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A N I N T E R N A T I O N A L J O U R N A L

A Simplified Model for *A. Niger* FS3 Growth during Phytase Formation in Solid State Fermentation

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ABSTRACT

A simplified model to describe fungal growth during citric pulp fermentation for phytase production was described for the first time. Experimental data for biomass growth were adjusted to classical mathematical growth models (Monod and Logistic). The Monod model predictions showed good agreement with the experimental results for biomass concentration during 96 hours of fermentation. Parameters such as yield of biomass from oxygen ($Y_{X/O}$), maintenance coefficient (m) and specific growth rate (μ) were compared showing a good correlation between the data and the model. An alternative method for biomass determination in this process was developed since a great correlation was found between biomass growth and enzyme formation.

Key words: Monod Model, logistic model, modelling, fungal growth, phytase formation, citric pulp

INTRODUCTION

Some kinetic models are used to describe growth kinetics in solid state fermentation (SSF) (Rodriguez-Leon et al., 1988; Viccini et al., 2001; Hamidi-Esfahani et al., 2007; Carvalho et al., 2006; Spier et al., 2007). The study of kinetics enables the discovery of many important parameters such as specific growth rate, process yield, process productivity, generated heat, process control criteria, strategy for the production of particular products, and industrial scale considerations (Pandey et al., 2000a,b; Pandey et al., 2001a,b). The typical characteristic of SSF is that the biomass is attached to the solid surface and therefore it is difficult to measure it directly. Some indirect measurements of biomass in SSF have been used because many SSF processes involve filamentous fungi and they are tightly bounded to the substrate. The detection of some cell compounds such as proteins or other cell components such as DNA, RNA, chitin, ergosterol, and glucosamine (Raimbault, 1981; Okasaki et al., 1980; Desgranges et al., 1991; Carvalho et al., 2006) or even the measurement of metabolic data such as CO_2 evolution rate and O_2 consumption rate (Bellon-Maurel, 2003; Spier et al., 2007; Nishio et al., 1979; Carrizalez et al., 1981) are interesting issues for growth kinetics and mathematical modelling of heat and mass transfer in SSF (Doelle and Mitchell, 1992).

The present work shows a simplified model to described fungal growth during phytase formation using citric pulp bran, one of the major

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agroindustrial residues produced in Brazil, costing US$ 10.00 per ton. It is obtained from the citrus-processing industry and may also be used for the production of enzymes such as phytases, thus offering an economic alternative to traditional phytase production. Low substrates such as agroindustrials residues are been studied for biomolecules production by solid state fermentation (Brand et al., 2006).

If the substrate concentration S(t) is a given function of time, then microorganism population growths according to the differential equation:

$$\frac{dX}{dt} = \mu(S(t))X(t) \qquad \text{Eq. 1}$$

Where X(t) denotes the microbial concentration at time t.

Monod model is an empirical law that follows the general equation presented above. It states that the specific growth rate μ depends on the growth limiting substrate S according to the following formula (Monod 1941):

$$\mu = \frac{\mu_{\max} S}{K_S + S} \qquad \text{Eq. 2}$$

Where μ_m and K are constants.

MATERIALS AND METHODS

Microorganism And Inoculum Preparation

A phytase fungal strain was isolated from soil samples collected at Parana State (Brazil) and identified as *A. niger* FS3 (Spier et al., 2008), grown on potato dextrose agar medium and kept at 4ºC. Citric pulp extract was prepared with 1:10 (milled citric pulp bran: water) supplemented with 0.5 $g.L^{-1}$ $NaNO_3$, diluted to 1:10 with ultra pure water, adjusted to pH 5.0 and sterilized.

SSF fermentation

SSF was performed in a glass column bioreactor (20 cm x 4 cm) using 0.8-2.0mm particles of citric pulp (Cargill, Brazil). The solution, consisting of 0.33 M Na-citrate buffer pH 5.0, contained 1.5% w/v urea, adjusted to 65% of the moisture and 1.0 N $mL.g^{-1}.min^{-1}$ aeration rate. Then 10% v/v of *A. niger* FS3 pellets suspension grown in 10% (w/v) citric pulp extract media was inoculated and thoroughly mixed, and 68-70 g were added to the columns. The columns were capped at both ends with cotton filters. They were connected to humidifiers and immersed in a water bath at 30 ºC for 96 h. Extracellular crude phytase was obtained from initial maceration and filtration using Whatman nº1 filter paper, and then the filtrate was centrifuged (4000 g, 15 min, 4 ºC). The supernatant was used in the phytase assay.

Biomass Determination In SSF

The biomass was estimated by ergosterol extraction based on the Seitz method (Seitz et al., 1979) with some modifications. The method consisted of a mixture composed of 1g of fermented substrate from each column, 4 mL of ethanol and 2 mL of NaOH 2M incubated in a water bath at 70ºC for 1h. Then, 4 mL of HCl 1M was added and the solution was agitated. Following this, 2 mL of 1M $KHCO_3$ and 4 mL of n-hexane were added, the mix was agitated vigorously, and then centrifuged at 4500 g for 5 min. After the addition of 4 mL of n-hexane, a second centrifugation was performed at 4500 g for 3 min. Then, after the addition of 2 mL of n-hexane, a final centrifugation (at 4500 g for 2 min) was performed. All supernatants were collected in a light-protected and open flask for solvent evaporation. The extracts were analysed in an HPLC Varian ProStar with a C_{18} column and a photodiode array detector set to 282 nm. An injection volume of 10 μL of sample was used. The mobile phases used were pure methanol (from 0 to 3 min), pure acetonitrile (from 3 to 10 minutes), and pure methanol again (from 10 to 15 minutes) with a 1 $mL.min^{-1}$ flow. Pure ergosterol (Sigma) solutions of 5000 and 1000 μg mL^{-1} were used as standards.

Simplified Model For Biomass Growth

The ordinary differential equations were solved by the analytic method of separation of variables. The initial condition (biomass concentration in the initial instant) was adjusted to correspond to the observed value (0.1027 g per g of dry substrate $g.gds^{-1}$). The parameters adjustment was performed by the minimization of the sum of quadratic differences (SQD) between the experimental and the predicted values by the model. The program used to help in this calculation was MS Excel (and the tool was MS Solver).

Phytase Activity Assay

Phytase activity was determined based on heinonen and lahti's (1981) method. The whole analysis was performed in triplicate and the mean values and standard deviations were reported.

RESULTS AND DISCUSSION

The biomass content increased during the process reaching a maximum value (0.9216 $g.gds^{-1}$ at 96 h of fermentation (Fig 1). Phytase synthesis also increased during this period with the fungal biomass reaching maximal production at 96 h (94-96 U gds^{-1}). Considerable respiratory activity was observed as a result of fungal metabolic activity. Therefore, enzyme synthesis can be assumed to be associated with the growth (Fig 1). Other studies also showed that phytase production is associated with growth (Krishna and Nokes, 2001).

Initially phytate concentration was 18.17 $mg.gds^{-1}$. After 96 h of fermentation, 7.10 mg/gds of residual phytate was found in the maximum phytase activity corresponding to 90 $U.gds^{-1}$ and biomass content was 0.92 $g.gds^{-1}$. Inorganic-P increased in the course of fermentation from 74 mg/100 gds to 108 mg/100gds.

Fig 1 shows the profile of total sugars (%) reduced from 36.23% to 11.34% during 96 h of fermentation, in a total of 24.89% of reduction.

According to Greiner (2005), in moulds, phytase production is growth associated, and enzyme activity increases from the onset of growth to the beginning of the stationary phase. However, the peak synthesis of *A. niger* FS3 phytase occurred in the stationary phase, possibly because of the limited conditions of nutrient or energy known to occur in the stationary phase, which could be responsible for the phytase induction. A limitation of inorganic phosphate could also be the reason to induce phytase production. Citric pulp presents low inorganic phosphate content (0.025µmol/g citric pulp), as observed by Spier et al. (2008) and Shieh et al. (1969). The production of extracellular fungal phytase was induced by a limiting concentration of inorganic phosphate in the growth medium. The enzyme yield declined during further incubation, possibly due to the reduced nutrient level of the medium, the presence of inorganic-P in high levels, and the consequent declining phase of *A. niger* FS3.

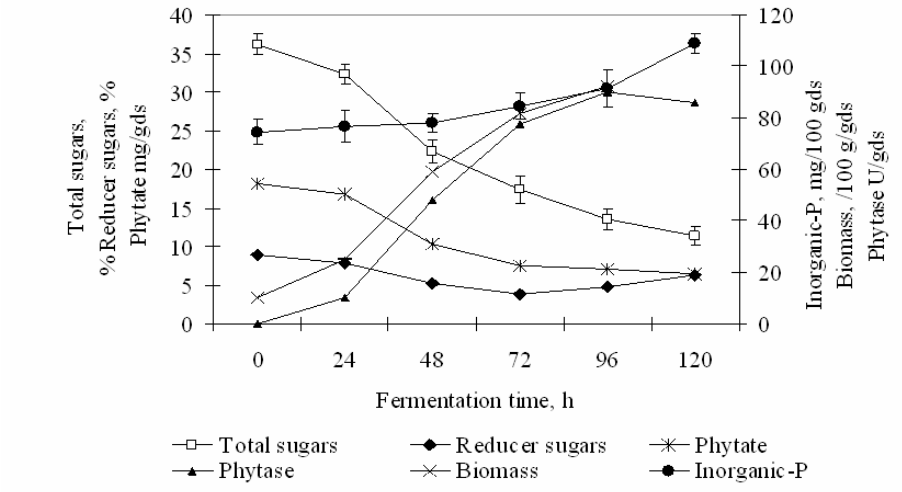

Figure 1 - Profile of substrate consumption – Na-phytate ($mg.gds^{-1}$), total sugars (%), biomass growth ($x10^{-2}$ g. gds^{-1}), phytase formation ($U.gds^{-1}$) and liberation of inorganic-P release (mg. $10gds^{-1}$ during citric pulp fermentation.

Phytase production increased more than 90 times (from 1.0 to 96 U gds^{-1}) during biomass growth. Phytase production reported in the literature showed that 58 $U.gds^{-1}$ were achieved in dry olive waste (Vassilev et al., 2007). Singh and Satyanarayana (2007) obtained 20.8 $U.gds^{-1}$ by *Sporotrichum thermophile* using sesame oil cake.

Mucor racemosus NRRL 1994 synthesised 44.5 U gds^{-1} in wheat bran and sesame oil cake (Roopesh et al., 2006). *A. ficcum* NRRL 3135 produced 25 U gds^{-1} using wheat bran as substrate (Bogar et al., 2003), and higher phytase yield (130 $U.gds^{-1}$) was obtained using released meal by an *A. niger* A-98 locally isolated (El-Batal and Karem, 2001).

Correlation Between Biomass And Phytase

A correlation analysis (not shown) of the data from the whole columns (biomass and phytase produced) – data presented in Table 1 showed good correlation (R^2 = 0.9863), which suggests a fairly proportional relationship between both factors. Therefore, a correlation of biomass concentration and phytase activity could be used to estimate biomass in further experiments.

The following equation was defined for biomass estimation:

$$Biomass = -10^{-5}Phy^2 + 0.0103Phy + 0.0692 \quad \text{Eq. 3}$$

$R^2 = 0.9863$

where *Biomass* is the biomass in the dry fermented citric pulp ($g.gds^{-1}$, *Phy* is the phytase activity in $U.gds^{-1}$.

That study may be a simple alternative analysis to ergosterol extraction and chromatography analysis. However, this expression should be used with care, since it probably depends on the microorganism used and on the cultivation conditions (Carvalho et al., 2006).

Table 1 - Kinetic of *A. niger* FS3 growth, phytase formation and substrate consumption during SSF.

Time (h)	Total sugars (%)	Reducer sugars (%)	Phytate (mg/gds)	Phytase (U/gds)	Biomass ($x10^{-2}$ g/gds)	Product (inorganic-P) mg/100gds
0	36.23	8.98	18.17	0.00	10.27	74.52
24	32.37	7.88	16.82	10.18	24.95	76.7
48	22.29	5.21	10.37	48.10	58.93	78.1
72	17.31	3.86	7.55	77.64	81.52	84.52
96	13.51	4.73	7.10	89.83	92.16	91.42
120	11.34	6.34	6.43	85.64	nd	108.77

The yield of product from substrate ($Y_{P/S}$) showed a peak at 72 h. It means that each gram of substrate consumed generated 594.55 U in the column at that time. Since phytase and biomass are correlated, the yield of biomass from substrate ($Y_{X/S}$) was maximal at 72 h of cultivation, with 1.42 g of fungal biomass being produced for each g of substrate consumed.

Simplified Model For Biomass Growth

Experimental data for biomass growth was adjusted to a simplified mathematical model. The specific cell growth rate is defined according to Eq. 4:

$$\mu = \frac{1}{X}\frac{dX}{dt} \quad \text{Eq. 4}$$

Following the hypothesis below:

$$\mu = f(X) \quad \text{Eq. 5}$$

The growth rate is a function of cell number presence only. The variables (substrate concentration, temperature, pH, aeration rate, presence of inhibitory components) do not interfere negatively in the process. The logistic model of growth frame in the hypothesis above assumed:

$$\mu = \mu_{max}\left(1 - \frac{X}{X_{max}}\right) \quad \text{Eq. 6}$$

In the equation 6, μ_{max} indicate the maximum specific cellular growth rate while X_{max} indicates the capacity of cell saturation, which means the maximum cell concentration expected. Admitting the logistic model, equation 7 is the solution of equation 1, the table 2 shows the optimized parameters values, and the figure 2 exhibit the best adjusted curve to the experimental data (with the parameter of table 2).

$$X = \frac{X_{max}}{1 + \frac{X_{max} - X_0}{X_0} e^{-u_{max} t}} \quad \text{Eq. 7}$$

The hypothesis of logistic model indicates that the biomass concentration (in function of time) is crescent and mono tonic and the maximum specific growth rate (μ_{max}) is equivalent to the specific growth rate in the initial instant (μ).
Thus, the logistic model optimized:

$$\mu(0) = 0.052$$

Considering the hypothesis that the substrate is limitant factor of growth, and that the others variables do not interfere in the process:

$$\mu = f(S) \quad \text{Eq. 8}$$

The Monod model is a classic example of equation groups with the form of equation 8:

$$\mu = \frac{\mu_{max} S}{K_S + S} \quad \text{Eq. 9}$$

In the equation 9, μ_{max} indicates the maximal specific rate of cell growth, and K_S is the constant of microorganism affinity by the substrate (total sugars).

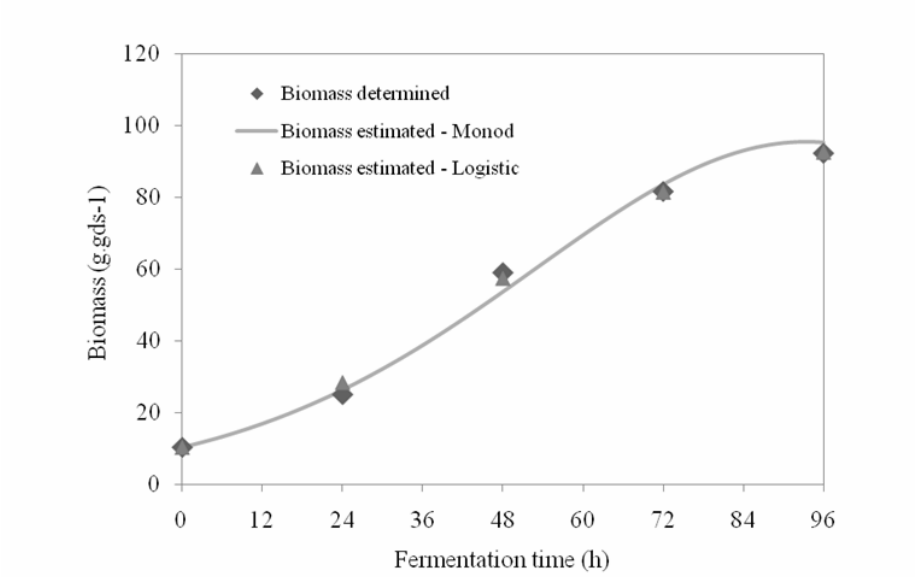

Figure 2 - Curve of biomass growth obtained in the simplified Monod and Logistic model.

Table 2 - Estimations of process parameters through the Logistic model

Process Parameters	Logistic model
μ_{max}	0.052
X_{Max}	98.0
R^2	0.997

Admitting the Monod kinetic, the equation 10 is the solution of the equation 1. The table 3 exhibits the values of optimized parameters, and the figure 2 shows the curve that better fits to the experimental data:

$$X = X_0 e^{\frac{\mu_{max} S}{K_S + S} t} \quad \text{Eq. 10}$$

At the initial instant of fermentation, the substrate concentration was 36.23%.

Considering this data, the values of Table 3 and the equation 10, it is possible to calculate the value of specific growth rate at the instant 0 according to the Monod kinetic:

$$\mu(0) = 0.043$$

The logistic model has a good adaptation to the experimental data to determined choose of parameters (R^2=0.997), therefore, the hipothesis of growth limitation due to cell growth itself (intraespecific competition) can not be discarded. The Monod model was also well fitted to the experimental data to determined choose of parameters (R^2=0.995), and consequently, it is recommendable not to discard the hypothesis of growth limitation due to substrate limitation. Monod model was also gave the best fit to the experimental data in studies by Gerard et al. (2006).

It is important to detach that biologic processes are extremely complexes, and they have numberless variables interfering in the process. The models proposed can not be validated without additional experiments.

Table 3 - Estimations of process parameters through the Monod model.

Process Parameters	Monod model
μ_{max}	0.077
K_s	29.4
R^2	0.995

CONCLUSIONS

The adjust of both of curves (Logistic and Monod) was good (R^2=0.997 and R^2=0.995). The Monod model predictions showed good agreement with the experimental results for biomass concentration during the course of fermentation for maximum phytase formation. It is fairly probable that both substrate concentration and biomass concentration have influence on microbial kinetic ($\mu = f(S, X)$), and even that another variables of cultivation have decisive roles in growth rate (inhibitors, sub products, temperature conditions, pH, among others). There are sufficient evidences that give support to future experiments with the finality to determine some kinetic parameters of cultivation. Those experiments would be the confirmation of these or eventually others more complex kinetic models.

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RESUMO

Um modelo simplificado para descrever o crescimento fúngico durante a fermentação em polpa citric para a produção da fitase foi descrita pela primeira vez. Dados experimentais para a formação de biomassa foram ajustados a modelos clássicos de crescimento microbiano (Monod e Logístico). O modelo Monod previsto mostrou boa correlação aos resultados experimentais para a concentração de biomassa até 96 horas de fermentação. Parâmetros como rendimento de biomassa a partir de oxigênio *($Y_{X/O}$)*, coeficiente de manutenção (m) e taxa específica de crescimento *(μ)* foram comparados mostrando uma boa correlação entre os dados e o modelo. Um método alternativo para a determinação de biomassa neste processo foi desenvolvido a partir de uma excelente correlação encontrado entre o crescimento microbiano e a formação da enzima.

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