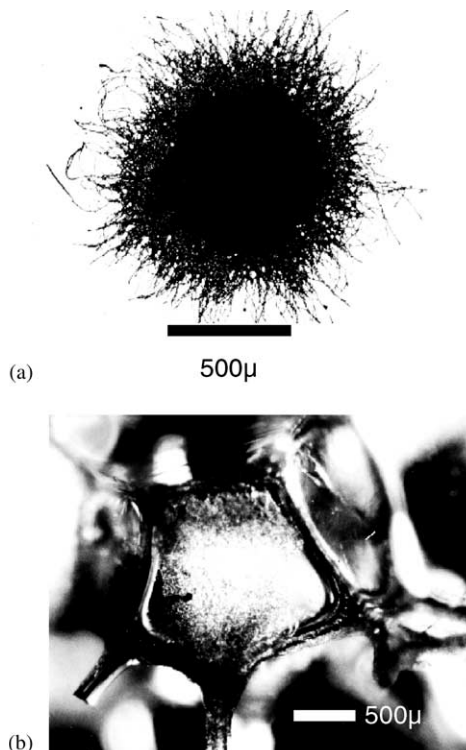

Fig. 12. (a) Micrograph of *A. niger* C28B25 growing under submerged culture conditions with 100 g of sucrose per liter after 24 h of incubation (50×). (b) Micrograph of *A. niger* C28B25 growing in polyurethane foam under solid state fermentation conditions after 6 h of culture (100×).

$$\frac{A}{V} \approx \frac{1}{h_W} \approx 1.67 \times 10^2\,\mathrm{cm}^{-1}$$

For example, 2.5 g of PUF loaded with 50 cm^3 of liquid broth will have an area

$$A = (1.67 \times 10^2\,\mathrm{cm}^{-1})(50\,\mathrm{cm}^3) = 8.35 \times 10^3\,\mathrm{cm}^2$$

This surface area exposed to passive gas exchanges is much larger than the usual surface area of the air to liquid interphase in a 250 cm^3 shake flask filled with 50 cm^3 of broth (ca. 20 cm^2). This geometrical consideration shows that SSF cultures have a much larger air to liquid interphase than conventional SmF culture, for example, 400 times higher. Thus, SSF cultures are grown initially in thin slabs of cell aggregates having a very large surface area for gas exchange, whereas SmF mold cultures are grown in pellets having large diameters and small surface area for gas exchange. For single cell cultures, such as yeast suspensions, it has been observed that SSF technique on PUF produces large cell aggregates (nearly 10^2 cells) spread out in the thin layers of liquid broth having a large A/V ratio. SmF cultures (shake flasks), however, produce small aggregates (ca. 10 cells) with a much smaller A/V ratio, as indicated above (unpublished results).

Such geometrical differences between SSF and SmF cultures may account for important physiological differences encountered experimentally and discussed below in terms of processes limited by diffusion of gasses, substrates and products.

3.5. A model to account for physiological differences between SSF and SmF cultures

Pirt [8] proposed that cell aggregates have strong diffusional limitations based on the mass balance of a heterogeneous reaction systems

$$\frac{\partial C}{\partial t} = D\nabla^2 C - q_S \rho(x, y, z) \tag{12}$$

where D is the diffusion constant of a solute having concentration, $C(x, y, z)$, and q_S the metabolic coefficient of biomass having density $\rho(x, y, z)$. To make things simpler, consider the steady state ($\partial C/\partial t = 0$) of a thin layer of biomass with coordinate x, uniform density ρ_0 and thickness h. Boundary conditions can be fixed as $C = C_0$ for $x = 0$, and $C = 0$ for $x = h$. Under these conditions the solution of the differential equation becomes

$$C(x) = C_0 \left(1 - \frac{q_S \rho x^2}{2DC_0}\right)\left(1 - \frac{x}{h}\right) \tag{13}$$

This equation shows that $C = 0$ when $x = h = [2DC_0/q_S\rho]^{1/2}$ as shown by Pirt (1975). This way it can be predicted that for oxygen, $h_G \approx 10^2\,\mu$M and for sugar consumption $h_S \approx 10^3\,\mu$M, assuming that for oxygen $C_0 = 6\times10^{-3}\,\mathrm{g\,l^{-1}}$ and $D = 5 \times 10^{-5}\,\mathrm{cm^2\,s^{-1}}$, and for glucose $C_0 = 10^2\,\mathrm{g\,l^{-1}}$ and $D = 1 \times 10^{-6}\,\mathrm{cm^2\,s^{-1}}$ with $q_S \approx 2 \times 10^{-4}\,\mathrm{s^{-1}}$ ($1\,\mathrm{h^{-1}}$) and $\rho = 0.3\,\mathrm{g\,cm^{-3}}$ (solid content of biomass).

As for SmF mold cultures in the pellet form, it is well known that the active aerobic layer has a thickness $h \approx 10^2\,\mu$M, which is in the same order of magnitude as the measured thickness of the experimental loose layer in Fig. 12a. Hence, whenever the diameter of the pellet becomes of the order of $10^3\,\mu$M, most of the pellet will be anoxic with a rather low-level of sugar inside the pellet core. When considering a mold culture made of disperse mycelia, most of those cells would be in full contact with a similar concentration of sugar and will be subjected to strong catabolite repression. Also, the small, A/V, ratio in a stirred tank will require vigorous stirring and forced aeration in order to compensate the large oxygen demand when sugar concentration is high.

For SSF cultures, geometrical and physical restrictions are quite different to those observed in SmF cultures. Oxygen diffusion is perpendicular to large cell aggregates, which are dispersed in a thin layer of broth. In such a system, sugar diffusion is horizontal along the thin layer (Fig. 12b). It is therefore possible to create concentration gradients of sugars within large cell aggregates because there is no mixing within the static layer of liquid broth, itself finely dispersed on the solid substrate. Furthermore, the relaxation times ($t = h^2/D$) for oxygen diffusion can be estimated in the order of a few seconds contrasting with nearly 1 h for sugar diffusion. This is consistent with a moving boundary problem where the biomass boundary moves at approximately the same rate ($1/\mu_M$ = hours) as the spreading rate of sugar diffusion. Oxygen diffusion, however, is equilibrated at a much faster rate.

The above facts seem to explain why mold cultures grown in shake flasks with high sugar concentration have low conversion values of, $Y_{X/S}$, and $Y_{P/X}$. Also, secondary proteolysis may be seen as a physiological adaptation to metabolic stress. In this sense, it may be that SSF cultures are in a better physiological condition and for such a reason, produce less proteolytic enzymes than in SmF cultures. Altogether, these factors seem to explain the physiological differences concerning enzyme production as outlined above.

4. Conclusions

In summary, SSF culture seems to be working, in a natural way, as a fed batch culture with fast oxygenation but slow sugar supply. The process has the added advantage of being a static process without mechanical energy expenditures. In contrast, SmF cultures work as homogeneous systems requiring large energy expenditures to supply oxygen at fast enough rates to cope with high oxygen demand. There is the need in such processes for automated fed batch supply of substrates in order to avoid catabolite repression.

Further study of SSF systems may profit from the use of microscopic techniques such as image analysis, microelectrodes, development of DNA probes with optical properties such as in situ hybridization of specific RNA messengers and the use of colored fusion proteins used as tags of specific protein products. Perhaps this way, heterogeneity of SSF systems will be transformed to its advantage to control microbial activity for enzyme production as compared to the handicap of homogeneous systems present in SmF cultures.

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