RELATION BIOMASS / RESPIRATION: THEORETICAL AND PRACTICAL ASPECTS

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Summary
We present here some theoretical consideration on the correlation model between Biomass and respiration. The case of the growth of Aspergillus niger on starchy substrate is discussed as an example of the application of the calculation of fermentation parameters . It is specially possible to calculate from the respiratory metabolism the specific growth rate, the maintenance, yields in biomass, metabolic products, and also heat evolved during the fermentation on the basis of Oxygen Uptake Rate, CO2 evolved and the consumed substrate. In a second part we detail the equipment and methodology of the lab standardised method we developed for all physiological studies and optimisation of the culture conditions for SSF of fungi. Finally we illustrate the technique by the case of *A. niger* and *Rhizopus oryzae* cultivated on starchy SSF for showing the software developed for automatic calculation from the on line data obtained by CPG.

I. Theoretical Aspects

1. General aspect of the fungal growth kinetics:

Exponential Model: Various models were proposed to fit up with the kinetics growth of micro-organisms. First of them were proposed by Monod for the unicellular growth of bacteria and can be written in the exponential equation:

$$dX / dt = \mu . X$$

where X is Biomass; t is Time and μ the specific growth rate . μ represents the biomass produce per hour and by g of biomass; it means that the growth rate is proportional to the actual biomass. In the integrated form:

$$X = X_0 . e^{\mu}.t$$

and the logarithmic form:

$$\ln X = \mu.t + \ln X_0$$

Then you can calculate the specific growth rate (μ = ln2/td) plotting the biomass on a log scale versus time. This type of exponential model is generally applied for unicellular bacteria or yeast when the number of cells growth exponentially .

Vegetative growth of mycelium: In the case of mycelium, the growth is of different mode without cellular division. The exponential form could be the result of a linear growth at the apex of the hyphae combined to the frequency of the branching point which increase the

number of hyphal apex. Trinci calculated that for A. nidulans, the apex grown exponentially until 120µm then the growth became linear and a new branching point appeared. Considering the biomass proportional to the total length of the mycelium, he global equations become :

$$X = k.\ L$$

$$dL/dt = k1\ .\ n \qquad \text{with} \qquad n = k2.L$$

where L= length of mycelium; k1 and k2 specific constants and n frequency of branching , which can be written :

$$dL/dt = k1.k2\ (L)$$

$$dX/dt = k.\ dL/dt = (k1.k2).\ k.L = k1.k2.\ X$$

$$dX/dt = \mu\ .\ X \quad (\text{with } \mu = k1.k2)$$

Thus, it is possible to explain the fungal growth as an exponential equation. This model fit well only in the first stage of the growth because rapidly, not all the total mycelium can grows without limitation, and after 3 to 5 generation-time, a part of the mycelial biomass can not participate to the growth rate.

Growth limitation:

The common cause of the growth limitation is the decrease of the substrate concentration. Monod (1942) proposed a relation between growth rate and substrate concentration:

$$\frac{dX}{dt} = \mu_{max} \cdot \frac{S}{(kS + S)} \cdot X$$

where the µmax is µ in the optimal condition, kS the saturation constant of the substrate en S the substrate concentration.

Cubic Model of growth:

Pirt (1966) proposed a cubic root model to explain the fungal growth in pellet form:

$$X^{-3} = k.\ t + X_0^{-3}$$

But this model fit up only in LSF and when the mycelium grow in pellet form and it do not fit well for SSF.

Logistic Model:

The mycelium is not homogeneous, and can depend of the distance to the apex, with vacuoles in oldest parts. The concept is based on the fact that the medium composition can produce a

defined maximum of biomass, when the biomass increase, the rate slow down in reason of appearing limiting factors. the general equation of logistic is as follows:

$$\frac{dX}{dt} = \mu . X \left(1 - \frac{X}{X_{max}} \right)$$

This type of model generally is well correlated with fungal growth (Edwards & Wilke, 1968), particularly with batch cultures.

Maintenance concept:

When the growth stops and the biomass remains constant, the biomass needs to consume energy and substrate to maintain its viability and to realise its basic metabolic activities like respiration, secondary metabolisms., turnover of proteins and active transport (Pirt, 1965).

The general equation is:

$$\frac{dS}{dt} = \frac{1}{Ys} . \frac{dX}{dt} + m. X$$

2. Stoechiometric equations of respiration and Biomass biosynthesis

General equations:

In the following, we suppose the growth of mycelium in the exponential form, and for constants coefficients, we used the data established for Aspergillus niger cultivated on starch substrate (Raimbault, 1981). The global equation for the biomass is the result of starch hydrolysis, respiration and biosynthesis:

Hydrolysis..... **1/n (C6H10O5)n + H2O ----> C6H12O6**

Respiration.. (a) **C6H12O6 + 6 O2 -----> 6 CO2 + 6 H2O** (- 673 Kcal/mole)

Biomass ..(b) **C6H12O6 + 0.84 NH4OH + 30.4 H2O---> 6(CH1.62O0.62N0.14; 5.6 H2O)**

Balance ..... **C6H12O6+ 2.1 O2+ 0.54 NH4OH+17.6 H2O ------>**
3.9 (CH1.62O0.63N0.14; 5.6 H2O) + 2.1 CO2

In the present case of A. niger on starch, the proportion of glucose consumed for respiration (a) is 35% and 65% for biosynthesis.

The mycelium composition in CHON was determined on the basis of the composition of mycelium cultivated on liquid medium on starch substrate. The coefficients a and b were calculated on the basis of total glucose consumed and the oxygen uptake following the equation of respiration. where S is the substrate , Ys the cellular yield, and m the maintenance coefficient.

Starting from the global equation, it is possible to calculate the metabolic heat production considering the exothermic reaction of respiration:

$$\frac{dO_2}{dt} = K \cdot \frac{dQ}{dt} \quad ; \quad \frac{dQ}{dt} = \frac{\Delta O_2\% \cdot F}{V O_2 \text{ mol}} \cdot 673 \text{ Kcal} \quad ; \quad \frac{dQ}{dt} = k \frac{dT}{dt}$$

Kinetics of Biomass:

Considering the direct relation between CO2 and O2, it is possible to get on line the evolution of the biomass, capturing data of Oxygen Uptake Rate or CO2 evolution:

$$\frac{dQ_2}{dt} = \frac{1}{Y_{o2}} \cdot \frac{dX}{dt} + m_{o2} \cdot X \quad ; \quad \frac{dCO_2}{dt} = \frac{1}{Y_{co2}} \cdot \frac{dX}{dt} + m_{co2} \cdot X$$

From this equation we can write considering $dX/dt = \mu.X$:

$$\frac{dO2}{dt} = \left(\frac{\mu}{Yo2} + mo2 \right) \cdot X$$

Considering that Δ O2% = OUR and F = Air Flow

$$\frac{d\,O2}{dt} = O2\% \cdot F = Yo2 \cdot X$$

The logarithmic form of this equation is:

$$\mathbf{Ln\ (O2\%) = Ln\ X + constant\ ,}$$

or

$$\mathbf{Ln\ (O2\%) = \mu \cdot t + constant}$$

Similarly we can write the same equation for the CO2 evolution (CO2%)

$$\mathbf{Ln\ (CO2\%) = \mu \cdot t + constant}$$

From the last two equation, it is thus possible to calculate the specific growth rate of the mycelium using on line data of gas composition evolving from the incubator, without

destroying the sample, observing the kinetic evolution of the same sample all the time of the fermentation. That represents a great advantage of the SSF.

3. Equipment & Methodology

In order to measure kinetics evolution of fungal biomass cultivated on SSF, we have developed a simple column incubator device which allows to control air flow and analysis on-line of the gas composition at the exit of the reactor (Raimbault, 1980; Alazard & Raimbault, 1981). The figure 1 shows the laboratory device designed to realise lab experimentation with 24 incubator with temperature and air flow control (Trejo-Hernandez, 1986 ; Oriol, 1987 ; Dufour, 1990 ; Saucedo-Castañeda, 1991 ; Soccol, 1992).

Glass column reactors (5) of 2 or 4 cm diameter and 20 cm length are filled with the inoculated and moistened solid substrate (100-150 g of WM). Incubators are put on a humidificator (3) and installed in controlled water bath (2 & 4). The air flow, pre-saturated in water, is controlled by microvalves (6). So, the air flow can be controlled for each column.

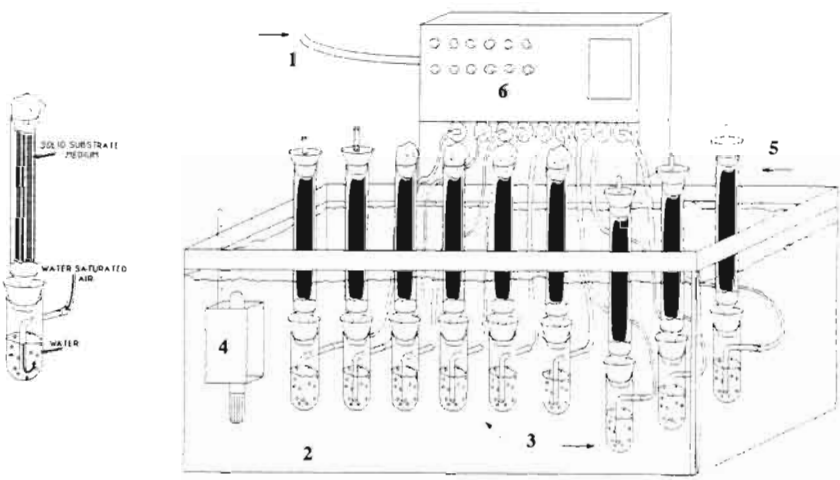

Figure 1 : Incubation device for aerobic SSF. (1): air input; (2) Thermoregulated water bath; (3) Humidficador; (4): Control heater; (5): Column incubator; 6: Microwalves.

To analyse gas composition, different techniques were developed including trapping CO2 in alkaline solution, paramagnetic analysis for oxygen, Infra Red analysis for CO2. We describe here (figure 2) the Gas Chromatography technique that we used with success during various years at the laboratory scale. Other alternative are also presented by other speakers during the course.

The equipment is composed by a gas chromatograph (CPG) equipped with thermal conductivity detector and Alltech column CTR1 (double concentric column : extern molecular screen 5 A° and internal Porapak as stationary phase .

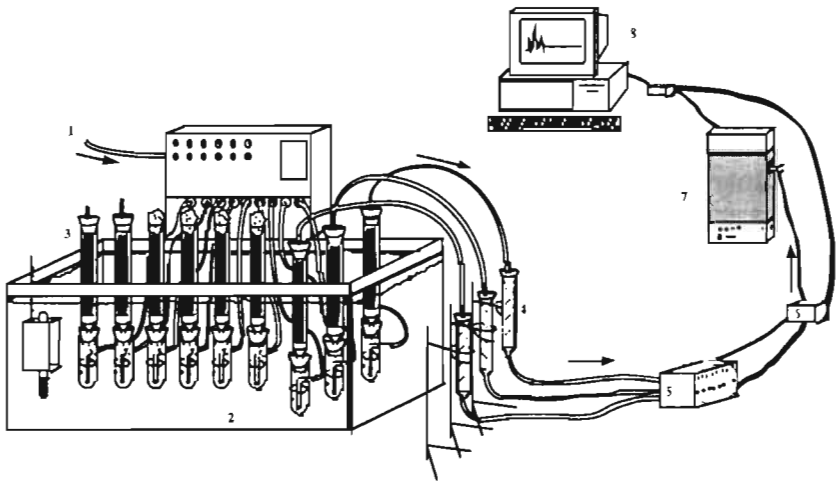

Figure 2: On line analysis equipment for gas measurement using CPG. 1 Air input; 2: Thermostatic; 3: Column incubator; 4 Silicagel ; 5: Sampler; 6 Interface for automatic injection.

The conditions of chromatograph are as follows:

Detector: Thermal conductivity
Detector temperature: 60°C
Column temperature: 60°C
Gas phase: Helium
Gas phase flow: 40 ml.min-1
Catharometer current: 120 mA
Helium pressure: 1 bar
Loop injection Volume: 200 µl
Gas for calibration: Air: , CO2 (0.0) / O2 (21.0) / N (79.0)
Mixture 1: CO2 (5.0) / O2 (5.0) / N (90.0)
Mixture 2: CO2 (10.0) / O2 (15.) / N (75.0)

I. Experimental Aspects

The experimental part of the course was performed at the Laboratory of Biotechnological Processes of the Chemical Technology Department of the UFPR. So, the training part concerned practice cultivation of two filamentous fungi (*Aspergillus oryzae* and *Rhizopus formosa*) on Cassava Bagasse, a Brazilian by-product of the industrial extraction of Cassava Starch and flour production for human consumption.

Inoculum preparation:

The two strains of fungi were cultivated in erlen flask on the surface of PDA medium during a week at 35°C. Then, conidia were cropped with a platinum loop in a laminar flux cabinet, in sterile tubes containing 10 ml of water, 1% of Tween and glass balls. The suspension is homogenised 15 min and successively diluted as necessary ($10^{-1}/10^{-7}$) for direct microscopic count in a Neubauer cell. The number of conidia in the cell, allows calculate the number of conidia in the original suspension, using the formula:

N (conidia/ml) = n (number of conidia in counting cell). (1/Dilution factor). (25. 10^4)

The adequate dilution is then prepared in order to get a good concentration of conidia allowing final inoculation of $1x10^7$ conodia/g of DM substrate. This suspension is kept at 4°C under constant agitation until utilisation (Soccol, 1991).

Substrate preparation:

In this experience, we used Cassava Bagasse, an industrial by-product obtained from the Lorenz Company (Quatro Pontes, SC, Brazil). The raw material was grounded in order to get a granulometry of 0,8-2,0 mm diameter particles. The material was dried at 55-60 °C in oven with circulating air during 12 hours.
This material was analysed in agreement to the recommended methods described in Analytical Normas of the Adolfo Lutz Institute (Sao Paulo, 1985). The starch was determined by the NS-00396/85 method (National Starch Chemical Corporation, 1985), using the Thermamyl commercial α-amylase (Thermamyl). Protein content was determined by the Stutzer method (Vervack, 1973). The following table shows the composition of the by-product:

Table 1. Physico-chemical composition of the Cassava Bagasse (Stertz, 1997)

Compound	Cassava Bagasse composition (g/100g DM)
Moisture Content	10.70
Proteins	1,60
Carbohydrates	63.40
Lipids	0,53
Fibres	22.20
Ashes	1.50

Preparation of the medium for Solid Substrate Fermentation:

The saline solution used to humidify flour contained:

- $(NH_4)_2 SO_4$..... 4,34 g
- KH_2PO_4 ..... 1,7 g
- Urea ..... 0, 83 g
- Water ..... 233 ml

The pH of the solution was adjusted to 5,8 with Na_2CO_3 (3N)

The volume of saline solution necessary to moisten the flour of cassava bagasse is calculated by the formula:

$$\text{Mass of Water (g)} = \frac{M\% \times \text{Mass of substrate (g)}}{(100 - M\%)}$$

For example, for 100 g of Cassava Bagasse substrate, and for a Moisture content of 65%, it would be used 185 ml of saline solution.

Analytical procedure:

In order to characterise kinetics biotransformation of the material by the fungal strains, samples are picked up at regular duration of the fermentation and physical-chemical determinations are performed. For that purpose, samples are treated following the flow sheet showed on the figure 3.

All analysis (pH, Moisture content, Ashes, Lipid, Fibres, Total acidity and sugars) were performed as recommended by Normas Analiticas do Intituto of Adolfo Lutz (Sao Paulo, 1985).

pH:

pH was determined under agitation electronically with pH meter, after homogenisation of the suspension of 1g in 10 ml of distillated water.

Moisture content:

Moisture content was determined from 5 gm of moistened sample by drying in a controlled oven during 24 h at 105°C. The Moisture Content is calculated by the formula :

$$M\ \% = \frac{(P2-P1)\,.\,100}{P2.}$$

with: M% = moisture content
P2 Weight of the pre-treated sample (5 g) ; P1 Weight after desiccation of the sample.

Carbohydrates and primary metabolites:

Sugar, organic acids, ethanol, were determinated by H.P.L.C.
The starch was determined by the NS-00396/85 method (National Starch Chemical Corporation, 1985), using the NOVO Nordisk « Thermamyl 120L » (liquid commercial α-amylase (Thermamyl). 4 grams of sample are added in 100 ml of water: autoclaved at room pressure during 1 h , and adjusted to pH 6,0-6,5 with a solution of NaOH (1N). When temperature is 95°C, 60 - 70 ppm of CacCl2 and 1 ml of Thermamyl Novo were added , and kept 15 min at this temperature then filtered on Whatman paper, washed and centrifuged ; finally the residual material is dried at 105-110 °C during 1 h 30 and residual weight is determined. Starch % of residual dry matter was calculated by the formula:

$$\text{Starch}\ \% = \frac{(PT - PR)\,.\,100}{PT}$$

were PT = Total mass and PR = Residual mass

For true Protein content the Stutzer method was used (Vervack, 1973).

Solid State fermentation cultivation in column:

The device showed in figures 1 and 2 was used for incubation and respirometric analysis, under following conditions:

Temperature of 35 °C
Flow rate of air flux : 100 ml / min
The composition of air can be observed directly on the computer screen .
After 24 hours and 48 hours samples columns are pick up for analysis.

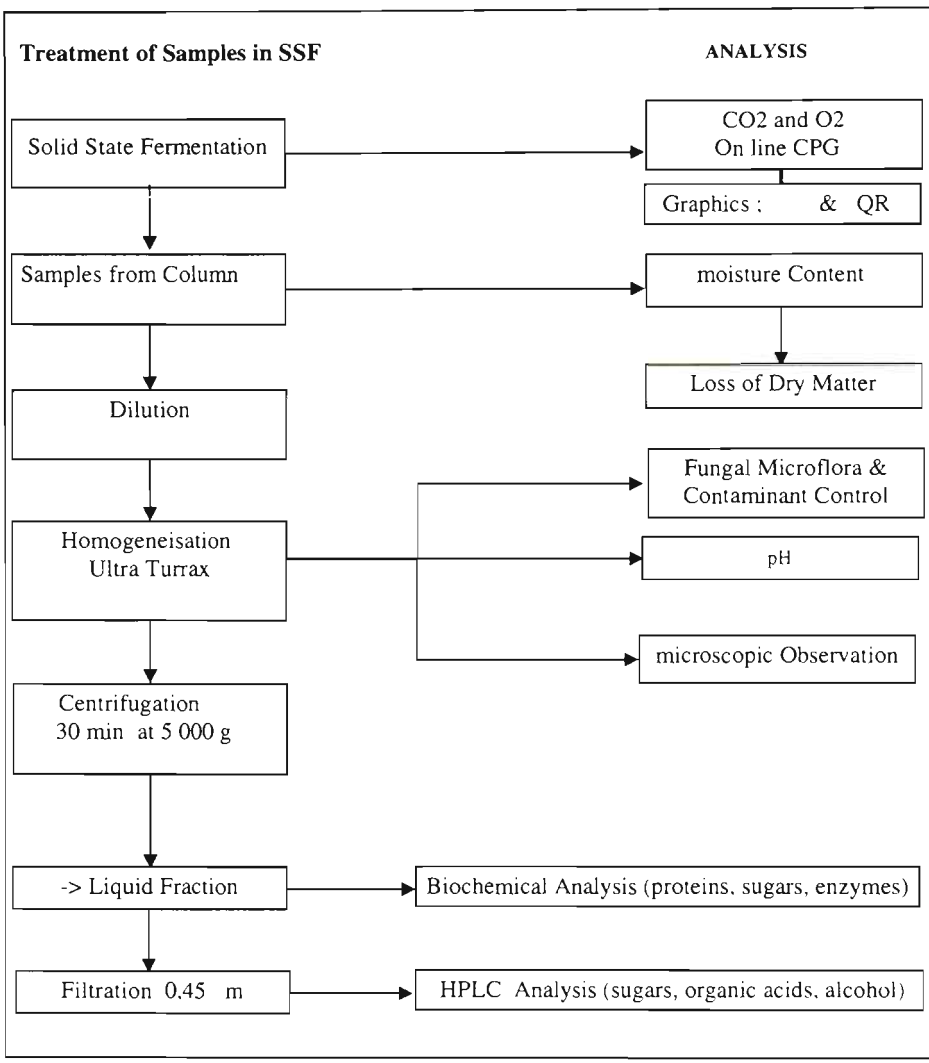

Figure 3. Flow sheet of samples treatment s for analysis in SSF

Typically the Table I represents results obtained from SSF cultivation of *Rhizopus oryzae* on Cassava. Figure 3 represent graphs obtained from *on line* data captured by the computer. It can be calculate directly the oxygen consumption and CO2 evolution, and also the specific growth rate. In addition, biochemical analysis allowed to correlate all fermentation parameters, and calculate all the balance of the biotransformation process. All that information is of importance for pilot and scale up further applications.

Table I.- Data of Solid Substrate Fermentation of Rhizopus oryrae MUCL 28168, cultivated on Cassava flour substrate. (Raimbault et al, 1995).

	INITIAL	FINAL
Total Mass of Material	142,20	128,40
Dry Matter Content	60,45	45,85
Air Flux of aeration	85,00	<----------
Total Dry Matter	85,96	58,87
Duration of the germinating	7 h	<----------
Time for Maximum Rate (	16 - 30	<----------
Maximum O2 Uptake Rate(ml/H/g	4,65	<----------
VMaximum CO2 Evolved Rate (ml/H/g	4,91	<----------
Mean of Respiratory	1,05	<----------
Total O2 uptake (g / g	0,19	<----------
Total CO2 evolved (g / g	0,28	<----------
Duration of the exponential	9 h	<----------
Specific Growth Rate (μ)	0,263	<----------
Protein (% DM	9,96	20,08
Total Sugar (% DM	46,54	15,05
Loss in Dry Matter	31,51	<----------
Yield Protein / Sugar (Y	0,105	<----------
Yield	0,77	<----------

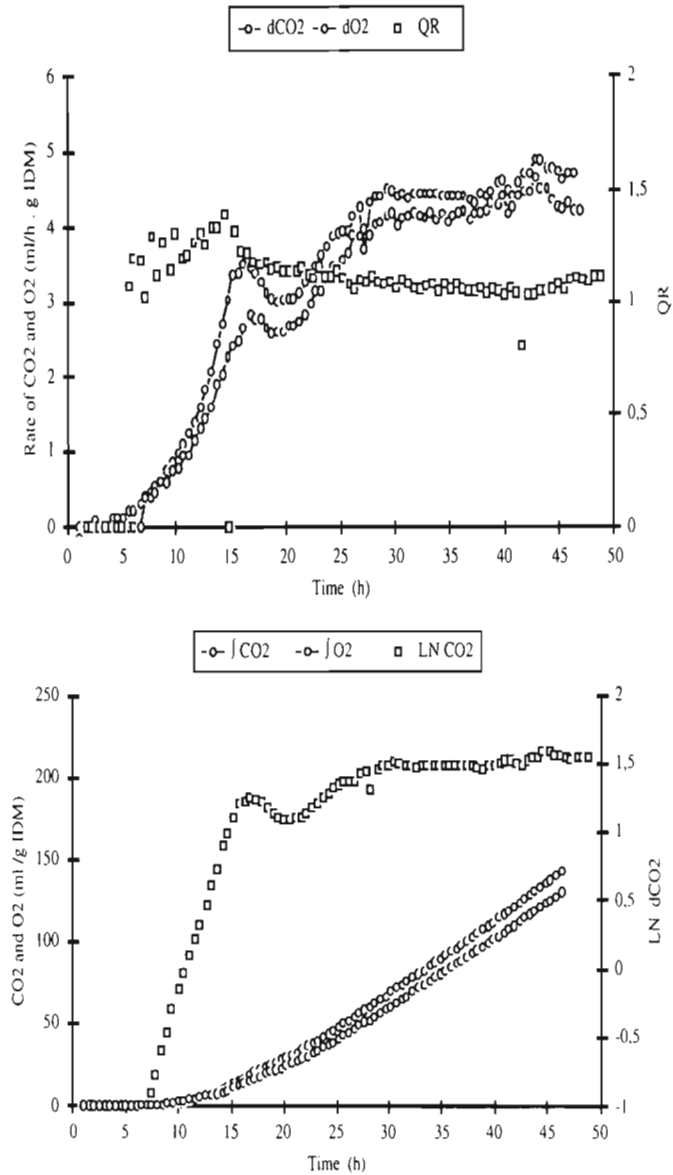

Figure 3. Kinetic of respiration characteristic during growth of Rhizopus oryzae on crude Cassava flour.

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