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Estimation of Fermentation Biomass Concentration by Measuring Culture Fluorescence

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The fluorescence of a fermentation culture was studied for its application as an estimator of biomass concentration. The measurement was obtained by irradiating the culture with ultraviolet light (366 nm) through a glass window and detecting fluorescent light at the window surface at 460 nm. It was estimated that over one-half of the fluorescent material was intercellular reduced nicotinamide adenine dinucleotide, with the remainder being reduced nicotinamide adenine dinucleotide phosphate and other unidentified intercellular and extracellular fluorophores. The culture fluorescence was found to be a function of biomass concentration, together with environmental factors, which presumably act at the cellular metabolic level to modify intercellular reduced nicotinamide adenine dinucleotide pools (e.g., dissolved oxygen tension, energy substrate concentration, and inhibitors). When these environmental conditions were controlled, a linear relationship was obtained between the log of the biomass concentration and the log of the fluorescence. Under these conditions, this relationship has considerable potential as a method to provide real-time biomass concentration estimates for process control and optimization since the fluorescence data is obtained on line. When environmental conditions are variable, the fluorescence data may be a sensitive index of overall culture activity because of its dependence on intercellular reduced nicotinamide adenine dinucleotide reserves and metabolic rates. This index may provide information about the period of maximum specific productivity for a specific microbial product.

A widely recognized problem in the analysis and control of fermentation processes is the inability to determine the biomass concentration in the culture on a real-time basis (9). This fact is a consequence of the long time periods associated with most chemical and physical assays. Methods employing the optical properties of cell suspensions have rapid response times, but suffer from numerous interferences such as medium solids, dense medium coloration, mycelial culture morphology, and growth on the optical surfaces, which are characteristic of many practical fermentations.

Since intercellular reduced nicotinamide adenine dinucleotide and reduced nicotinamide adenine dinucleotide phosphate [NAD(P)H] fluoresce at 460 nm when cells are irradiated by 366-nm light, this property has been used extensively by biochemists in studying the transitions in reduced pyridine nucleotide concentrations in response to changes in cellular environment. Duysens and Amesz (4) first used this phenomenon to confirm that starved yeast cells have less reduced nicotinamide adenine dinucleotide (NADH) than actively growing cells, a conclusion that was first suggested by less sensitive absorption spectroscopy methods (2). Chance et al. (3) used fluorescence to study damped NADH oscillations in response to changes in cell environment. Harrison and Chance (5) applied the technique to continuous cultures of cells. They demonstrated that intercellular NADH increases in yeast cells during the transition from aerobic to anaerobic growth. The absence of oxygen prevents oxidative phosphorylation from oxidizing high-energy NADH to low-energy nicotinamide adenine dinucleotide (NAD), thereby causing a net increase in NADH and fluorescence. These results were confirmed chemically.

The purpose of the research described in this paper was to determine the relationship between the concentration of cells and the amount of culture fluorescence (D. W. Zabriskie, Ph.D. thesis, University of Pennsylvania, Philadelphia, 1976).

MATERIALS AND METHODS

Culture fluorometer. The culture fluorometer used was an adaptation of the electronics (7) and

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fermentor assembly described earlier (5) and is shown schematically in Fig. 1. The fluorometer was mounted on a vessel observation port located beneath the culture surface. The culture was irradiated with light near 366 nm by using a fluorescent ultraviolet lamp and an optical filter (CS-7-60, Corning Optical Products, Inc., Corning, N.Y.). This light excited the culture, causing it to fluoresce near 460 nm. The fluorescent light was measured using a photomultiplier that was filtered to screen out the exciting light wavelengths and other fluorescent peaks (CS-5-57 and CS-3-73, Corning Optical Products). Tap water was circulated through the mounting assembly to remove the heat generated by the lamp.

Fermentation conditions. Three aerobic batch fermentations using growth conditions characteristic of industrial processes were studied. Bakers' yeast (*Saccharomyces cerevisiae* ATCC 7754) was grown at 30°C (pH 5.0) on a medium composed of 2% corn steep liquid, 8.0 g of $(NH_4)_2 HPO_4$ per liter, and glucose. The sugar was added on demand to prevent glucose repression and excessive conversion to ethanol. Each pulse was sufficient to bring the broth to a concentration of 1.0 g/liter. A pulse was added when glucose became limiting, as shown by a sudden drop in the carbon dioxide evolution rate. After the maximum growth rate had been reached, a period of diauxic growth was initiated by withholding glucose and allowing the assimilation of accumulated ethanol and acetate byproducts. Glucose additions were resumed after the exhaustion of the metabolizable intermediates as determined by a sudden decline in the carbon dioxide evolution rate. The biomass concentration was determined by dry-weight analysis (Zabriskie, Ph.D. thesis).

The second fermentation used a species of *Streptomyces,* which is used in the industrial production of enzymes. The culture was grown at 30°C (pH 6.0) on a medium similar to the one used to grow the yeast. Glucose, however, was added batchwise (26.4 g/liter) since it has no repressive effects on this microorganism under these conditions. The biomass concentration was determined by dry-weight analysis.

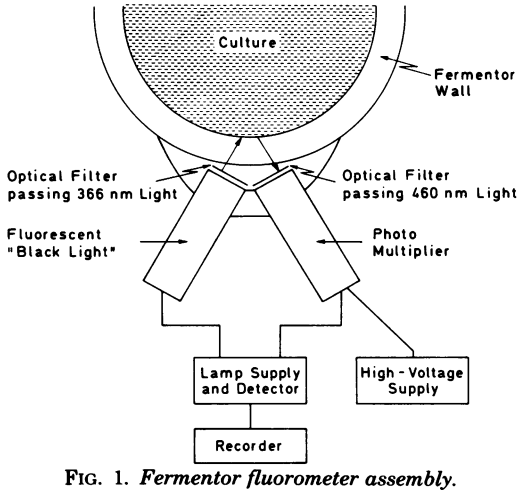

FIG. 1. *Fermentor fluorometer assembly.*

The third fermentation used a thermophilic species of *Thermoactinomyces,* which is currently being investigated for its potential to produce single cell protein from cellulosic materials (1). It was grown at 55°C (pH 7.2) in a 5% suspension of cellulose (Avicel, PH 102, FMC, Inc., Philadelphia, Pa.). The soluble components of the defined medium included (per liter): NaCl, 1.5 g; ethylenediaminetetraacetic acid, 50 mg; $MgSO_4 \cdot 7H_2O$, 0.2 g; $ZnSO_4 \cdot 7H_2O$, 8 mg; $FeSO_4 \cdot 7H_2O$, 20 mg; $MnSO_4 \cdot 4H_2O$, 20 mg; $CaCl_2$, 20 mg; $(NH_4)_2SO_4$, 31 g; thiamine, 1.0 mg; biotin, 1.0 mg; KH_2PO_4, 9.1 g; and yeast extract, 0.1 g. Since the biomass solids and cellulose solids cannot be separated, a dry-weight analysis for biomass concentration determination had to be replaced by chemical assays for materials present in the cells and absent from the cellulose. This was accomplished by using modified micro-Kjeldahl and Lowry procedures to measure the concentration of organic nitrogen and protein, respectively, after the culture solids were washed thoroughly with distilled water (Zabriskie, Ph.D. thesis).

All fermentations were grown in an aseptic 70-liter batch fermentor. Environmental conditions were monitored and controlled by an instrumentation package, which included analyzers for dissolved oxygen (Johnson type, New Brunswick Scientific Co., Edison, N.J.), gaseous oxygen concentration (paramagnetic wind type, model 802, Mine Safety Appliances, Inc., Pittsburgh, Pa.), gaseous carbon dioxide concentration (infrared absorption type, model 303, Mine Safety Appliances), pH, temperature, and gas flow rates. The dissolved oxygen was controlled above limiting conditions for all fermentations, except during the yeast oxygen depletion experiments.

RESULTS

Response to fluorophore concentration. The response of the culture fluorometer to fluorophore concentration was evaluated by using solutions of NADH in 0.05 M phosphate buffer or quinine in 0.05 M H_2SO_4. The fermentor was filled with fluorophore-free buffer. Aliquots of concentrated fluorophore solutions were added, and the fluorometer readings were recorded. Typical results are shown in Fig. 2.

The non-linearity of the data is due primarily to the inner filter effect (8). As the exciting wavelength light passes through the sample, the light is absorbed according to the Beer-Lambert Law:

$$I = I_o e^{-kCL} \quad (1)$$

where I is light intensity, k the molar extinction coefficient, C is the concentration of absorbing species, and L is the path length for light absorption. When the concentration of absorbing species is high, it leads to a gradient of exciting light intensity within the sample. When the sample is located far from the light source, it is effectively filtered from the exciting wavelength, thereby reducing its fluorescent capability. Un-

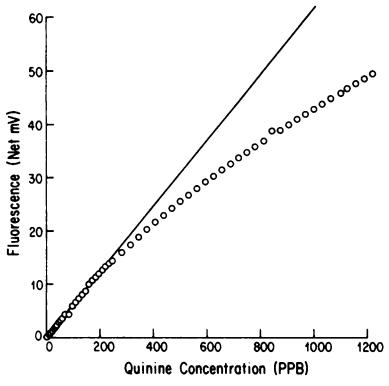

FIG. 2. *Fermentor fluorometer response to quinine concentration.*

der these conditions, the total fluorescence, f, generated in all directions, is given by (8):

$$f = I_o(1 - e^{-kCL})\phi_f \qquad (2)$$

where ϕ_f is the fluorescent efficiency of the fluorophore. Therefore, fluorescence and fluorophore concentration are only approximately linear at low fluorophore concentrations.

Generally, fluorometers do not measure the total amount of fluorescence generated by a sample as given by the previous equation, but, rather, the amount contained in a comparatively small solid angle. Therefore, the geometry of the detector system determines a fluorometer's response to concentration with inner filter effect aberrations. Log-log plots have been shown to be useful in correlating fluorescence and concentration data from instruments that irradiate and measure the fluorescence through the same surface on the sample holder (surface detector type [8]).

The physical situation for the fermentor fluorometer is much more complex. This configuration does not have a defined boundary for the sample as do fluorometers with sample cuvettes. The exciting light penetrates the sample to whatever distance it can before it is completely absorbed or scattered. This penetration depth varies with the fluorophore concentration. The presence of cells and solids scatters the incident light out of the area measured by the fluorometer. The heterogeneity of the culture medium favors a secondary inner filter effect in which the fluorescent light is absorbed by the sample before it passes out of the sample to the detector. This heterogeneity also can cause quenching phenomena, which divert light energy absorbed by a fluorophore into channels other than the emission processes responsible for luminescence. These include internal energy conversion, intersystem crossing, energy transfer, and the deactivation caused by colliding with other molecules of solutes (8).

Since the fermentor fluorometer is a surface detector type, the data of Fig. 2 were replotted on log-log coordinates (Fig. 3). Despite the system's complexity, the results were effectively linearized by the coordinate transformation suggested by the elementary surface detector system, yielding a correlating equation of the form:

$$C = [\exp(-b)f_{net}]^{1/a} \qquad (3)$$

Constants a and b are the respective slope and intercept of the linear least squares line through the data plotted on the coordinates shown in Fig. 3. The quantity f_{net} is the difference between the observed fluorescence and the background signal obtained in the absence of fluorophore.

Environmental conditions affecting fluorescence. Several experiments were performed to evaluate the responses of the fluorometer to changes in culture conditions that were shown by other investigators to influence yeast cell fluorescence (4, 5). In the experiments described below, f_{net} is the difference between the observed culture fluorescence and the value obtained before inoculation of the fermentor. Figure 4 shows that decreasing the dissolved oxygen below 45% saturation, where oxygen becomes growth limiting, caused an increase in fluorescence, as observed by Harrison and Chance (5). When the dissolved oxygen was restored to above 45%, the fluorescence decreased to its original value.

Cell starvation reduced culture fluorescence. Figure 5 presents data from an actively growing yeast fermentation in which the energy substrate, glucose, was permitted to deplete. As it became limiting, the CO_2 evolution rate declined corresponding to a decreasing respiration rate and was followed closely by decreasing culture fluorescence. The inability of the cells to pro-

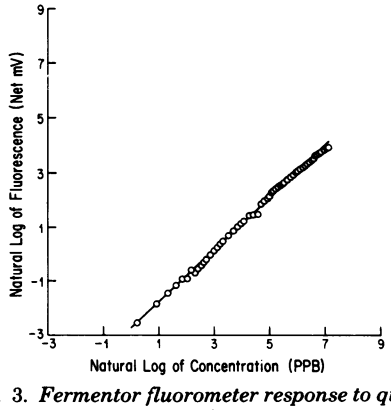

FIG. 3. *Fermentor fluorometer response to quinine concentration (log-log coordinates).*

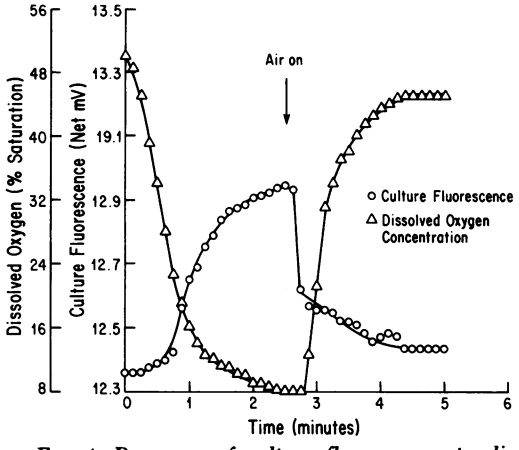

FIG. 4. *Response of culture fluorescence to dissolved-oxygen depletion in a bakers' yeast fermentation.*

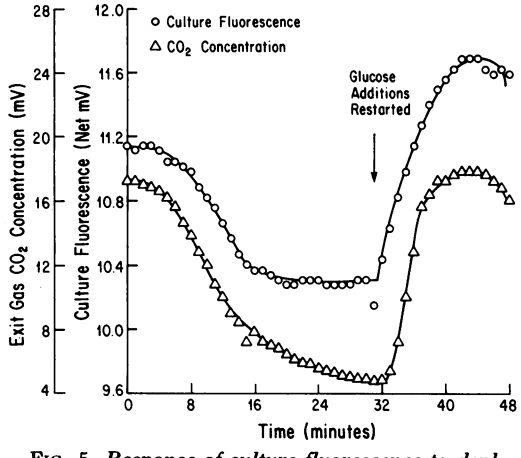

FIG. 5. *Response of culture fluorescence to depletion of energy substrate (glucose) in an aerobic bakers' yeast fermentation.*

duce energy by respiration during glucose starvation caused reductions in the high-energy NADH pools of the cell. When glucose additions were resumed, the respiration rate and fluorescence returned to their original levels.

Other factors affecting fluorescence include temperature and pH. Fluorescence increased dramatically as temperature decreased (temperature coefficient = 1.2 − 1.5 per 10°C), which emphasizes the need for precise temperature control in fluorescent sample measurement. Fluorescence decreased with increasing pH. Levels of pH are thought to affect fluorescence by changing the ionic state of some fluorophores or by acting on the solvent and other absorbing species in the sample to alter the inner filter effect.

The response of the culture fluorometer was generally insensitive to changes in agitation and aeration rate, provided the dissolved oxygen remained above limiting levels (~45% saturation). Gas bubbles contributed to the overall noise levels in the signal. This noise was minimized by using an active filter on the output of the fluorometer and by irradiating the culture and measuring the fluorescence over a large surface area (1⅜ inch-diameter disk).

Quantitation of NADH content of yeast culture fluorophores. To approximate the NADH portion of the fluorophores responsible for culture fluorescence, iodoacetate was added to a culture of actively growing yeast with a biomass concentration of 12.6 g/liter. The concentration of iodoacetate was 1.5 mM, which was sufficient to block the Embden-Meyerhof-Parnas pathway and prevent any further production of intercellular energy. This causes a depletion of intercellular NADH reserves followed by cell death. The effect of adding this potent inhibitor caused a 12.5% decrease in culture fluorescence (Fig. 6), similar in magnitude to the decrease obtained in the glucose starvation experiment.

The estimate of NADH content of the culture fluorophores is obtained from the calibration curve shown in Fig. 7. If it is assumed that the fluorophores present are directly proportional to the amount of biomass, then the abscissa of Fig. 7 may be thought of in terms of culture fluorophore concentration. It can then be seen that a 12.5% reduction in culture fluorescence caused by the exhaustion of intercellular NADH corresponds with a 50% reduction in fluorophore concentration. This observation suggests that 50% of the culture fluorophore content is intercellular NADH. The actual NADH contribution to cul-

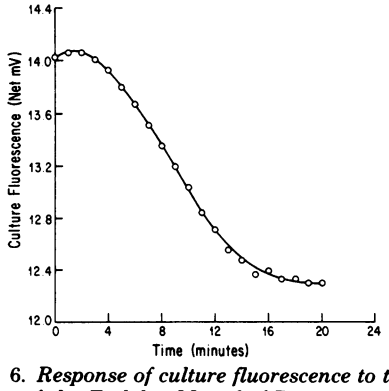

FIG. 6. *Response of culture fluorescence to the inhibition of the Embden-Meyerhof-Parnas metabolic pathway by iodoacetate in an aerobic bakers' yeast fermentation.*

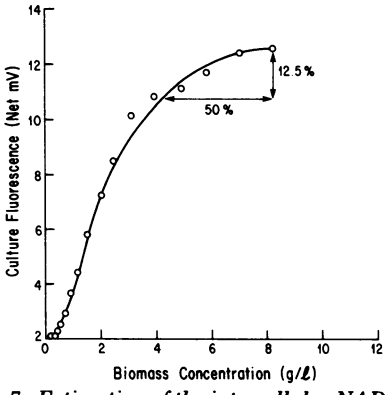

FIG. 7. *Estimation of the intercellular NADH portion of the culture fluorophores.*

ture fluorescence may exceed 50% since the blocking of the cellular energy-generating apparatus probably affects mitochondrial NADH pools. Therefore, NADH present in the cell cytoplasm is not accounted for by this approximation procedure (6).

The sources of non-NADH fluorescence are open to speculation. Many biological compounds which are found in cellular material fluoresce (e.g., deoxyribonucleic acid, ribonucleic acid, proteins, cytochromes, some vitamins, hormones, amino acids, and nucleotides). However, only a few are known to fluoresce under the conditions selected to optimize NADH fluorescence used in these experiments. Some of this residual fluorescence is due to intercellular NADPH. Some medium nutrients fluoresce, causing the medium to fluoresce before inoculation. Other fluorophores may be released into the medium by growing cells, such as pyridoxal enzymes and flavins, suggested by other investigators (6).

The presence of extracellular fluorophores was confirmed by measuring the fluorescence of cell-free broth and whole-broth samples. A modification of the instrument described above, which could accommodate a standard optical cuvette, was used to make these measurements. Direct comparison of these results with those from the original instrument cannot be made, and, therefore, the measurements are expressed in arbitrary units (percent span, Fig. 8). The data for the whole-broth and cell-free broth samples have similar trends with respect to fermentation time (Fig. 8). The removal of the cells was accompanied by a decrease in the fluorescent intensity of the sample.

Biomass concentration results. Typical results of culture fluorescence and biomass concentration for a yeast fermentation are presented in Fig. 7. The nonlinear relationship was similar to that obtained from experiments using soluble fluorophores (Fig. 2) and suggested that a log-log representation of the data might be appropriate. Figure 9 shows the results for another *S. cerevisiae* fermentation in which oxygen and carbohydrate nutrients were present in excess to minimize non-biomass-related fluorescence phenomena. Culture pH and temperature were controlled at constant levels.

The linearity of the data suggested that equation 3 could be used to correlate the biomass concentration and culture fluorescence data. After determining the slope, a, and intercept, b, of the line on the log-log plots (Fig. 9), equation 3 was used to compute estimates for biomass concentration from the fluorescence data. The estimates and laboratory data were compared for the yeast fermentation (Fig. 10) and a *Streptomyces* sp. fermentation (Fig. 11). The respective dispersions of the estimates from the biomass concentration data were 15 and 36%, calculated as coefficients of variation.

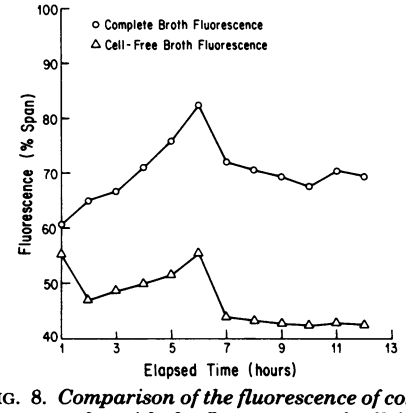

FIG. 8. *Comparison of the fluorescence of complete culture samples with the fluorescence of cell-free medium samples.*

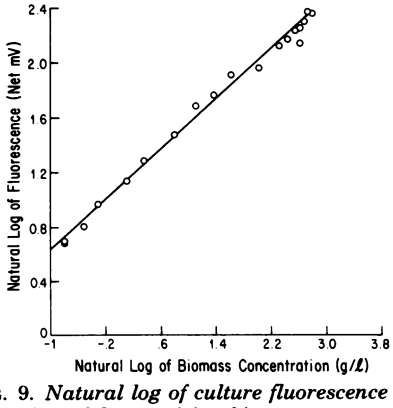

FIG. 9. *Natural log of culture fluorescence versus natural log of S. cerevisiae biomass concentration.*

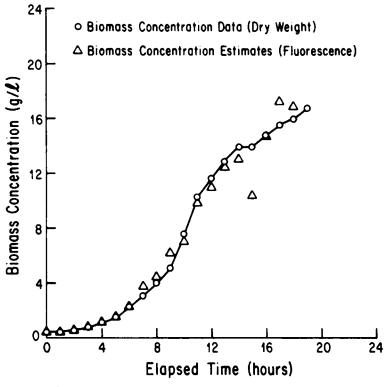

FIG. 10. *S. cerevisiae biomass concentration data and estimates derived from culture fluorescence measurements.*

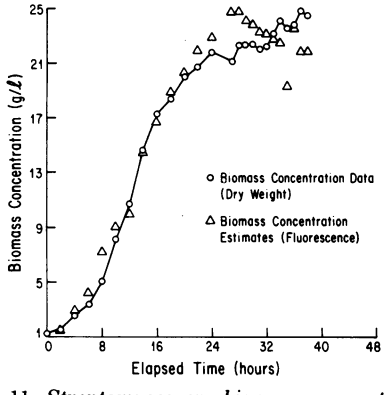

FIG. 11. *Streptomyces sp. biomass concentration data and estimates derived from culture fluorescence measurements.*

The fluorescence behavior of the cellulose fermentation (Fig. 12) was quite different from the previous experiments. At hours 13 to 15, the culture fluorescence decreased, even though biomass concentration continued to increase. Since the pH was maintained constant and dissolved oxygen was controlled above 50% saturation, the inflection period appears to reflect a metabolic shift in the culture, perhaps initiated by a substrate limitation or inhibition by a product or intermediate. Although this trend did not permit biomass concentration estimation as in the previous experiments, the reproducibility of the phenomenon suggests that it may reflect an important metabolic transition that may have implications of its own in the overall control and optimization of the process.

Real-time estimation of biomass concentration. The potential application of this technique is its use to estimate the biomass concentration in real time, using parameters *a* and *b* derived from a previous experiment, with equation 3 and the on-line culture fluorescence data. However, experiments which were conducted to evaluate this procedure did not provide accurate approximations. The cause of this problem was found to be the poor stability of the fluorescent ultraviolet lamp in the instrument. A decay in lamp intensity changes the values of *a* and *b* during the course of a long experiment and from one experiment to another.

A series of experiments was conducted to study the long-term stability characteristics of the lamp using samples of constant fluorophore concentration. The signal generated and drifted up and down the scale, with a gradual overall downward excursion corresponding to the aging of the lamp. Drift rates of 0.24 to 0.72 mV/day were common. Fermentations of the duration of these studies produced total fluorescence changes of 2 to 15 mV. It can be seen that the instrument drift can be a substantial portion of the overall signal change during a fermentation. This effect becomes especially important at high cell densities when a small change in fluorescence corresponds to a large equivalent change in biomass concentration due to the form of equation 3.

DISCUSSION

These results suggest that the measurement of culture fluorescence will be able to provide accurate real-time estimates for fermentation biomass concentration under controlled environmental conditions. Before this potential can be completely evaluated, compensation must be made for the drifting intensity of the exciting ultraviolet lamp. A new instrument is being developed in our laboratory at the State University of New York at Buffalo, which will eliminate

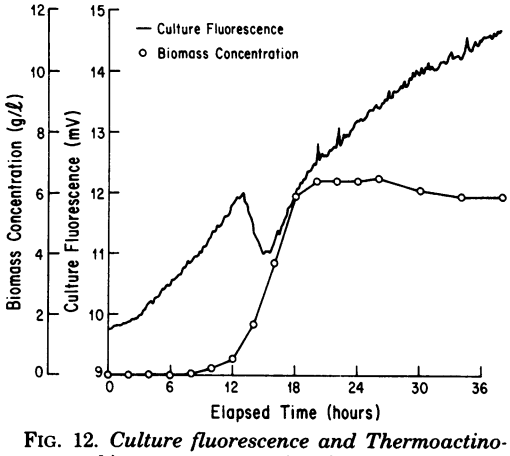

FIG. 12. *Culture fluorescence and Thermoactinomyces sp. biomass concentration data.*

lamp stability effects by using a double-beam approach, analogous to the ratio measurement principle used in double-beam spectrophotometers. This new instrument will enable a much more detailed analysis of the fluorescence signal with biomass concentration and cell metabolic activity.

It has also been shown that fluroescence is a strong function of the microbial environment. Although this poses a problem in using fluorescence to estimate biomass concentration in fermentations in which the environment cannot be controlled, this characteristic may prove to be a valuable attribute. Evidence suggests that culture fluorescence is a measure of culture metabolic activity, i.e., the product of biomass concentration and the relative rate of metabolic activity of each cell. It is, therefore, reasonable to speculate that culture activity may prove to be a more important parameter to monitor and regulate than biomass concentration, when optimizing and controlling fermentations that produce products related to cell metabolic processes.

ACKNOWLEDGMENTS

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