Method To Estimate Growth of *Trichoderma reesei* and *Aspergillus wentii* in Mixed Culture on Cellulosic Substrates

T. PANDA, V. S. BISARIA,* AND T. K. GHOSE

Biochemical Engineering Research Centre, Indian Institute of Technology, Delhi, Hauz Khas, New Delhi-110 016, India

Received 4 April 1988/Accepted 6 December 1988

A simple differential method based on measurement of an intracellular pigment of *Aspergillus wentii* was developed for estimation of the individual growths of two fungi, *Trichoderma reesei* and *A. wentii*, in mixed-culture fermentation of an insoluble cellulosic substrate.

*Trichoderma reesei* is one of the most potent producers of endoglucanase (EC 3.2.1.4) and cellobiohydrolase (EC 3.2.1.91) components of the cellulase enzyme system (2, 10). For practical saccharification of pretreated lignocellulosic residues, β-glucosidase (EC 3.2.1.21) (the third component of cellulase) and xylanase are also required at an optimum level (7). The deficiency of β-glucosidase in the culture filtrate of *T. reesei* can be overcome by supplementing it with β-glucosidase of *Aspergillus* species, which are reported to produce high levels of this enzyme (1).

To get enhanced levels of cellulase and xylanase, a mixed-culture process involving *T. reesei* D1-6 and *A. wentii* Pt 2804 was studied. The mixed-culture process produced higher activities of cellulase and xylanase when *A. wentii* was inoculated 15 h after *T. reesei*. The effect of the interaction of the two cultures and of the metabolites produced by them on the production of cellulase and xylanase activities has been previously reported (6, 8, 13-15). The levels of cellulase and xylanase activities produced in the mixed culture were found to be a function of the individual biomass concentrations of the two cultures. To optimize the enzyme levels, it is therefore necessary to regulate the biomass concentrations of the two fungi in the culture broth. This paper reports a simple differential method for estimating the individual growths of the two fungi in a mixed culture, which is based on measurement of an intracellular pigment produced by *A. wentii*. The method will also be useful for exploring the effects of various parameters on the growth behaviors of the two fungi in a mixed population.

*T. reesei* D1-6 and *A. wentii* Pt 2804 were used in the studies (8). The optimized medium (14) for production of cellulase and xylanase by the mixed culture of *T. reesei* and *A. wentii* was used in a 5-liter batch stirred-tank bioreactor fitted with monitoring and control devices for pH, temperature, dissolved oxygen, and agitation. Fermentations were performed at the optimum conditions reported earlier (8), with 40 g of microcrystalline cellulose powder per liter or 20 g of sodium carboxymethyl cellulose (type 7L2; DS = 0.7; Hercules Inc.). Other conditions were a pH (after a natural rise and fall) controlled at 4.8, aeration at 1 vvm, agitation at 250 rpm, and a working volume, 3 liters.

DNA was estimated by the Burton method (5), following extraction as described by Ghose and Sahai (9), and cellulose was measured by the anthrone reagent method (18).

The intracellular pigment from the *A. wentii* mycelium was extracted as follows. The mycelium of *A. wentii* and the residual cellulose obtained after centrifugation in 10 ml of culture broth were washed with distilled water, treated with 20 ml of a 95% (vol/vol) ethanol-water mixture (1:1) at pH 5.0 and 80 to 85°C for 30 min, followed by centrifugation at 3,000 rpm for 20 min. This extraction procedure was repeated three times. The supernatants from each centrifugation step were pooled and kept at 4°C for 5 h. The chilled mixture was centrifuged at 3,000 rpm for 15 min at 4°C, and the resulting supernatant was concentrated under vacuum at 50°C. The residue thus obtained was dissolved in distilled water, adjusted to pH 5.0, and recrystallized. Lipids were removed as described by Bligh and Dyer (3), and the remaining residue was dissolved in a known volume of an ethanol-water mixture at pH 5.0 (adjusted with dilute HCl) and kept at 4°C. The residue was found to be free of carbohydrates (analyzed by the dinitrosalicylic acid method [11] after acid hydrolysis), proteins (4), and lipids (16).

Analysis of DNA content throughout the fermentation cycle of *A. wentii* in a single culture showed that its mycelium contained 2.0% DNA on a dry-weight basis. This relationship was found to hold for up to 170 h of fermentation with sodium carboxymethyl cellulose or microcrystalline cellulose powder as the substrate. It was also observed that *A. wentii* synthesized an intracellular pigment with an absorption maximum at 470 nm. The time course of pigment formation (Fig. 1) followed a pattern similar to that of cell weight and DNA content in the fermentation range of 10 to 170 h. No correlation was observed after 170 h, possibly because of lysis of the mycelium, resulting in dilution of the pigment.

The pigment produced by *A. wentii* was extracted from 25 ml of culture broth and suspended in 25 ml of an ethanol-water mixture (pH 5.0). The standard plot of absorbance at 470 nm as a straight-line relationship up to 0.48 mg of pigment per ml, with a slope of 1.0. With the other 25-ml portion of the culture broth, dry cell weight was determined. The relationships of pigment versus *A. reesei* and dry cell weight versus pigment gave the following relationship between cell weight concentration and *A. reesei*: 

\[
X = 10.1 \cdot A_{470}
\]

This relationship was used to determine the cell growth (*X*) of *A. wentii* in grams per liter. When the *A. reesei* was higher than 0.48, the sample was suitably diluted before the *A. reesei* was measured.

The cell growth of *T. reesei* in the mixed culture was determined as follows. On the basis of the values of pigment (*P*) and DNA (*D*) concentrations in *A. wentii* (Fig. 1), it was observed that the average ratio of *D* to *P* was 0.2 and that

* Corresponding author.

† Present address: Division of Biochemical Engineering, Department of Chemical Engineering, Indian Institute of Technology, Madras-600 036, India.
that of X to D was 50.5 between 10 and 170 h. In the mixed-culture mycelium, the total contents of DNA and the pigment were first determined. The cell weight of A. wentii in the mixed culture was evaluated on the basis of the relationship given above, and the DNA content was measured on the basis of the relationship of D to P. The cell weight of T. reesei in the mixed culture was then estimated by subtracting the DNA content of A. wentii from that of the mixed-culture mycelium and converting it to its mycelial dry weight. The amount of DNA in the T. reesei mycelium was 1.5% on a dry-weight basis up to 170 h of fermentation.

The validity of the pigment-DNA method was verified by mixing the mycelia of T. reesei and A. wentii grown in single cultures on cellulosic substrates in different proportions and estimating their total cell weights by two different approaches. (i) Total cell weight in the mixed culture was determined by measuring the dry weight of cells. Subtraction of the amount of cellulose from the total weight of the mycelium plus cellulose gave the total cell weight. (ii) Total cell weight in the mixed culture was determined by estimating the cell weight of A. wentii by the pigment method and the cell weight of T. reesei by the DNA method (described above) and adding the cell weights. It was found that estimation of total cell weight by the two approaches produced identical results (Fig. 2A). This was verified by the regression of the datum points (correlation coefficient, 0.9968). The applicability of the method was further confirmed by estimating the growth of the two fungi during actual mixed (dual)-culture fermentation (Fig. 2B). The cell weight of the mixed culture was estimated similarly by the two approaches.

Direct estimation of fungal growth in fermentations with insoluble cellulosic substrates is not possible. Indirect methods are, however, available for determining fungal growth in single pure cultures on these substrates (9, 12, 17). There have been no previous reports on growth estimation of individual fungi in a mixed culture on cellulosic substrates. The method reported here presents a simple differential approach to quantify the growth of T. reesei and A. wentii in a mixed-culture fermentation on cellulose. A. wentii produced a growth-associated intracellular pigment which could be extracted and monitored at 470 nm, enabling the measurement of fungal growth. The other fungus, T. reesei, did not produce any such metabolite absorbing at this wavelength and therefore did not interfere with the estimation of A. wentii growth. The characterization of the A. wentii pigment will be reported elsewhere.

In a mixed culture the growth of the participating organisms is not the sum total of their growths in single cultures, since the metabolites produced by one organism could affect the growth of the other. However, since growth and pigment formation were found to be concurrent events in A. wentii, any change in growth would be expected to result in a similar change in pigment formation. The validity of the approach

![FIG. 1. Production of mycelium, DNA, and pigment by A. wentii. Symbols: ○, dry mycelial weight; □, DNA; △, pigment.](http://aem.asm.org/)
used in this investigation was substantiated by estimating the growth of each individual fungus in a simulated mixed-culture mycelium and in a mixed-culture fermentation. Although the approach described here is specific for *T. reesei* and *A. wentii*, it should prove useful for devising suitable methods for estimating the growth of other individual fungi in mixed cultures.

**LITERATURE CITED**