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Biomass estimation in solid state fermentation

II. On-line measurements

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Summary. Two methods for on-line biomass measurement were tested with success: (a) infrared (IR) estimation of cell components (glucosamine and ergosterol) and medium residues (sucrose and nitrogen) directly on solid medium; (b) the CO_2 evolution rate during cultivation. These methods were very satisfactory for following biomass changes during a defined process. The IR measurements correlated well with manual methods. The correlation between CO_2 and glucosamine measurements was very satisfactory with good precision. However, they did not permit comparison between processes.

Introduction

Previously, Desgranges et al. (1991) tested manual biomass measurements in solid state fermentation (SSF) to determine which cell constituents gave satisfactory measures of biomass. The concentrations of two cell constituents, glucosamine and ergosterol, gave information absolutely necessary for control of the fermentation process. However, these manual methods are time-consuming and cannot give instantaneous results, an important consideration for industrial applications. We therefore investigated automated methods using infrared (IR) measurements of glucosamine, ergosterol and also substrates. This method was previously utilized with success in our laboratory (Durand and Chereau 1988) for protein determination in sugar beet pulp.

However, the value of IR analysis can be limited, because in some cases glucosamine estimation is not possible due to interference from the culture medium. In order to estimate biomass, other methods must be used, such as on-line measurement of the CO_2 production rate, as mentioned by Narahara et al. (1982).

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Materials and methods

Microorganism and inoculum preparation

The microorganism was *Beauveria bassiana* strain no. 147, from the INRA collection (La Minière, France). The inoculum preparation used was the same as described previously (Desgranges et al. 1991).

SSF conditions

We used clay granules wetted with CG1, CG2, CG3 or CG4 media as the cultivation support (Desgranges et al. 1991). These solutions contained sucrose and non-sugar residue (NSR) in different concentrations.

The cultures were carried out in laboratory-scale reactors of 50-l capacity (Durand et al. 1988).

Analytical procedures

Manual determination. For manual determination of glucosamine, ergosterol, sucrose and conidia, we used the methods described previously (Desgranges et al. 1991).

For nitrogen consumption, a 0.75 g sample was washed with 75 ml of 10% trichloroacetic acid, under agitation for 20 min. The solution was filtered, left for 30 min at room temperature and centrifuged for 10 min at 5000 rpm. This method allowed reproducible recovery of only 66% of total medium nitrogen (100% of mineral nitrogen and 34% of organic nitrogen). Nitrogen determination was carried out by a classic Kjeldhal method.

IR analysis. The apparatus used was an InfraAlyzer 400 (Technicon, Domont, France). Its principle is founded on the measurement of reflected light by the matrix surface at specific wavelengths. It has 19 filters distributed on a wavelength range between 1400 and 2400 nm. The amount of the measured component is calculated by the mathematical relationship:

$$C = F_0 + F_1 \log \frac{1}{R_1} + \ldots + F_i \log \frac{1}{R_i} + \ldots + F_n \log \frac{1}{R_n}$$

where C is the component amount; F_0 to F_n, the calculation constants; R_1 to R_n, the reflection values; 1 to n, the filter number selected by the computer; and n a number between 1 and 19. This relationship is established by calibration of the apparatus.

For the calibration, 42 samples were analysed for each component. They were taken at different times (0–48 h) of a culture on CG1 medium. The manual measurements of glucosamine, ergosterol, sucrose and nitrogen were determined twice for each sample, and each sample was measured twice by the IR analyser. We placed the sample, without any treatment, in a cupel (model 1.127.IRXX.01 Technicon). All data (manual values of each component and log values) were stored on a computer.

Multiple linear regressions were applied in order to choose the wavelength combinations specific for each component and to calculate their constants, F_0 to F_n.

Measurements of CO_2 production. The CO_2 produced was measured on-line by an IR analyser (model URAS-3G, Hartman and Braun, Metz, France). For each culture, four replicate CO_2 measurements were made. The data were stored on a microcomputer every 2 h. Then, the total CO_2 produced was calculated and the results expressed in µmoles per gram of granule dry matter (DM), taking into account the air-flow rate.

Results

IR measurements

The filter selection and regression coefficient for each constituent are given in Table 1. The multiple regression coefficients were very good for glucosamine, sucrose and ergosterol. For nitrogen it was low but the manual method was not very satisfactory in any case. The maximum deviations between the results obtained by regression and the theoretical values were for glucosamine, 65 µg/g DM; ergosterol, 7.5 µg/g DM; sucrose, 0.5 mg/g DM; and nitrogen, 0.7 mg/g DM.

All these calculations were made with the standard samples. To check the reliability of this IR measurement, we then considered test samples taken from different cultivations. When we tested the standardization, we noted that the test values were inscribed in the calibration point set. For glucosamine, the IR measurements were slightly higher than the manual values in the middle of the range. For high sucrose concentrations the IR values were lower.

Table 1. Filter selections for infrared measurement

Constituent	Number of filters selected	Wavelength selected (nm)	Regression coefficient
Glucosamine	2	2100 1445	0.960
Ergosterol	4	1982 1778 1940 1734	0.925
Sucrose	3	2336 2139 1722	0.960
Nitrogen	4	2270 2208 2100 1445	0.840

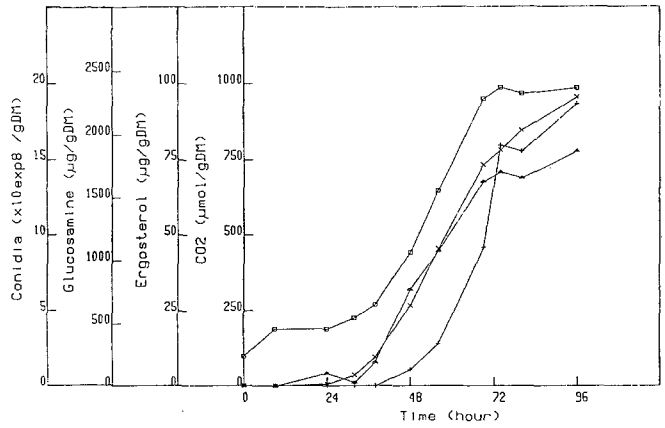

Fig. 1. Growth kinetics of *Beauveria bassiana* on CG2 medium: ⊡, glucosamine; △, ergosterol; ×, CO_2; +, conidia. DM, dry matter

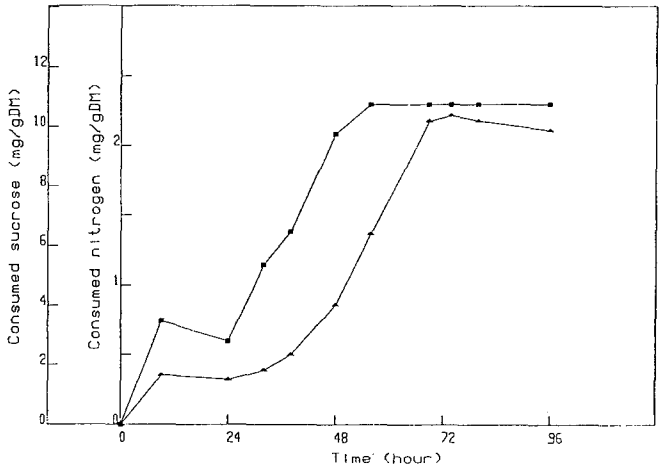

Fig. 2. Growth kinetics of *B. bassiana* on CG2 medium: ■, sucrose consumed; ▲, nitrogen consumed

Despite these small differences between the manual methods and IR measurements, we obtained good representations of fungal growth. For example, Figs. 1 and 2 show the growth kinetics of *B. bassiana* on CG1 medium, measured by glucosamine, ergosterol or sucrose and nitrogen consumption IR analysis. There were three phases. First, a latent phase lasted about 24 h for glucosamine or ergosterol production and sucrose or nitrogen consumption. Second, an exponential growth phase lasted for 24 h. Finally, the stationary phase started at 72 h of cultivation time for all parameters except for ergosterol production.

CO_2 measurement

Kinetics of CO_2 production. Figure 1 also shows the mean of four growth curves of *B. bassiana* cultivated on CG1 medium, obtained by CO_2 measurement. The comparison of the curves indicates that CO_2 production appeared before the other constituents (glucosamine or ergosterol). At 24 h of cultivation, the CO_2 rate was sig-

Table 2. Correlation between glucosamine and CO_2 for CG1, CG2, CG3 and CG4 media

Medium	Regression equation	Correlation coefficient
CG1	$Y=0.25x-73.69$	0.992
CG2	$Y=0.50x-112.81$	0.981
CG3	$Y=0.54x-149.64$	0.968
CG4	$Y=0.34x-52.51$	0.994

x, glucosamine; Y, CO_2

Table 3. Correlation between glucosamine and CO_2 for six cultures on CG1 medium

Culture number	Regression equation	Correlation coefficient
1	$Y=2.46x-2432.97$	0.985
2	$Y=1.72x-1236.54$	0.950
3	$Y=1.53x-1074.00$	0.891
4	$Y=0.94x-896.55$	0.773
5	$Y=1.39x-1181.14$	0.985
6	$Y=1.68x-1568.20$	0.980

x, glucosamine; Y, CO_2

nificantly different at the zero point from glucosamine and ergosterol production or sucrose and nitrogen consumption. The CO_2 measurement is more sensitive than the other measurements. Moreover, fungal respiration continued after 72 h of cultivation when the other parameters did not change, except for the sporulation rate.

CO_2 production on different cultivation media. The calculation of the regression for each medium (CG1, CG2, CG3 and CG4) between glucosamine and CO_2 showed a good correlation. Indeed, in Table 2 all the correlation coefficients were higher than 0.96 and the probability α lower than 1%. Nevertheless, comparison of the four regression equations indicated that the amount of CO_2 produced per unit of glucosamine was different for the four media. The origins of the straight lines were the same but the slopes were different.

Variation of the respiration with culture conditions. Six replicates of the *B. bassiana* cultivation on solid CG1 medium were carried out under similar physical culture conditions (temperature and aeration). The changes in fungal biomass was estimated by IR for glucosamine and CO_2 production. The results of the linear regression are represented in Table 3, which shows that the respiration of culture 2 was identical with replicates 3, 5 and 6. However, culture 1 had a higher respiration rate (indicated by the slopes of the regression equation in Table 3) and culture 4 a lower rate.

Discussion

The IR measurements were as accurate as the manual biochemical methods. We estimated the precision for glucosamine at 200 µg/g DM, close to the colorimetric method (Ride and Drysdale 1971). For ergosterol it was about 15 µg/g DM, which agreed with the recovery procedure used and HPLC analysis (Desgranges et al. 1991; Cahagnier 1984). When the manual values were very high (about 100 µg/g DM), the IR values were inaccurate. Such values were obtained in the last phase of fungal development when the mycelium sporulated.

For sucrose, the precision was 2 mg/g DM. This value was also the precision of the extraction and the enzymatic method. If sucrose estimation by IR analysis appeared to be perfect, some results could be aberrant, especially for samples taken at the end of cultivation (120 h) when the fungal biomass was high and sucrose concentration low.

The nitrogen IR determination was the least satisfactory. The precision of this method was of 0.7 mg/g DM. It was very high in view of the low nitrogen consumption (about 2.1 mg/g DM after 120 h of cultivation). The lack of accuracy derived from the manual method for nitrogen estimation. Effectively, the extraction procedure was not efficient enough to desorb all the nitrogen fixed on the clay and the Kjeldahl method was not sensitive enough to estimate small nitrogen quantities.

IR measurements are well adapted for fast fungal biomass estimation in SSF. Indeed, with our method, we achieved IR analysis directly on the clay granules without any pretreatment such as drying or grinding. However, it is necessary to take the samples and introduce them into the analyser by handling. In future, we envisage a completely automatic system allowing online measurements. Bellon and Boisde (unpublished work, 1989) described the use of optical fibres coupled with a near-IR spectrometer in the food industry. It is certainly possible to adapt this system to our reactors.

The use of IR measurements is only possible if manual biochemical methods for biomass estimation are possible. For example, glucosamine estimation is not adapted to media containing insoluble nitrogen. In that case, we must consider another biomass estimation method, the measurement of CO_2 production.

CO_2 production correlated well with the other parameters. Moreover, it can indicate even low physiological activity, because the method is very sensitive. Narahara et al. (1982) described the same results in the cultivation of *Aspergillus niger* on rice. Two closely similar media (CG2 and CG3) had the same slope (the difference was not significant). However, like the glucosamine and ergosterol measurements, CO_2 estimation cannot allow comparison of the growth of fungi cultivated on solid media differing in composition.

We have not calculated the linear regression between CO_2 and the other parameters (ergosterol production and sucrose and nitrogen consumption) because we showed previously (Desgranges et al. 1991) that they are correlated with glucosamine amount. The

CO_2 was consequently correlated to these other biomass indicators.

The results in Table 3 indicated that the CO_2 evolution rate varied also with the fermentation conditions. For example, the difference observed in the culture 1 could well be explained by a less aged inoculum than the others (and so a faster germination phase). In the case of culture 4, the difference was due to defective air humidity regulation during the first 12 h of cultivation. In the other cases, the deviation between each replicate was equal to the precision of the glucosamine IR method. Thus, cultivations carried out under exactly the same conditions had the same CO_2 production rates.

Different authors (Sugama et Okasaki 1979; Narahara et al. 1982) have mentioned that CO_2 production was correlated with biomass and we agree with them. However, it is very important to note that this CO_2 measurement can be only applied for a defined process.

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