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Development of an On-Line Sensor for Bioreactor Operation

Scientific Note

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INTRODUCTION

Control and optimization of bioreactor operation are strongly influenced by the quality of the sensors available for crucial response variables. Direct on-line sensors are generally available for the physical variables, such as dissolved oxygen level, temperature, speed of agitation, and pH. However, on-line sensors for key variables, such as composition (e.g., concentrations of biomass, substrates, and products), and those for the physiological state of the biomass are either not available or are relatively unreliable. In many cases, these variables are analyzed off-line, which often results in significant delay, thus making on-line feedback control infeasible. Development of an on-line sensor that would give information on these key variables would alleviate the bottleneck to effective control of such processes.

Several attempts at developing new on-line biosensors have been reported. They include enzyme-embedded probes (*1–3*) that would react with one of the reaction broth components to yield an indirect measurement of the concentration of that component. An approach based on light

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emission is the fluorescence approach. The use of fluorescence measurement of cellular NADH as an indicator of biomass appears promising (*4–9*). One problem associated with this approach is its nonspecificity and research continuous in its investigation (*10–12*). Another optical-based approach is the on-line turbidimetric measurement approach, in which a broth stream is cycled through a spectrophotometer that measures the optical density (OD) of the broth. Through correlation, the biomass concentration can be inferred. A drawback in this approach is also in its nonspecificity in that all particles present in the broth, such as dead cells and lysis debris, would contribute to the OD reading (*13*). In addition, linearity of correlation is only valid for low-cell-concentration broth. As cell concentration increases, the relationship between cell density and turbidity deviates from linearity rapidly (*13*). Presented here are preliminary results of using a new approach based on the intensity of bioluminescent emission from cells of interest, which is correlated to the biomass concentration in the reactor. This approach has the potential of being noninvasive and specific for the target cells of interest.

During the past few years, the bioluminescent *lux* genes, originally isolated from either the firefly *Photinus pyralis* or marine bacteria *Vibrio, Photobacterium, Alteromonas,*and *Xenorhabdus* have been cloned into several microorganisms for applied measurement purposes (*14–18*). Bioluminescence has excited the curiosity of people over the centuries because of its unique beauty, although the practical applications of this naturally occurring phenomenon have been developed only recently.

The *lux* A and B genes of the *lux* structure genes code for the α and β subunits of the luciferase enzyme. The *lux* C, D, and E genes code for the polypeptides of the fatty acid reductase system responsible for the regeneration of the aldehyde substrate for luciferase from the fatty acid product (*19–21*). Luciferase, a mixed-function oxidase, catalyzes light emission of the *lux* system. In bioluminescent bacteria, the luciferase catalyzes the oxidation of a long-chain aldehyde and $FMNH_2$, resulting in the emission of visible blue-green light. The bioluminescent reaction is most active between 23 and 30°C. In general, the bioluminescent activity is regulated, among others, by the endogenous synthesis and accumulation of the so-called autoinducer molecules (*16,22*). A study by Stouthamer (*23*) showed that the cellular energy required to support the bioluminescence is negligible, when compared to the overall cellular demand for ATP.

The strain RB1151, used in these experiments, is an *E. coli* DH5α strain containing the pUTK4 plasmid, constructed by one of the authors (R. B.). The pUTK4 plasmid was created using the pUCD615 *lux* fusion plasmid (*24*), and the pUC18 plasmid (generally used as a *lac*Z fusion plasmid) containing the *lac* promoter, a strong promoter when derepressed. The construction is shown in Fig. 1. In this strain, the *lac*I repressor gene is absent, thus making the *lux* gene activity constitutive. Further, construction is such that the *lux* gene is capable of *de novo* synthesis of the aldehyde required for the bioluminescent reaction. The ligated plasmids were

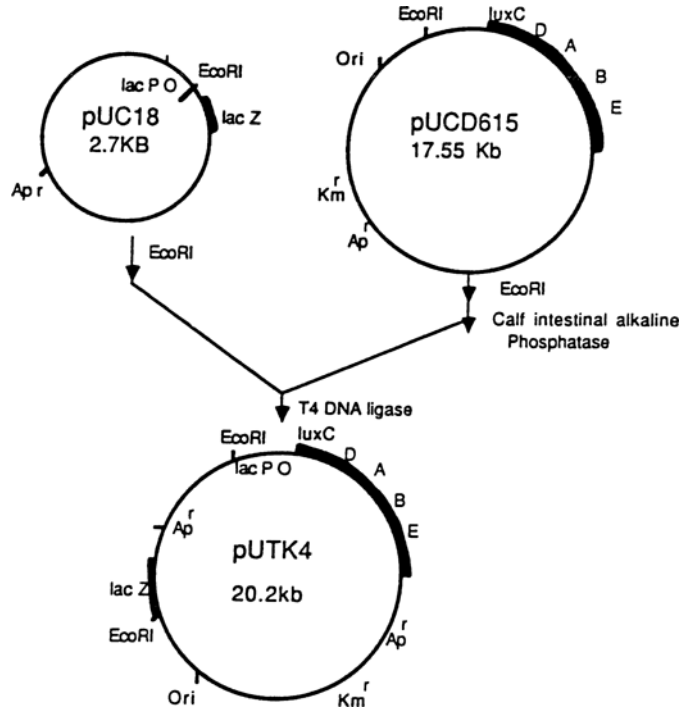

Fig. 1. Construction of bioluminescent plasmid pUTK4, including the cloning strategy. *Km*^{r} denotes kanamycin resistance; *Ap*^{r} denotes ampicillin resistance; ori denotes origin of replication.

transformed into *E. coli* DH5α. Selection was on LB agar with ampicillin. The light-producing colonies were easily seen after the plates were cooled down to 25°C. The orientation of the pUC18 insert was ascertained using restriction enzyme digestion and agarose gel electrophoresis.

Prior work with cells harboring the *lux* gene cassette suggests that there is a direct correlation between the amount of biomass and the amount of emitted light (*25*). If such a correlation can indeed be established, then it has the potential to be used as an on-line sensor to provide biomass concentration information during bioreactor operation. In addition, when a gene product is the desired final product, the *lux* gene may provide a convenient way of monitoring its synthesis progress. For example, the *lux* gene may be cloned adjacent and be subjected to the control of the protein gene of interest, such that when the transcription and translation of the protein gene become active, bioluminescence genes also become active. Monitoring the kinetics of bioluminescence would provide a convenient handle for monitoring the synthesis of the protein of interest. Any disturbance to its synthesis would lead to a shift in the corresponding bioluminescence profile. Finally, feedback control of the protein synthesis would then be achieved through the bioluminescence measurement.

Of course, it is recognized that initial cloning work is required to introduce the *lux* gene cassette into the host cell of interest. With the increasing employment of recombinant cells in the production of pharmaceutical, food, and agriculture products, the cloning work involved in introducing the *lux* gene may not prove to be inhibitive. If successful, the increased profit margin derived from using such on-line information may very easily offset the added cost involved.

The objective of the current work is to develop a prototype quantitative correlation model between the intensity of the emitted light and the biomass concentration for a batch operation process. Batch operation is first selected as the process to be analyzed because of its popularity in industry. Biomass is chosen for the correlation because of the relative ease of its off-line measurement by a variety of methods (e.g., OD, dry weight, and direct count).

MATERIALS AND METHODS

Bacterial Strains

The *E. coli* strain RB1151 used here is derived from *E. coli* DH5α (*rec lac* Nal^r, Bethestha Research Laboratory). The construction is described previously. The stability of this recombinant clone has been observed for over hundreds of subclonings in the presence of ampicillin.

Medium

A modified LB (Luria-Bertani) medium was used for culture work: Bacto-tryptone 10 g/L; yeast extract 5 g/L; sodium chloride 10 g/L. After autoclaving, filter-sterilized ampicillin was added to the medium to a final concentration of 50 mg/L.

Growth Conditions

Overnight RB1151 cultures were grown in LB broth in a 25°C water bath with constant shaking at 250 rpm with antibiotic selection. Cell growth was monitored by light scattering, using a Klett meter (Klett Manufacturing Co., NY).

Operating Parameters

Each experiment was performed in a 3-L bioreactor (Applikon Inc.) at an initial broth vol of 1.5 L. An exponential-phase culture (100 Klett U) was used to inoculate the LB medium at 1:20 dilution. During growth, temperature was maintained at 25°C and pH at 7.2. Impeller speed and air flow were controlled by the computer in order to maintain a desired dissolved oxygen level of 80% or above. A batch run was continued for a period of 24–32 h, by which time the stationary phase of the culture had been well attained. Intermittent sampling of the cell density by a Klett meter was performed using aseptic techniques.

Light-Detection System

Light production from growing cultures in the bioreactor was measured by a photomultiplier via a flow-through system designed at the Chemical Engineering Department of UTK. It is a light-tight steel cylinder containing a photomultiplier tube juxtaposed with a flow-through glass cuvet (0.75 mL Suprasil Micro Spectrophotometer Cell, with a light path of 10 mm $\pm$ 0.01, and an aperture [mm] of 11×6.5). The culture broth is continuously pumped to and from the reactor through the cuvet by a peristaltic pump (flow rate at 33 mL/min). The tube connections were made so that the creation of turbulent flow or bubbles is minimized. The output of the photomultiplier tube is in the form of a voltage potential. The power supply voltage used to drive the photomultiplier tube is set at 500 V. This voltage supply generally results in a voltage output reading between 0 and 600 mV during the course of a batch run of the reactor. The background noise of the detection system is generally around 0.5 mV. The flow-through cuvet and all the connecting tubes were autoclaved prior to usage, maintaining sterility.

RESULTS AND DISCUSSION

Cell mass and bioluminescent intensity information were monitored and recorded over a period of about 24–32 h during a batch run of *E. coli* strain RB1151. A total of 11 runs were carried out. Figure 2(A) shows the growth profiles of three of the experiments at 1:20 inoculation dilutions. Figure 2(B) shows the corresponding bioluminescence profiles. Figure 3 is a typical light-emission profile superimposed onto the biomass curve. The biomass curve has the typical sigmoid-shaped kinetics associated with batch growth. In contrast, light emission starts from a low level, goes through a maximum, and then rapidly declines. Toward the late log phase, it is evident that only a very low level of light was observed, in spite of the fact that biomass concentration was reaching maximum. Comparing the two curves, it is immediately evident that the two curves, as they stand, do not have a direct correlation, but rather that the light-emission curve appears to have a more direct correlation with the slope (or first derivative) of the biomass curve. The slope of the biomass curve reflects the rate of biomass growth. The slope of the biomass curve shall be referred to as the growth rate curve. This correlation hypothesis is based on the observation that the light emission starts and ends at a low level, corresponding to the relatively low-growth regions of the lag and late-log phases of growth. The emission goes through a maximum corresponding to the region of logarithmic growth where the growth rate is at highest. The above observation implies that the light-emission phenomenon is correlated with the process of growth, and not to the total amount of biomass itself. This is physiologically plausible, taking into account the

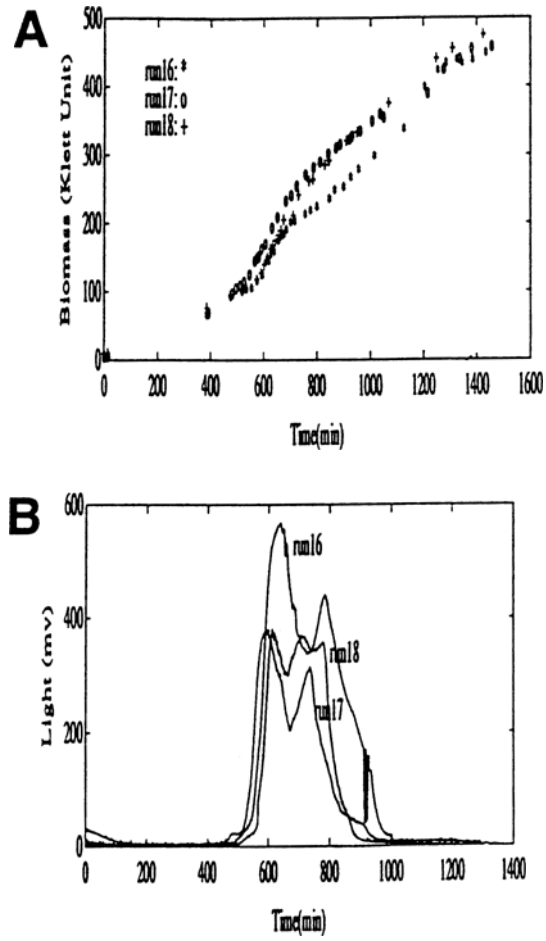

Fig. 2. A: Growth curves for *E. coli* RB1151 at pH 7.2, temperature 26.0°C. B: Bioluminescence comparisons under identical operating conditions of three batch runs.

underlying mechanism of bioluminescence emission, in that reactions involving the luciferase enzymes, oxygen, NADPH, the flavin mononucleotide, $FMNH_2$, and ATP are responsible for producing the light emission, and that the intensity of emitted light would be dependent on the relative production and turnover rates of these enzymes and molecules. The relative turnover rates, in turn, are directly related to the rate of growth of the biomass. When the net growth rate is close to zero, as in the stationary phase, the net turnover rate of these enzymes would also be close to zero. As a result, no light emission would be evident. It can be seen further that the greatest demand for oxygen is at the time of maximum growth rate or maximum light emission. If, during this time, dissolved oxygen level is not adequate, one observes an immediate decline in the emitted light intensity.

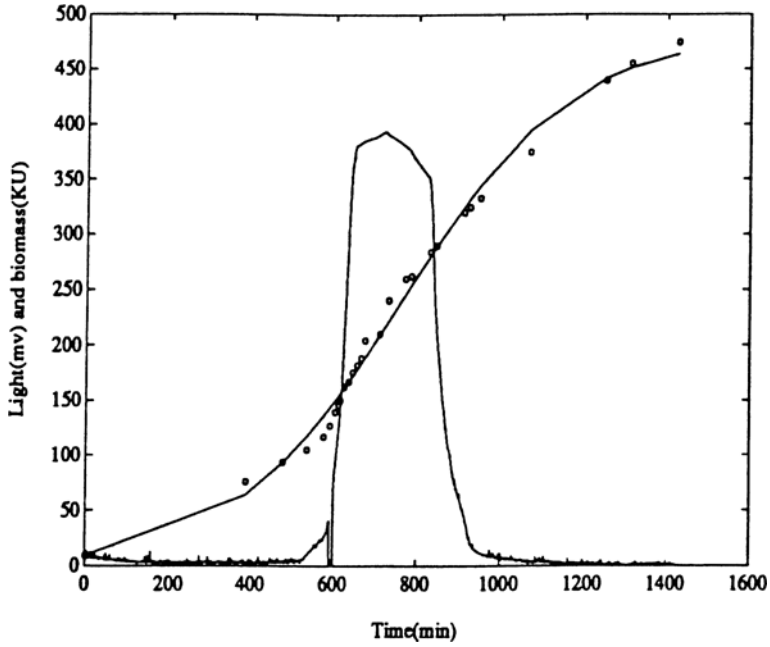

Fig. 3. Growth and bioluminescence kinetics of *E. coli* RB1151 grown in LB medium with ampicillin. Growth measured in Klett unit (open circle) and light intensity as millivolts. Circles indicate growth, and solid line indicates light emission.

Several general characteristics shared by all the light-emission profiles from the eleven experiments are also evident. These are detailed below.

1. There is a lag period following inoculation, before the onset of light emission, perhaps indicating the requirement for a threshold level of biomass for light emission or perhaps indicating the lack of sensitivity of our photomultiplier to the low level of light emission at this growth phase.
2. The overall time to light peak after inoculation as well as the total period of observable light emission are relatively constant, reflecting the constant duration of the period of the exponential growth phase.
3. Dips or valleys can be seen during the period of maximum light emission if the level of dissolved oxygen drops to below approx 60%, indicating the extreme sensitivity of the bioluminescence to the requirement for oxygen. This lack of oxygen may be attributed to the transient flow required for the broth stream to be pumped through the flow-through cuvet to be presented to the light-detection system. The transient time was approx 15 s. During high-growth-rate period, a high level of dissolved oxygen is required to support the bioluminescent

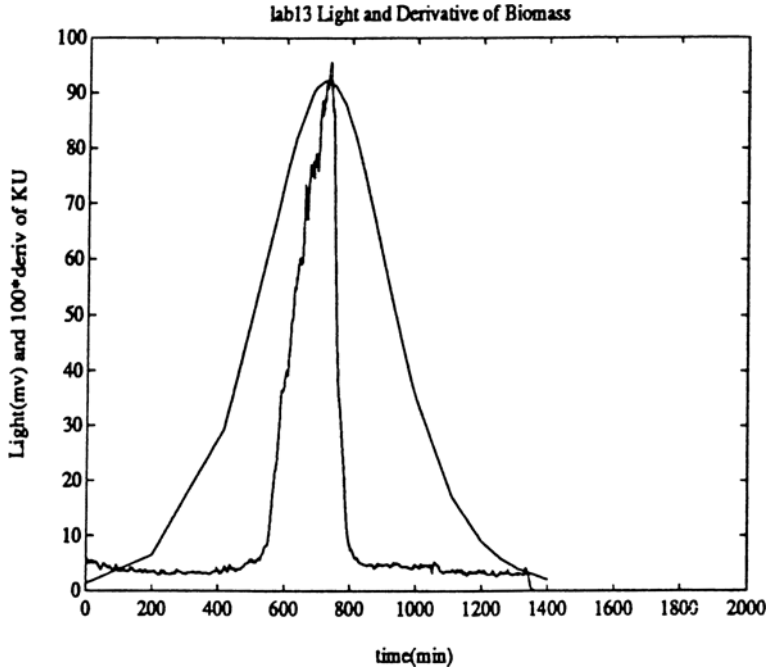

Fig. 4. Comparison of the growth-rate curve and bioluminescence curve; the curves are fitted with Gaussian probability distributive functions. (The upper curve is the growth-rate curve; the lower is the bioluminescence curve.)

reaction, and 15 s of deprivation of oxygen may very well cause the light emission intensity to decline.

4. High variability of the maximum light intensity level, as measured by our photomultiplier, was observed among the 11 experiments. This variability may be caused by the lack of consistent photomultiplier calibration from batch to batch.

The last point enumerated above precludes us from obtaining an outright consistent numerical correlation between the biomass growth rate and the corresponding light-emission kinetics, even though consistent interbatch biomass growth kinetics were obtained. Qualitative correlation does evidently exist because of the similarity of light-emission profiles from batch to batch. The possibility exists that the peak light variation is an experimental artifact rather than inherent with the cell-batch system. In order to test this hypothesis, the light-emission curves were normalized against the respective peak levels to eliminate the interbatch variations artifically. If after normalization, a consistent correlation is obtained, then one may conclude that indeed there is an inherent correlation between the biomass growth rate and light emission, and that interbatch light variation is an artifact of instrumentation.

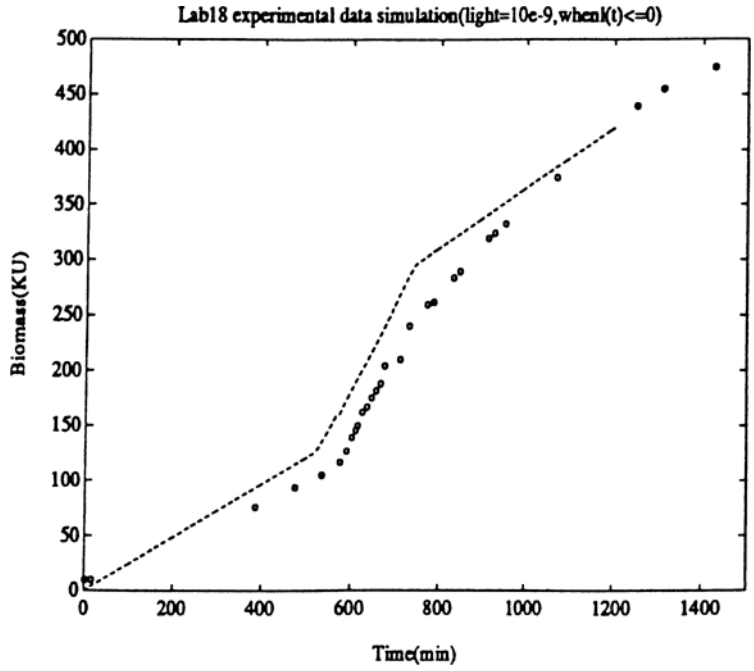

Fig. 5. On-line prediction of biomass using the fitted Gaussian distribution curve based on the measured light intensity during the batch culture of *E. coli*; (○) experimental biomass measured off-line (–) biomass predicted from the correlation model.

The light-emission curves of four experiments were normalized and an attempt was made to correlate the normalized light curves to the corresponding growth-rate curves via fitting with Gaussian probability distribution functions, which are characterized by two parameters, the mean μ and the variance σ (*28*). The values for the parameter μ for the two fitted curves coincide because the maximum of the light-emission curve almost always corresponds to the point of maximum growth rate. A constant ratio between the two σ's of the fitted curves was indeed derived from the normalized data. Finally, this correlation was validated on an independent experimental data set (normalized). Figure 5 compares the actual and the model-predicted results. Light-emission intensity data was logged at 1-min intervals. For this validation study, from each normalized light measurement, the corresponding growth rate is first calculated from the correlation model. Then, the growth rate is integrated discreetly to yield the corresponding total biomass level. The fit appears to be compatible. The ability to derive a consistent correlation when normalized, but not when raw data is used suggests that an inherent correlation does exist (although a nonlinear one) between the light-emission level and the biomass rate of growth. A possible reason to account for our failure to

elicit the correlation when using raw-batch experimental data may be the inconsistent calibration of our light-detection instrumentations. We are currently working on improving our instrumentation capability.

Several reports (*25,29–31*) have documented the observation of linear correlation between biomass and light emission during the exponential phase of a biomass growth system. Therefore, we investigated the linear correlation using our batch data limiting the time interval to approx 3 h during the exponential growth phase. A linear relationship does appear to be evident. This observation suggests that a more clear-cut linear correlation may be expected to be derived for cell systems growing in the exponential growth phase, which is the predominate growth phase in a continuous stirred-tank bioreactor, in which the dilution rate would determine the final steady-state specific growth rate. Our future plan then is to run continuous culture and attempt to do the correlation again. In addition, continuous mode of operation is expected to produce a higher yield in productivity in the long run, although it is recognized that at present, it is not the routine choice of mode of operation in industry because of a number of problems that may occur in continuous operation.

CONCLUSION

For effective feedback control and optimization of bioreactor processes, the availability of on-line sensors providing information on key process variables is indispensable. We report preliminary results of the development of a new biosensor based on bioluminescent measurement that has the potential to be used to infer biomass or product concentration information on-line. Batch operation was first used as a starting system. We attempted to correlate the bioluminescent intensity to the biomass concentration for the entire range of the batch operation. A consistent quantitative correlation between the biomass concentration and the light intensity cannot be obtained, mainly because of the inconsistency of the level of emitted light from batch to batch. This was largely owing to the lack of consistent calibration of the photomultiplier detection system between batches. We are currently trying to remedy this problem. In contrast, when normalized data were used, a fairly consistent correlation model was derived relating the light emission to the biomass concentration, further strengthening the conclusion that the lack of photomultiplier calibration resulted in the variation of the light-emission profiles between batches. Batch experimental results suggest that a cleaner linear correlation model exists for cells growing in the logarithmic phase. Thus, our plan is to switch to continuous cultures and attempt the correlation study again. A successful correlation would yield invaluable on-line information to enable effective implementation of feedback control and optimization of the bioreactor processes.

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