To the Graduate Council:

I am submitting herewith a thesis written by Bo Huang entitled "DEVELOPMENT OF AN ON-LINE BIOSENSOR USING BIOLUMINESCENCE TECHNOLOGY." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

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We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor
and Dean of The Graduate School

DEVELOPMENT OF AN ON-LINE BIOSENSOR USING BIOLUMINESCENCE TECHNOLOGY

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Bo Huang

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To my parents

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ABSTRACT

During the operation of any bioreactor process, the most crucial elements are the microbial biomass and their metabolic state of growth. One of the major inhibiting factors in the development and implementation of sophisticated bioreactor control regimes has been the lack of suitable on-line biosensors for measuring such parameters. The objective of the current work is to develop and evaluate a novel on-line biosensor based on the light emission capabilities of a recombinant host cell used in the bioreactor process. The cells are genetically engineered to include the *lux* gene operon, originally isolated from bioluminescent marine bacteria, such as *Vibrio fischeri.* The *lux*AB gene product is the enzyme *luciferase*, which catalyzes intracellular reactions leading to the emission of visible light at 490 nm wavelength. The focus of this work was to develop a correlation between the intensity of emitted light from a prototype *E. coli* culture and the corresponding cell biomass concentration. The bioluminescence was to be monitored to give inferred measurement of the cell biomass concentration in a bioreactor process. A qualitative correlation between the intensity of emitted light and the cell biomass shows that bioluminescence appears to be a reflection of the relative rate of growth rather than the absolute amount of biomass, in that the maximum light emission always coincides with the point of maximum growth rate. Due to the inconsistency in the emitted light intensity from batch to batch, a consistent quantitative correlation was not successful. However, with the inconsistency removed by normalization, correlation results suggest that the light intensity variation from batch to batch may be an artifact rather than inherent. We predict that a more consistent correlation in continuous culture runs may be expected in the future.

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CHAPTER 1

INTRODUCTION

1.1 Motivation

The control and optimization of a bioreactor operation is strongly influenced by the quality of the sensors available for crucial response variables. Direct on-line sensors are generally available for the physical-chemical variables, such as the dissolved oxygen level, temperature, speed of agitation, foam, and pH. Since these sensors respond relatively quickly, proportional plus integral feedback controllers often are suitable for these variables. However, on-line sensors for the composition (e.g, concentrations of biomass, substrates, nutrients, and ions) and for the physiological state of the biomass are either unavailable or are relatively unreliable. In many cases, these variables are analyzed off-line, resulting in a significant gap between the time when samples are taken and the time when the results are available. The time lag is often longer than the time constant of the process, thus making on-line feedback control infeasible.

The potential maximum rate of product synthesis during fermentation is partially determined by two variables: (1) biomass concentration and (2) the metabolic state of the cells. Since these state variables cannot be measured routinely on-line with currently available sensors, the estimation of these variables is often relied upon to optimize and control the reactor process. The development of new on-line sensors that allow measurement of such parameters is urgently needed.

1.2 Statement of the Problem

The inadequacy of available on-line biosensors and the lack of insights into the biological pathway indicate a problem that can be stated as follows:

> *Develop an on-line biosensor, based on the intensity of light emitted by the microorganisms in a bioreactor process, that can provide indirect, correlated on-line information on the cell biomass concentration.*

This on-line measurement will provide the feedback information necessary for the implementation of control strategies required for the optimization of bioprocesses.

CHAPTER 2

BACKGROUND

2.1 Review of Biosensors

2.1.1 General Review of Biosensors

Biosensors are required to supply information for the effective control of bioreactor operations, such as fermentation processes. A biosensor is defined as a sensor that detects biological material as a primary recognition system and signal generator. Biosensors for on-line process control offer the facility of sophisticated data-handling and control techniques, but present a very difficult design challenge [1]. Good review papers on biosensors are available [22, 40, 45, 51, 56]. Briefly, biosensors can be divided into: off-line, such as gas chromatography and mass spectrometry; and on-line, which can be further divided into three categories:

- Intermittent sensors that require injection of a portion of a sample stream into the instrument;
- Continuous sensors that permit the sample to flow continuously through the instrument;
- Non-invasive sensors in which the chemical analysis is done *in situ*, directly inside the process line, using a probe that is chemically sensitive.

The disadvantage of using on-line sensors is their inherent need to divert part of the reactor broth through a flow loop in order to present it to the analyzing instruments. The diversion constitutes a disturbance to the overall system in that delays, contaminations, or variations in the process characteristics may be introduced. These problems are further complicated by the on-line sensor's need to have separate hardware to perform the sampling, concentration, separation, and sensing/identification steps. A major limitation to the successful application of existing biosensors is that cleaning procedures are required to maintain sterility, especially in large-scale industrial bioreactor systems. Therefore, the problem to be overcome centers on the ability of on-line sensors to be maintained sterile without loss of sensitivity.

The noninvasive sensors do not come into direct contact with the culture system, thereby eliminating many sampling and sterilization problems. Examples of noninvasive sensors are the fluorescence sensors which use fiber optics [24], near-infrared(NIR) spectroscopy from 700nm to 1000nm [37], and ultrasound [52]. Of these methods, the fluorescence sensor is the most applicable to the bioreactor process.

In a recent article on sensor development [58], the following statement was made by Van Brunt assessing the role of sensors in the development process, ". . . you scale up to 10, 100, 1000 liters. You put the organisms in the tank and pray. Today we can monitor the pH, temperature, dissolved oxygen, and maybe something else, if we are lucky. You try to get things in balance with only a few parameters. We have just scratched the surface of on-line monitoring". This statement is perhaps an overly conservative estimate of the current technology, but it does show that the new sensors in bioreactor processes have not made possible the implementation of advanced control strategies. These limitations are especially evident in conjunction with the new fermentation-based products

from biotechnology.

2.1.2 General Review of Fluorescence Sensor

For the last ten years, much effort has been focused on the design of instruments capable of on-line measurement of biomass concentration and growth rate. A number of significant advances have been made in spectroscopic analyses, specifically fluorescence and infrared spectroscopy. These advances are coupled with advances in fiber optics and in computer-aided analysis. Among several approaches, the measurement of NADH-dependent culture fluorescence appears to be promising. Since the discovery by Duysens and Amesz (1957) [11] of culture fluorescence, fluorescent measurements have been attempted by several investigators. Humphrey's group at Lehigh University began using fluorometry to monitor intracellular NAD(P)H levels 20 years ago [19]. Zabriskie and Humphrey (1978) [62] showed that the fluorescence signal can be used as an estimate of biomass concentration. Meyer and Beyeler (1984) [34] developed process control strategies based on the NADH content of microbial cells. During the last ten years, two different types of fluorometers, designed for the *in situ* monitoring of microbial cells, have appeared on the market (BioChem Technology, Malvern, PA, USA and Ingold AG, Urdorf, Switzerland). Ristroph et al. [42] determined the timing of substrate feed in a fed-batch reactor by monitoring the culture fluorescence kinetics. Einsele et al. [14] used the fluorometric approach to determine mixing times in a bioreactor as well as to study the kinetics of substrate uptake. More research continues in the investigation of this approach [54, 59].

The fluorometric approach includes several drawbacks: The interpretation of an NAD(P)H fluorescence signal from the whole culture broth is complex and complicated,

depending on the medium, the oxygen level and the substrate. Also, the cells and the growth media contain a number of fluorophores, such as pyridoxine, tryptophan, and riboflavin, in addition to NAD(P)H. The behavior and fluorescence intensity of various fluorophores differ in the various fermentation systems. Applying fluorometric methods to a bioreactor is a complex installation process. Miniaturization and standardization of a fluorescence sensor must be performed in order to equip a fluorescence probe for every standard bioreactor.

2.2 Review of Bioluminescence

Bioluminescence is a natural phenomenon associated with several species, notably the firefly (*Photinus pyralis*) and marine microorganisms that consist of four major genera: *Vibrio, Photobacterium, Alteromonas,* and *Xenorhabdus.* Bioluminescence has intrigued people over the centuries with its unique beauty, although the practical applications of this naturally occurring phenomenon have only been recently developed.

A fascinating account of early work is reported in [61] which refers the monograph of Harvey [20]. The first systematic studies on bioluminescence were done by Robert Boyle in 1667, who observed that the bioluminescence of some species of fungi required the presence of air and described the dimming of "shining wood" during evacuation of its container. In the last few years, significant advances have been made in cloning the genes from the bioluminescence operons (*lux* gene cassette) of a number of marine bacteria [2, 5, 15, 36] into other host cells. The specific functions of the polypeptide products of the structural genes (*lux* A-E) have been identified [33] (See Table 2.1.): the *lux*A and B genes code for the α and β subunits of the enzyme *luciferase*; and the *lux* C,

D and E genes code for the polypeptides of the fatty acid reductase system, responsible for the synthesis of the fatty aldehyde substrate for the luciferase catalyzed reaction [8] [23] [17]. (See Figure 2.1.)

Table 2.1: Enzymes and genes from the bioluminescence systems (Source Meighen, E.A., *Ann. Rev. Microbiology.* 1988)

Gene	*Polypeptide*	*Mass(kd)*	*Functions*
luxA	*Luciferase α subunit*	40 – 44	*Catalyzes light reaction :*
luxB	*Luciferase β subunit*	35 – 40	$FMNH_2 + O_2 + aldehyde$
			$\rightarrow\rightarrow$ *light*
luxC	*reductase*	53 – 58	*NADPH – dependent reduction of*
			fatty acyl groups of aldehyde
luxD	*Acyl – transferase*	32 – 34	*Generation of fatty acids for*
			the luminescent system
luxE	*Synthetase*	42 – 50	*ATP – dependent activation of*
			fatty acids

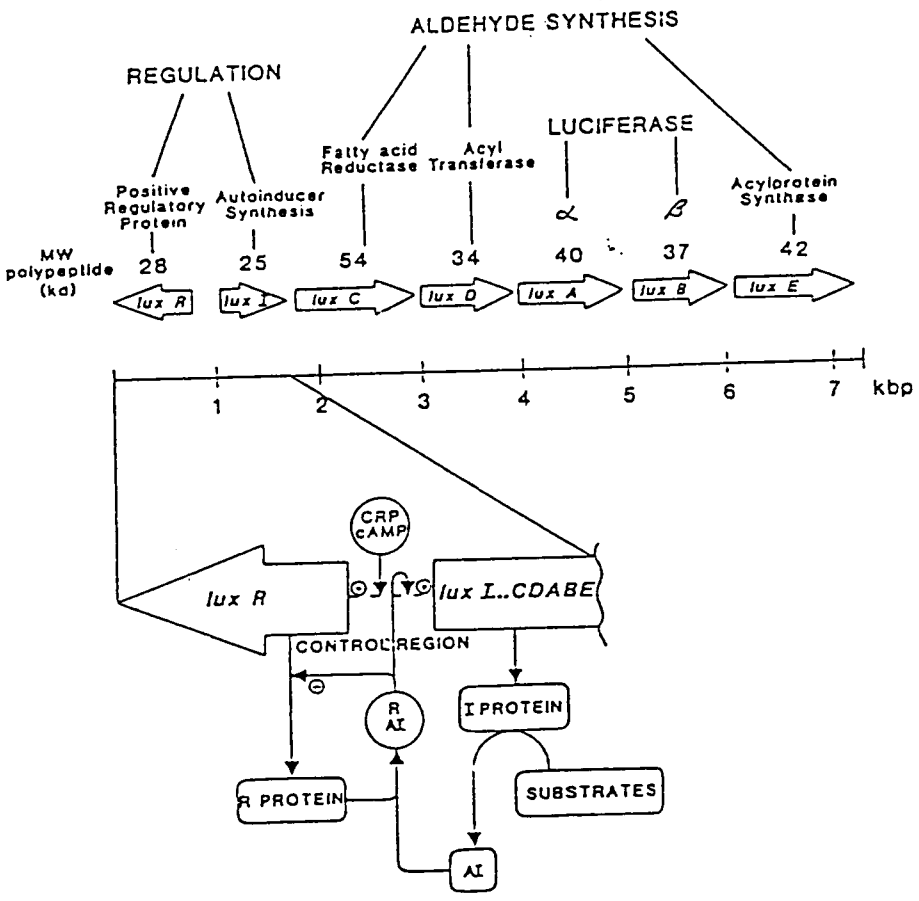


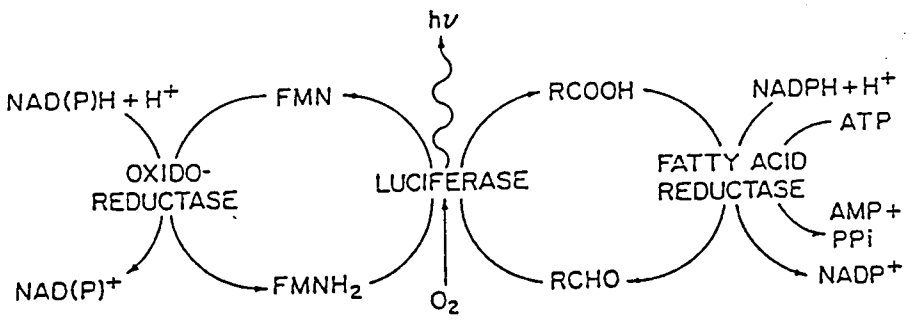
Figure 2.1: Regulation system and physical map of the *lux* regulon of *Vibrio fischeri* strain. (From Baldwin, T.O., *J. of Bioluminescence & Chemiluminescence.* Vol. 4, p327)

2.2.1 Bioluminescence Reaction

Luciferase, a mixed function oxidase consisting of α and β units, catalyzes the light emission of the *lux* system. In bioluminescent bacteria, luciferase catalyzes the oxidation of a long-chain aldehyde and $FMNH_2$ (flavin mononucleotide, reduced form), resulting in the emission of visible blue-green light. The reactions are presented below, and the details are shown in Figure 2.2.

$$R-CHO + FMNH_2 + O_2 \xrightarrow{LuxAB} R-COOH + FMN + H_2O + h\nu$$

$$R-CHO + NADP + AMP + PPi + H_2O \xleftarrow{luxCDE} R-COOH + NADPH_2 + ATP$$



(From Showalter, R.E. J. of Bacterial, June 1990, p2947)

Figure 2.2: Components of the bioluminescence reactions in *Vibrio fischeri*. R-CHO - a long chain aldehyde substrate; RCOOH - a long chain fatty acid, hν refers to light.

In fireflies, luciferase requires the luciferin compound along with O_2 and ATP. The bacterial system requires an aldehyde substrate, $FMNH_2$ and O_2. While both the luciferin and the aldehyde substrate are relatively toxic and must be used with care, the aldehyde crosses cell membranes more easily than the luciferin, and thus can be used in smaller amounts in *in vivo* experiments.

Bioluminescent bacteria emit light under regulated conditions, and at different intensities depending upon the specific cellular content of the luciferase and substrates driving the reactions. Light production by most species of luminous bacteria is strongly influenced by the density of the cell culture. The light emission per cell can be as much as 1000-fold higher in dense cultures than in dilute cultures. Density-dependent regulation of luminescence has been investigated most thoroughly with *Vibrio fischeri* [12]. The research confirmed the presence of both an inhibitor in the fresh complete medium and an activator in the conditioned minimal medium. Luciferase synthesis is repressed when an exponentially growing and brightly bioluminescent culture is inoculated into a fresh medium, and the induction does not take place until the culture has reached a certain cell density level. Kempner and Hanson [25] showed that a conditioned medium that shows no lag in light production can be prepared by removing the bacteria from a culture in which induction has taken place, and the condition is caused by the removal of an inhibitor contained in a fresh complete medium. Nealson [15] observed that in a minimal medium an activator which leads to the induction of the luciferase enzyme is released into the medium by the bacteria, and this compound is responsible for the bioluminescence. They described this compound as an "autoinducer". The autoinducer for the *V. fischeri* luminescence system is N-β-ketocaproylhomoserine lactone [13]. The enzyme function responsible for synthesis of the autoinducer of *V. fischeri* has not been

identified.

2.2.2 Regulation of the *lux* Operons

The *lux* system of *V. fischeri* is under the control of a complex regulatory network, the general features of which have been established by the efforts of Engebrecht and Silverman [15, 17]. The regulon consists of two divergently transcribed operons and at least five structural and two regulatory genes . The leftward operon ($operon_L$) has only a single gene, *lux*R, which encodes a positive regulatory protein. The rightward operon ($operon_R$) has at least six genes (*lux*ICDABE) and encodes at least six polypeptides. The products of *lux*R and *lux*I appear to function together to stimulate transcription of $operon_R$. *lux*I product is required to synthesize the autoinduction compound. Transcription of $operon_L$ is stimulated by cAMP-CRP(cAMP receptor protein), and the $operon_L$ itself appears to be under the control of a complex autoregulatory process. In addition to these complex autoregulatory loops, the *lux* system appears to be modulated by O_2 levels and possibly by *htp*R(σ^{32}) [33].

Induction will occur only if specific conditions are met. First, the cell density must be sufficiently high for the local concentration of the autoinducer to be adequate for binding to the *luxR* protein. Second, the growth condition must be such that the cells have elevated levels of cAMP. Either condition can be present individually without causing induction; for induction of bioluminescence to occur, both conditions must be satisfied [10]. The details of the role of *lux*I protein are not clear. From the literature, the *lux* regulon is the only regulatory system in which an end-product causes positive feedback via an induced transcriptional activation.

V. harveyi bioluminescence is partially controlled by catabolite repression. The ex-

pression of the luciferase and fatty acid reductase is decreased by adding glucose, and their functions could be restored by the addition of cAMP. In *V. fischeri*, catabolite repression by glucose had been observed in phosphate-limited chemostat culture [18]. In *E.coli* with the *V. fischeri* lux system, it was found to be necessary to have cAMP and CRP for high expression of the left operon (containing *luxR*) [46]. At the same time, the right operon was inhibited in the absence of the *lux*R gene product. This may explain why the repression by glucose can be overcome by both cAMP and the autoinducer.

2.2.3 pUTK4 Plasmid Construction

The strain of bacteria RB1151 used in this research is a DH5α strain of *E. coli* containing the pUTK4 plasmid, constructed by Robert Burlage at the Center for Environmental Biotechnology of the University of Tennessee at Knoxville. The pUTK4 plasmid was created using the pUCD615 *lux* fusion plasmid that was first made by Clarence Kado of the University of California, Davis [44] [47], and the pUC18 plasmid, generally used as a *lacZ* fusion plasmid. The pUC18 plasmid contains the *lac* promoter, which is a strong promoter when derepressed. The construction of pUTK4 is shown in Figure 2.3.

In the RB1151 strain, the *lac* promoter is always derepressed. The pUTK4 plasmid contains a large deletion in the *lac* operon and has no *lacI* repressor gene, so RB1151 is a constitutive light-producing strain. It has a fragment of the *lac*Z gene left, which is necessary for α-complementation of *lacZ* fusions. Both pUCD615 and pUC18 plasmids were cut with EcoRI enzyme, and the pUCD615 plasmid was dephosphorylated with calf intestinal alkaline phosphatase. These two plasmids were then ligated with T4 DNA ligase, followed by transformation into DH5α. Selection was on LB agar with ampicillin. The light-producing colonies were easily seen after the plates were cooled down from

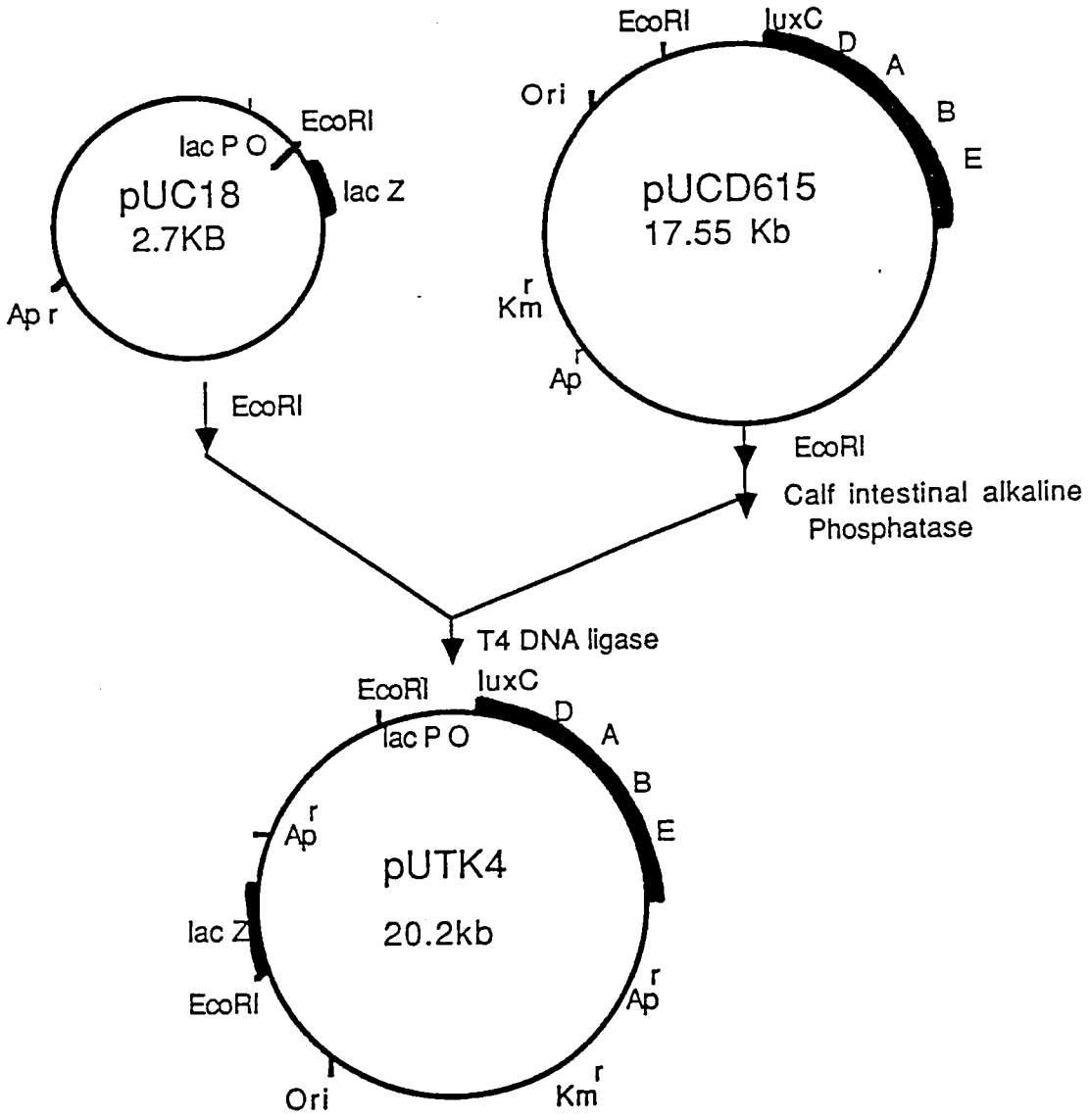


Figure 2.3: Construction of bioluminescent plasmid pUTK4, including the cloning strategy. Km^r denotes Kanamycin resistence; Ap^r denotes Ampicillin resistence; ori denotes origin of replication.

37°C to 25°C. The orientation of the pUC18 insert was ascertained by using restriction enzyme digestion and agarose gel electrophoresis. The plasmid contains two origins of replication (*ori*).

The pUCD615 plasmid contains a promoterless *lux* operon which has multiple cloning sites located at approximately 600 base pairs upstream of the start codon of *lux*C. Unique restriction sites for *Bam*HI, *Eco*RI, *Sma*I and *Xba*I, as well as two closely linked *Sal*I sites allow the cloning of a large variety of promoter-active fragments. Thus pUCD615 was designed to fuse a promoter of interest to the bacterial bioluminescence genes (*lux*), which allowed the study of promoter activity in a variety of bacterial species.

The *lux* system in the strain RB1151 consists of the structural genes of *lux*CDABE and has no regulatory genes *lux*R and *lux*I. So it is a constitutive strain, in that it does not require the autoinducer to initiate the bioluminescence reaction.

2.2.4 Previous Work with Bioluminescent Recombinant Clones

Several investigators ([5, 26]) have used the *lux* genes from *V. harveyi* in their genetic fusions and were successful in producing light in such disparate biological systems as *E.coli, Saccharomyces cerevisiae, Nicotiana plumbaginifolia* leaf protoplasts, and a lysate of rabbit reticulocytes. They used a monocistronic *lux*AB gene generated by site-specific mutagenesis. This gene created a luciferase that is a single gene product. So it can express luciferase under the control of a single promoter in eukaryotes as well as in prokaryotes. These groups have demonstrated the potential applicability of the fused bacterial luciferase gene as a reporter for genes expressible in both prokaryotic and eukaryotic systems. Kirchner et al. [26] also used *lux*A and *lux*B gene fusion, wherein they extend the scope of application of the bacterial luciferase system by making it more

convenient to use in eukaryotes. Kado's group (at the University of California, Davis [47] [48] [49]) cloned genes of *V. fischeri* into *E.coli.* They used bioluminescence as a marker for inactivation in cloning to compare the relative strengths of different promoters, to estimate bacterial number, and to follow the spread of bacteria in plants. In contrast, bacterial luciferase has been expressed in plants only by placing the *lux*A and *lux*B genes downstream of two separate eukaryotic promoter elements [28]. Similarly, the expression of firefly luciferase has been widely applied in a variety of eukaryotic organisms including plants, yeast, monkey cells (CV-1 cells), and transgenic mice [38] [39].

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 [29]. If such a correlation can indeed be established, it has the potential to be used as an on-line sensor to provide information on biomass concentration during bioreactor operations. Of course, it is recognized that initial cloning work is required to introduce the *lux* gene cassette into the host cell of interest. The cloning work involved in introducing the *lux* gene may not prove to inhibit the increasing employment of recombinant cells in the production of pharmaceutical, food, and agricultural products. If successful, the increased profit margin derived from using such on-line information may offset the added cost.

CHAPTER 3

EXPERIMENTAL SETUP AND METHODS

3.1 Experimental Setup

All studies were carried out batchwise in a 3-litre (working volume 1.8-litre), fully instrumented bioreactor, manufactured by Applikon of Foster City, California. The instrumentation has the capability of controlling pH, temperature, dissolved oxygen and foam through simple feedback control loops. For the purpose of data acquisition and future process control implementation, the bioreactor was interfaced to a Sun workstation. The diagram of experimental set up is presented in Figure 3.1. On-line data acquisition at one minute intervals of light intensity, pH, dissolved oxygen level, and flow rate started after inoculation. A 5-ml biomass sample was taken for off-line Klett unit measurements at 15 to 30 minute intervals during exponential growth phase, and less frequently afterwards.

3.2 Materials and Methods

Bacterial strain: The *E.coli* strain RB1151 used here is *E. coli* DH5α (*rec lac* Nal^r, Bethestha Research Laboratory). It contains the pUTK4 plasmid, constructed by Robert Burlage of the Center for Environmental Biotechnology at the University of Tennessee, Knoxville.

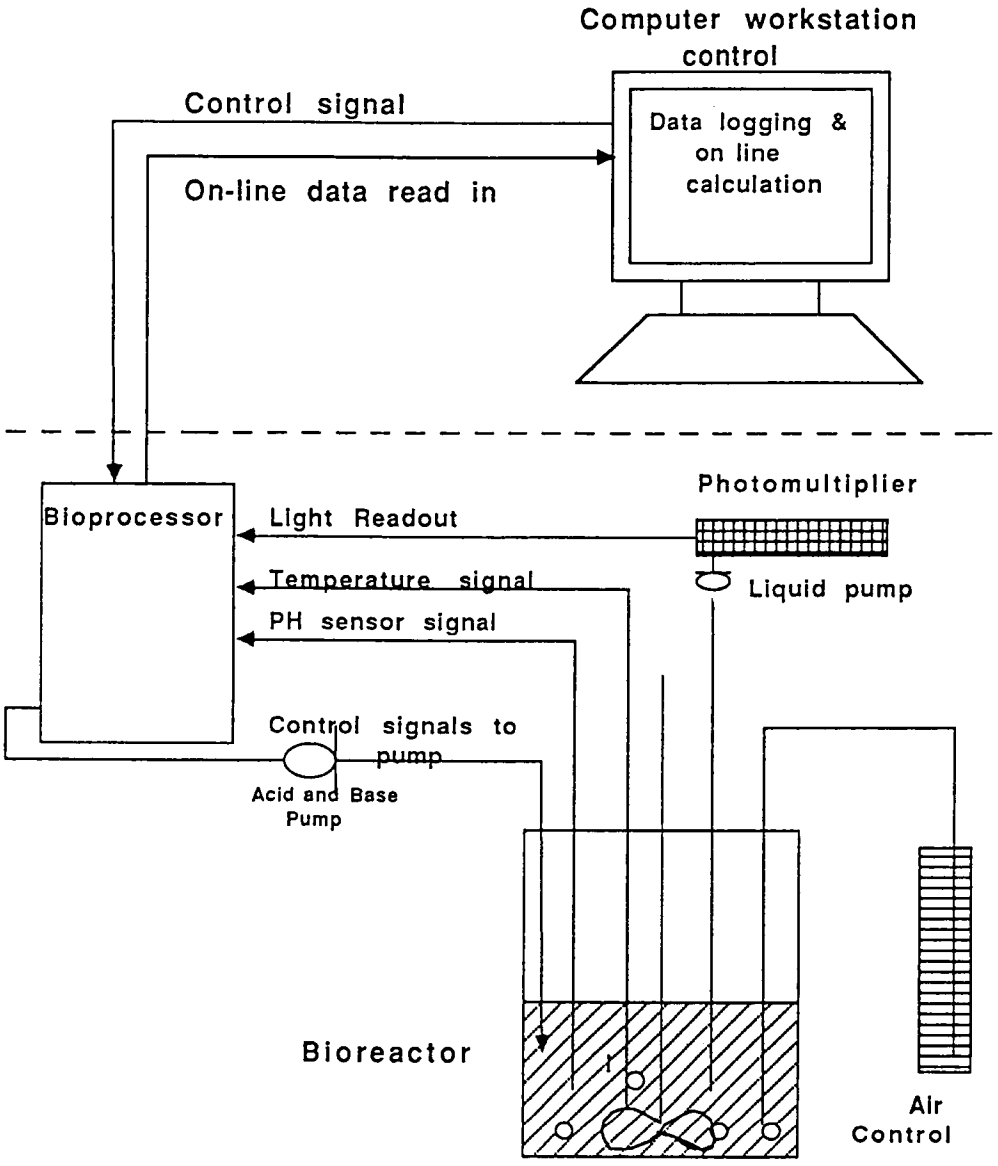


Figure 3.1: Diagram of bioreactor, controller and computer analysis system

Medium: LB (Luria-Bertani) medium was used for all 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. pH was adjusted to 7.2 by using 1 N NaOH solution.

Growth conditions: Overnight RB1151 cultures were grown in LB broth in 250-ml flasks, in a 26°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., New York).

Operating parameters: Each batch experiment was performed in a 3-litre bioreactor (Applikon Inc.) at an initial broth volume of 1.5 litre. A 75-ml exponential phase growing culture (at 100 Klett units) was used to inoculate the sterilized LB medium at 1:20 dilution. During growth, temperature was maintained at 26°C, pH at 7.2, and air flow at 1-10 LM (l/min); impeller speed ranges from 300 to 800 rpm (under computer control). The dissolved oxygen level was controlled by a simple feedback controller loop through the bioprocessor to be at 80% or above of saturation level. The dissolved oxygen level was sensed by a dissolved oxygen probe (the auxiliaries of the applikon bioreactor), which in turn sent the information to the bioprocessor to be compared to the desired setpoint. A low dissolved oxygen level was compensated first by increasing the impeller stirring speed up to a specified allowable maximum rate. If the desired dissolved oxygen level was still not met after the impeller speed had been increased to the maximum allowable maximum speed, for instance, during the cell exponential growth phase, then the bioprocessor increased the flow rate of the inlet air. The built-in air mass flow controller allows up to 5 l/min flow of air. When the demand for oxygen called for more flow, a manually controlled additional air flow was introduced, until the dissolved oxygen

level was at or above the desired setpoint level. A typical batch run was continued for a period of 24 hours, well beyond the time when stationary phase of the culture was attained. Intermittent sampling of the cell density (Klett units) was performed using aseptic techniques.

Data analysis software: All numerical and simulation work was carried out with the software Matlab [32] on a Sun workstation. The computer programs are documented in the appendix.

3.3 Biomass Measurement

Off-line biomass measurement provides a baseline against which on-line bioluminescence is to be correlated, several methods were used for the off-line measurement of the biomass. The methods employed include:

- **Light scattering measurement by a Klett meter.** This method provides a convenient means of measuring growth. Since the linearity range of a Klett meter is larger than that of a spectrophotometer (which measures the absorbance of light), this approach reduces the error of inaccuracy at high cell concentrations.

- **Light absorbance measured by spectrophotometer.** Measurement was at 660nm. However, the cell culture had to be diluted after reaching an OD of 0.5, in order to stay within the linear response range.

- **Dry weight.** This measurement was less accurate than those made by OD measurement. However, the dry weight approach is the standard biomass measurement approach, and is frequently used to follow the growth process.

- **Direct cell count.** Cell count using a Petroff-Hausser cell counter in a microscope was carried out. This approach was tedious, and large numbers of bacteria cells had to be counted in order to provide a statistically significant result.

3.4 Light Detection System

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

CHAPTER 4

EXPERIMENTAL RESULTS AND DATA ANALYSIS

4.1 Experimental Results

The feasibility of using the light emission of bioluminescent bacteria cells as a sensitive sensor for biomass concentration in a bioreactor was explored. Stabilization of light production by the bioluminescent *E. coli* recombinant clones was first accomplished by searching for the optimal environmental growth conditions.

4.1.1 Biomass Measurement

The correlations between cell biomass, as measured by Klett meter, optical density, dry weight, and cell numbers, are shown in Figures 4.1, 4.2, and 4.3. Reproducible biomass growth curves, using the same inoculum size, were obtained for batch runs to provide baseline information for correlation work to be performed later.

4.1.2 Control of Cell Inoculum

The initial inoculum size for a batch bioreactor run was standardized in order to obtain reproducible and consistent growth curves. In addition, environmental conditions, such as temperature, pH, dissolved oxygen, and various nutrient concentration levels, were all maintained at constant levels, so that they did not affect the metabolic states and the relative bioluminescence level.

Figure 4.4 shows the growth kinetics with different inoculum size at the specified

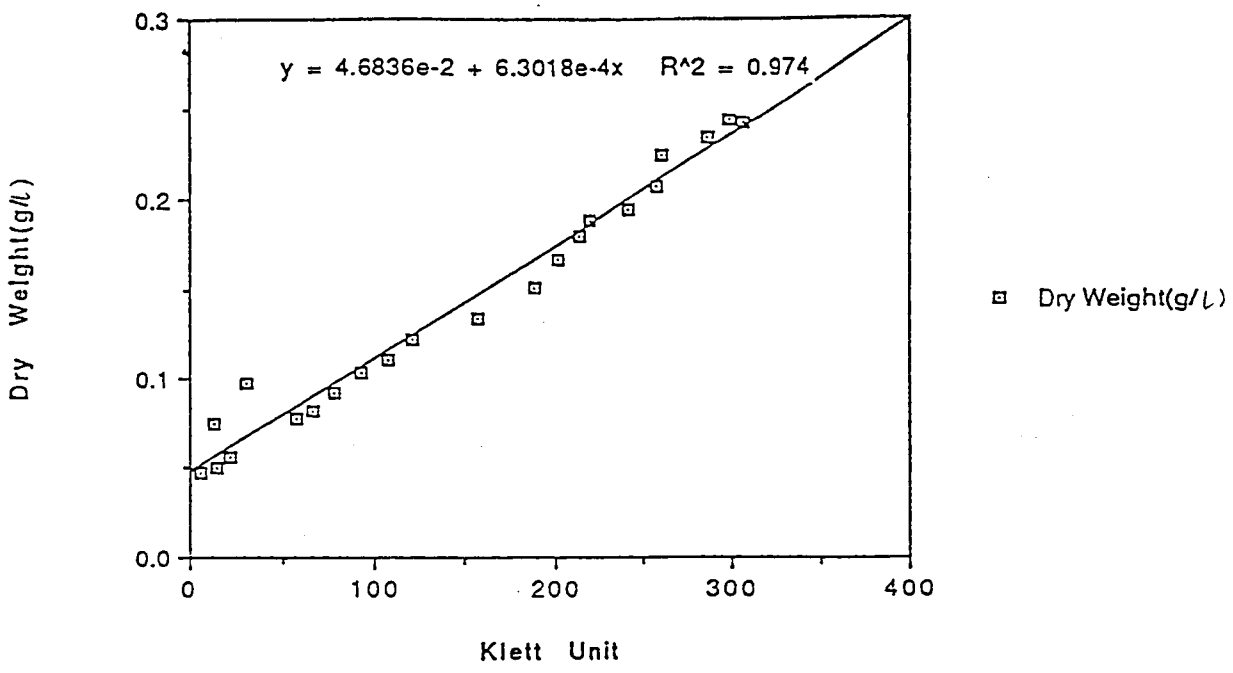


Figure 4.1: Dry weight correlated with Klett unit of *E. coli* RB1151.

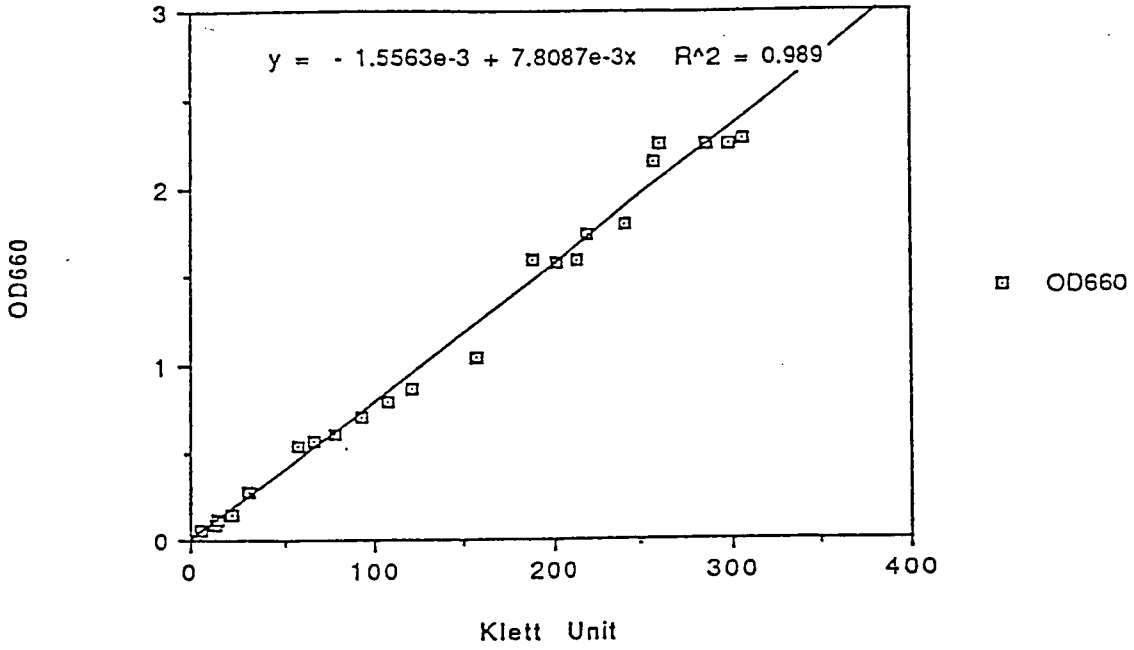


Figure 4.2: OD correlated with Klett unit of *E. coli* RB1151.

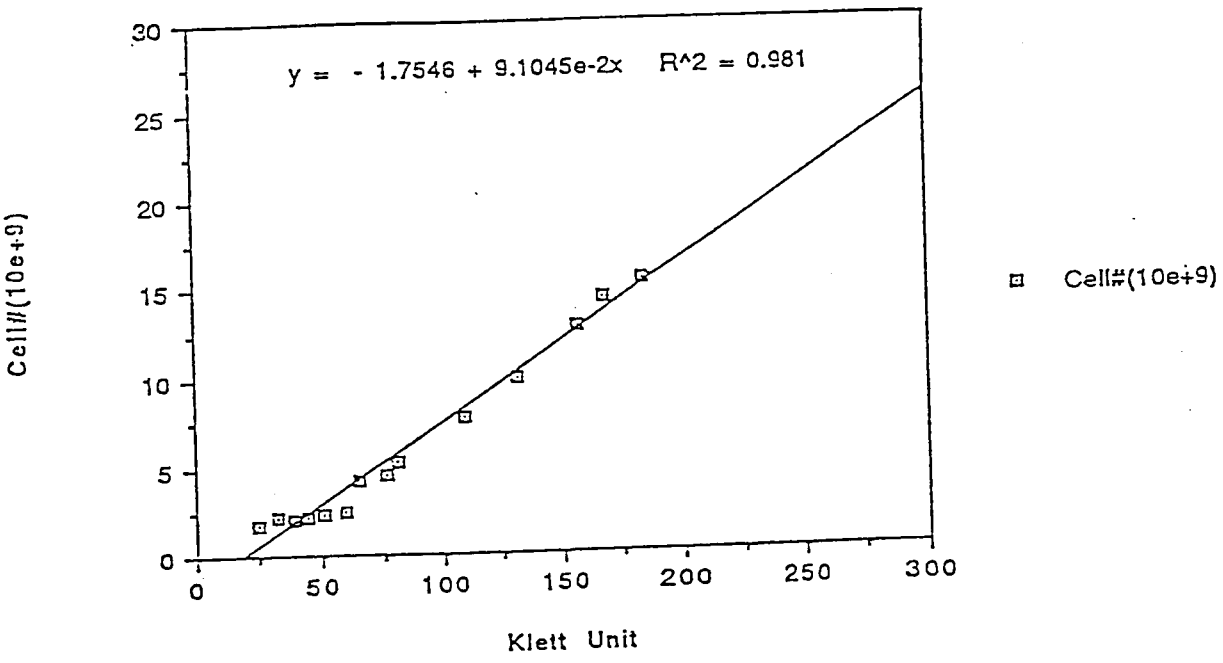


Figure 4.3: The relationship between direct count cell number and OD of *E. coli* R-B1151. A serial dilution was made in complete LB medium and OD was measured by spectrophotometer at 660 nm.

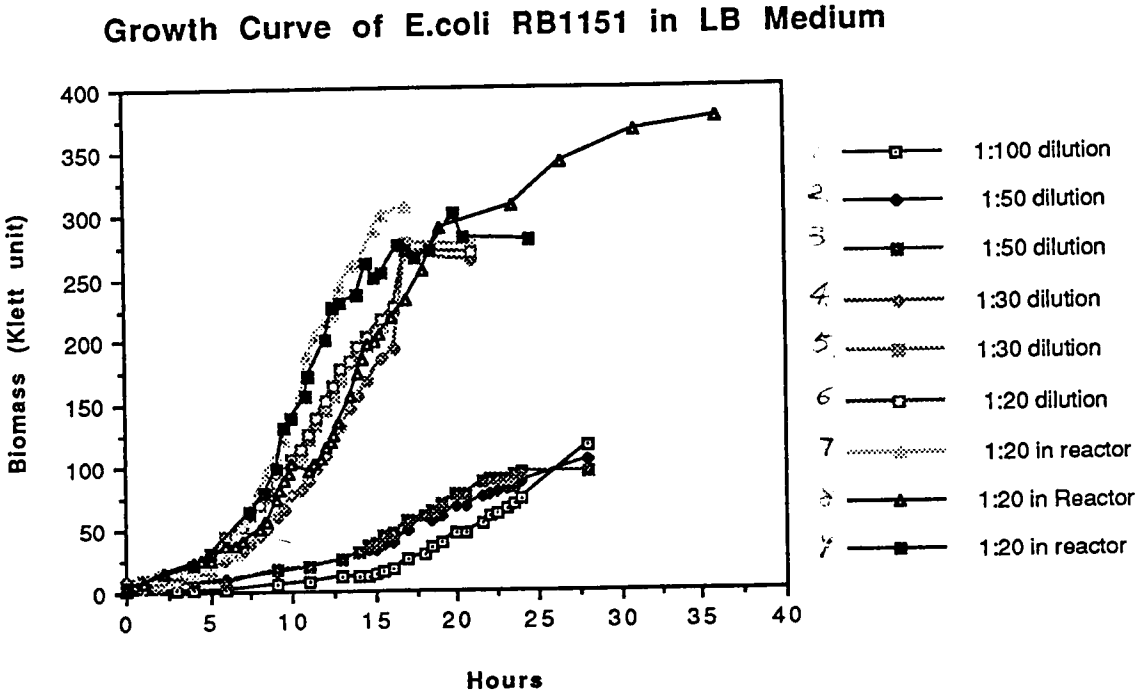


Figure 4.4: Bacteria growth at different inoculum. The recombinant *E. coli* RB1151 cells were grown at 26°*C* in LB containing 50 μg/ml ampicillin in shaking flask or 1.5-litre bioreactor. Aliquotes were withdrawn at the time indicated and measured for turbidity (Klett units). Curve 1 to 6 were carried out in 300-ml flasks, curve 7,8,9 were in 1.5-litre bioreactor.

growth conditions. *E.coli* RB1151 cells were grown at $26°C$ in LB broth containing ampicillin (50 mg/l). Curves 1, 2, and 3 show the growth curves of the cells in a 300-ml shaking Nephelo flask, with the low inoculum at a 1:100 or 1:50 dilution in an overnight culture (100 Klett unit). Curves 4, 5, and 6 show the growth of cells in a 300-ml shaking Nephelo flask, with a 1:20 to 1:30 dilution inoculum. Curves 7, 8, and 9 show the growth curves of cells grown in 1.5 litre of culture broth in a bioreactor with 75-ml inoculum (1:20 dilution). The operating conditions were maintained at constant, with on-line feedback control of temperature, pH, and dissolved oxygen. As can be seen from the figure, constant dilution inocula resulted in consistent growth curves.

To facilitate quantitative comparisons, the inoculum preparation steps for each bioreactor run were fixed. First, overnight *E. coli* RB1151 cultures in LB broth in 300-ml flasks were prepared from one or two glowing colonies on a LB agar plate. The 300-ml flasks were kept in a water bath, with constant shaking at 250 rpm and antibiotic selection. Cell growth was monitored by a Klett meter until it reached 100 Klett Units. Then a 75-ml inoculum of this culture was added to 1.5 litres of sterile LB medium in the bioreactor. Figure 4.5 shows that growth follows the same kinetics using these controlled inoculum preparation steps. However, the relative levels of the corresponding light kinetics appear to be more variable from batch to batch, as shown in Figure 4.6.

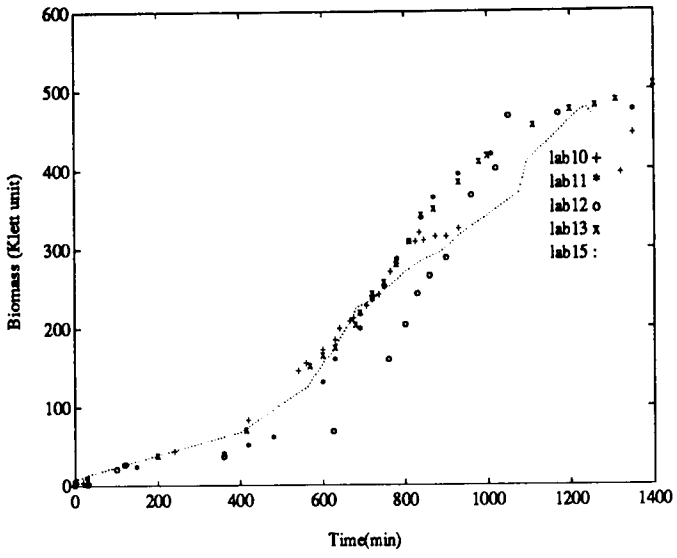


Figure 4.5: *E. coli* growth curves of batch runs.

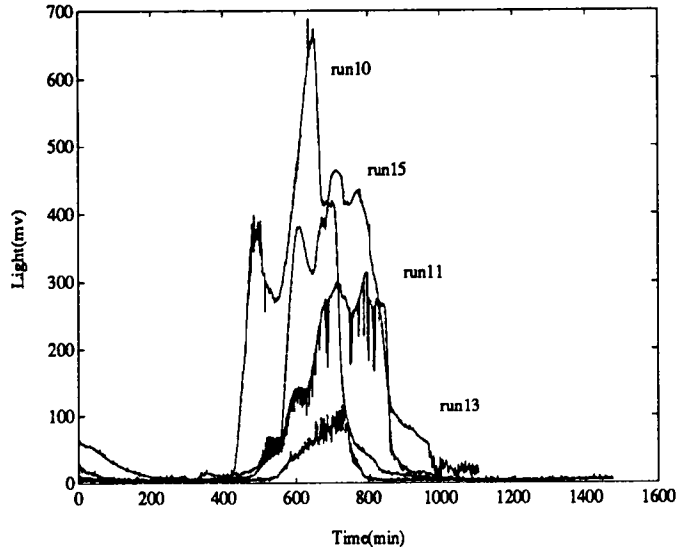


Figure 4.6: *E. coli* bioluminescence profiles of four batch runs.

4.1.3 Control of Operating Parameters

Environmental conditions greatly affect the light emission of bioluminescent bacteria. During cell growth, the physical parameters, such as temperature, pH, and dissolved oxygen, were controlled at their optimal values. The reactor operating parameters for all experiments are presented in Table 4.1.

Table 4.1: Operating parameters of a bioreactor for batch runs of *E.coli* RB1151.

OperatingParameters	*Setpoint*
Agitation	Min : 300 rpm
	Max : 800 rpm
Air Flow	0.5 – 10 l/min
Temperature	26°C
pH	7.2
Dissolved Oxygen	*above* 80%

Dissolved Oxygen

The control of dissolved oxygen can be observed from Figure 4.7. During operation, both oxygen and substrate depletion had an immediate effect on the level of bioluminescence.

Figure 4.8 shows one of the earlier experiments, in which dissolved O_2 was normally controlled only at 50% of saturation level. An early dissolved O_2 drop caused a sharp

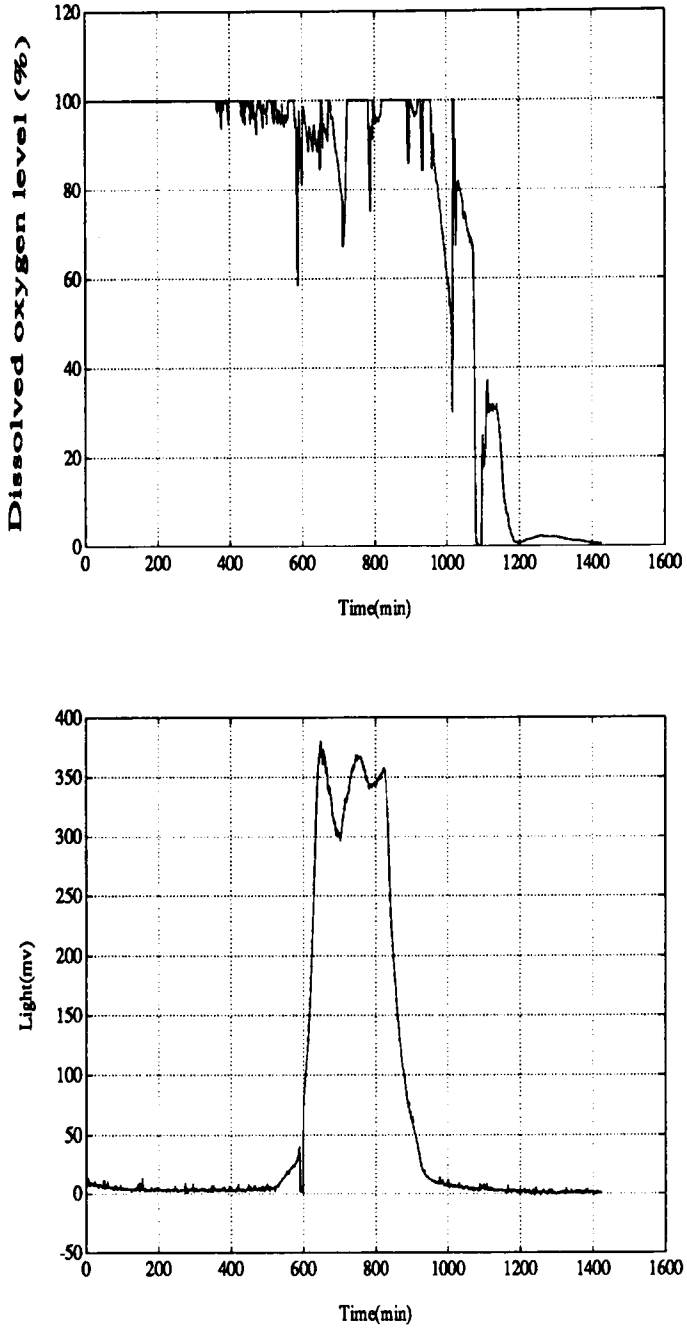


Figure 4.7: Effect of dissolved O_2 level on cell bioluminescence for the batch run #lab18.

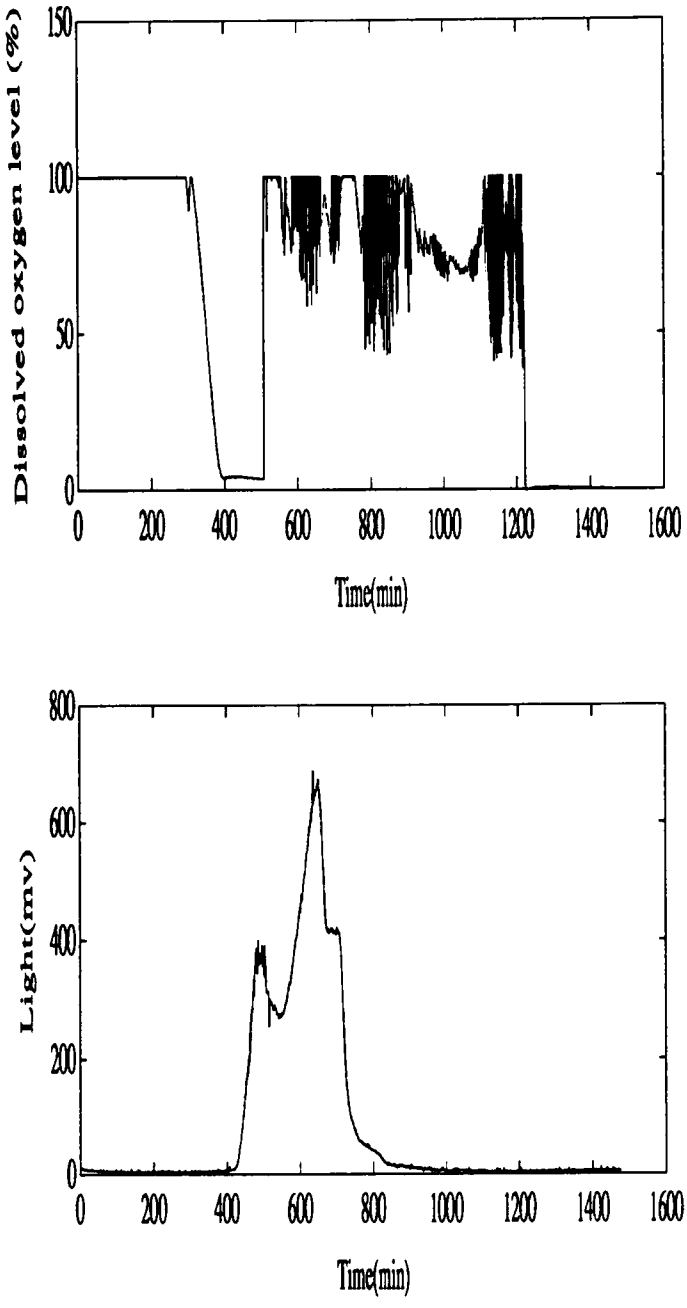


Figure 4.8: Effect of dissolved O_2 level on cell bioluminescence for the batch run #lab10.

drop in the level of light intensity. As can be seen from Figure 4.9, with an increase in cell biomass more O_2 was correspondingly required, especially during the exponential growth phase. The maximum value of automatic air flow rate allowed by the bioprocessor mass flow controller was only 5 l/min, while the required air flow rate could reach as high as 10 l/min when the light intensity of cells was at its peak value. Manual control of the air flow rate was carried out when demand for air flow exceeded 5 l/min. Due to this careful, additional, manual control of dissolved O_2, full luciferase activity was ensured. Even with good oxygen control, a brief drop to a 50% dissolved oxygen level for a few minutes would adversely affect the light intensity during the period of peak oxygen demand. This is shown in Figure 4.10.

Temperature and pH

As indicated above, the functioning of the *lux* system gene products derived from *V. fischeri* was temperature limited, and did not function at temperatures above $33°C$. Some researchers have used $28°C$ when working with *E.coli* harboring *lux* genes and encountered no problems. Low temperature growth can ensure good light emission for poorly bioluminescent cells, but a compromise has to be made to allow for a reasonable doubling time, so that cultures can be grown within acceptable time frames. We chose to work at $26°C$ in our experiments. From our observations, the bacteria can be grown at any reasonable temperature below $37°C$, and can be cooled down to room temperature for one or two generations prior to light measurement. For the sake of consistency, a constant temperature was maintained. A temperature difference of $\pm$ $1°C$ may cause undesirable, significant changes in light production. To achieve stable light emission, we also controlled the pH at 7.2 to keep cells in optimal growth condition and to maintain

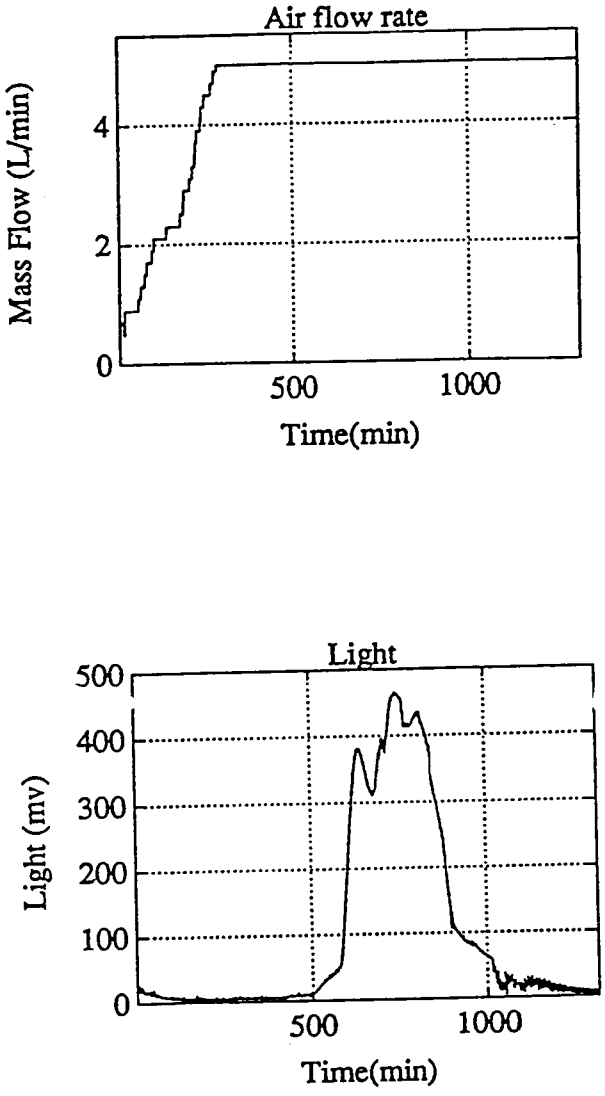


Figure 4.9: Increased air flow rate requirement to maintain adequate dissolved oxygen level for ensuring full bioluminescence

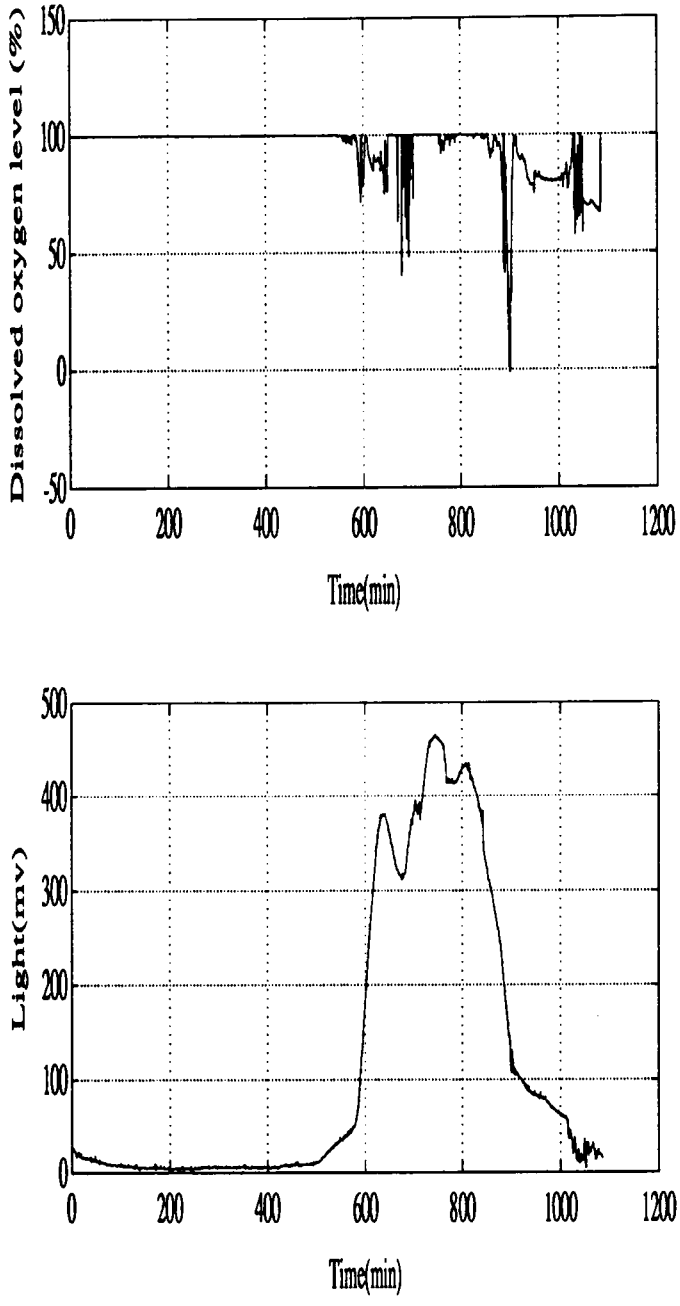


Figure 4.10: Effect of dissolved O_2 level on cell bioluminescence for batch run #lab15.

stable luciferase activity. A fluctuation of ± 0.5 pH may also cause changes in the bioluminescence behavior. Figure 4.11 shows the control of pH and temperature during a typical batch run of 24 hrs.

4.1.4 Bioluminescence Measurement

Cell mass and bioluminescent intensity information were monitored and recorded over a period of about 24 hrs during batch runs of *E. coli* strain RB1151. A total of 12 runs were carried out. We expected to obtain consistent bioluminescence profiles after we attained tight control over the operation parameters. The light output signal turned out to be very sensitive to both the temperature and dissolved oxygen levels, but more so to the latter. The saturation level of dissolved oxygen required for consistent bioluminescence profiles was explored. It was found that 80% or greater level of dissolved oxygen in the bioreactor is required to maintain consistent light emission (See Figure 4.7).

The growth dynamic profiles were in close proximity to each other, indicating an achievement of tight control of the biomass concentrations from batch to batch. Figure 4.12 shows the bioluminescence profiles corresponding to the growth curves of Figure 4.13.

Figure 4.14 is a typical light emission profile superimposed onto the corresponding growth curve. In contrast to the monotonic increase of biomass concentration as a function of time, light emission starts from a low level, then goes through a maximum, followed by a decline phase. Toward the late log phase, it was evident that only a very low level of light was observed, in spite of the fact that biomass concentration was reaching maximum.

Several general characteristics shared by all the light emission profiles from the twelve

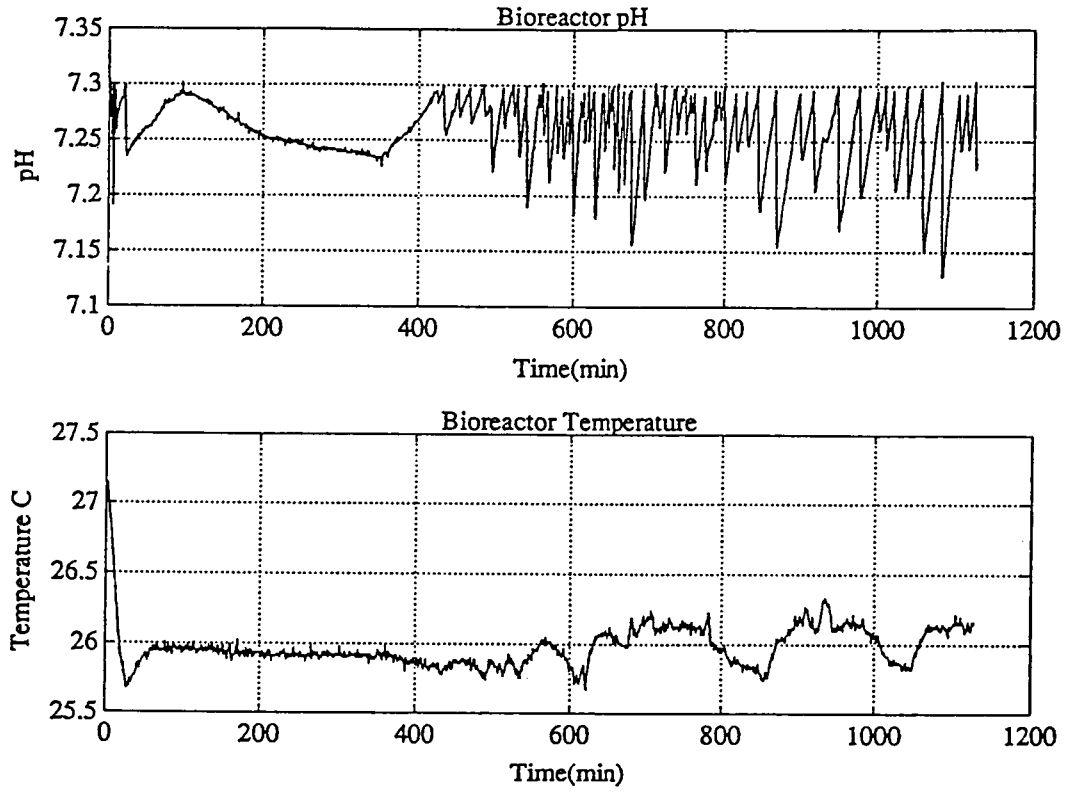


Figure 4.11: On-line feedback control of pH and temperature at 7.3 and 26°C respectively

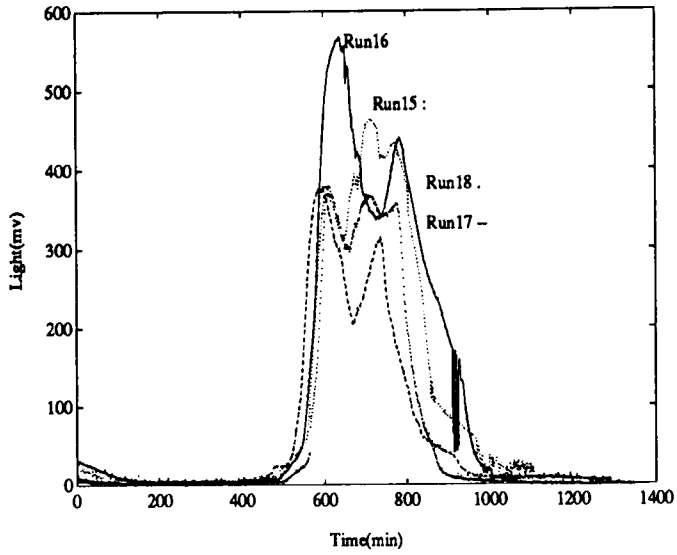


Figure 4.12: Bioluminescence comparisons under identical operating conditions of three batch runs.

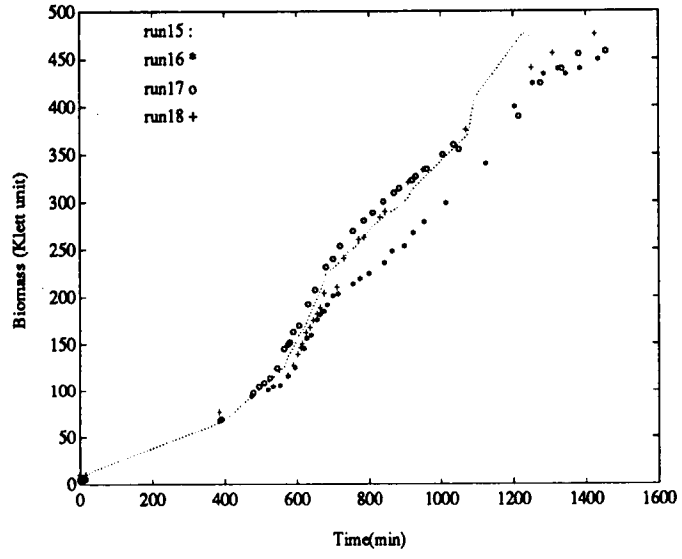


Figure 4.13: Growth Curve for *E.coli* RB1151 at pH=7.2, Temp=26.0.

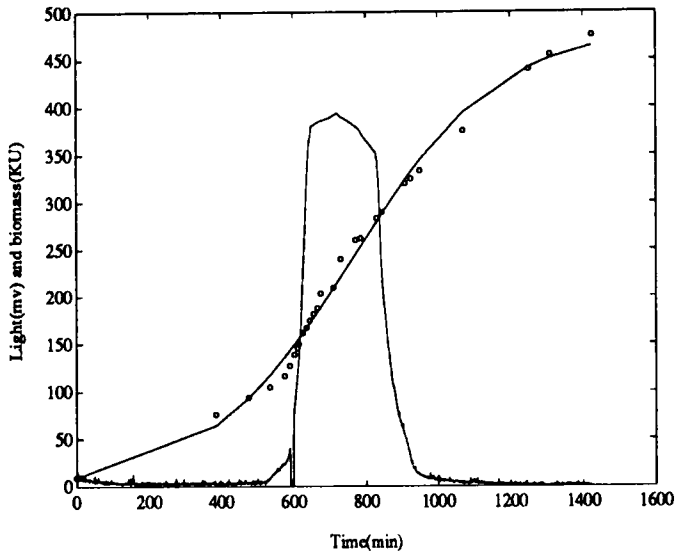


Figure 4.14: Growth and bioluminescence kinetics of *E. coli* RB1151 grown in LB medium with ampicillin. Growth is measured in Klett units (open circle) and light intensity as millivolts. Circles indicate growth and the solid line indicates light emission.

experiments were also evident. The detailed data for the twelve batch runs is summarized in Table 4.2. Several general qualitative conclusions can be drawn from data on the batch runs:

1. The time of the luminescence peak corresponded closely to the time of the maximum growth rate.

2. The overall time to the luminescence peak after inoculation, as well as the total period of observable light emission, were relatively constant among the batch runs, reflecting the constant duration of the period of the exponential growth phase.

3. There was a lag period following inoculation, before the onset of light emission, suggesting the requirement for a threshold level of biomass for light emission.

4. Dips or valleys were seen during the period of maximum light emission, if the level of dissolved oxygen droped below about 60%, indicating the extreme sensitivity of the bioluminescence to the requirement for oxygen.

5. High variability of the maximum light intensity level, as measured by the photomultiplier, was observed among the twelve experiments.

Conclusion (5.) precluded us from getting a consistent numerical correlation between the amount of biomass and the corresponding light emission, even though consistent biomass growth was obtained. Qualitative correlation does evidently exist between the kinetics of biomass growth and that of light emission during the batch growth. Point (1.) above implies that the phenomenon of light emission is associated with the **rate** of growth, not with the absolute level of biomass present. In all twelve experiments, the maximum light

Table 4.2: Summary of light emission profiles of eleven batch runs of *E. coli* RB1151 strain.

Run No.	Time to Peak (min)	Peak Height (mv)	Width to left half height (min)	Time (min) to commence of light	Period of detectable light(min)	Biomass (ku) at which light begins	Light Peak(mv) at maximum growth rate
Lab18	600	389		450	545	102	yes
Lab17	590	390		450	545	90-98	not sure
Lab16	685	570	100	430	560	90-95	not sure
Lab15	711	464.75	130	400-480	520-600	120-140	not sure
Lab 13	728	116.96	100	400-480	480-520	75-100	yes
Lab 12	750-780	312	94.1				yes
Lab 11	796	314.45	122.2	420-430	590-610	75-80	yes
lab 10	637	687.38	145	400-420	580-600	80-90	yes
Lab 9	630-670			450-480	480-540	125	yes
Lab 8	789	600	93.8	430-450	540-600	60-80	yes
Lab 7	690-740	161.7	130	400-480	480-560	80-100	
Mean	733.7		116.4	437	550	95.1	
Standard Deviation	50.7		19.2	19.1	37	21	

lab18-lab7 power supply was at 500 volts.

emission peak occurs in the neighborhood of the point of maximum growth rate, at the point of inflection of the growth curve.

4.2 Data Analysis

Several approaches based on experimental data were tried in an attempt to correlate the cell growth and bioluminescence kinetics. Due to the inconsistencies in the emitted light intensity from batch to batch, a quantitative correlation model that is based directly on the data obtained cannot be established. However, since such a strong qualitative correlation does exist, we surmise that a consistent quantitative correlation might emerge if one removed the high variability of the light emission profiles from batch to batch by normalizing them, such that the maximum is always scaled to 1. The motivation for this approach is that we were not sure if the inconsistent light emission profile is an artifact, due to inadequate control over the instrumentation, or if it is inherent. If, after normalization, a consistent numerical correlation does emerge, then we may possibly conclude that the instrumentation procedures need to be improved to remove the high degree of light emission variation from batch to batch. The details of such analysis are shown in the next section.

4.2.1 Derivative Analysis

The general observation from the above batch runs shows that the light intensity reaches a maximum level when the cells are in the mid-log phase. (See Figure 4.14.) It suggests that we can take the first derivative of the growth kinetics to obtain the rate profile to see if the light intensity is growth rate related. Figure 4.15 shows a comparison of the

derivative of the biomass growth kinetics and the bioluminescence profile of one batch run. From a mathematical point of view, the maximum point of the biomass derivative curve is the point of inflection of the biomass growth curve, and it corresponds to the point of maximum light emission.

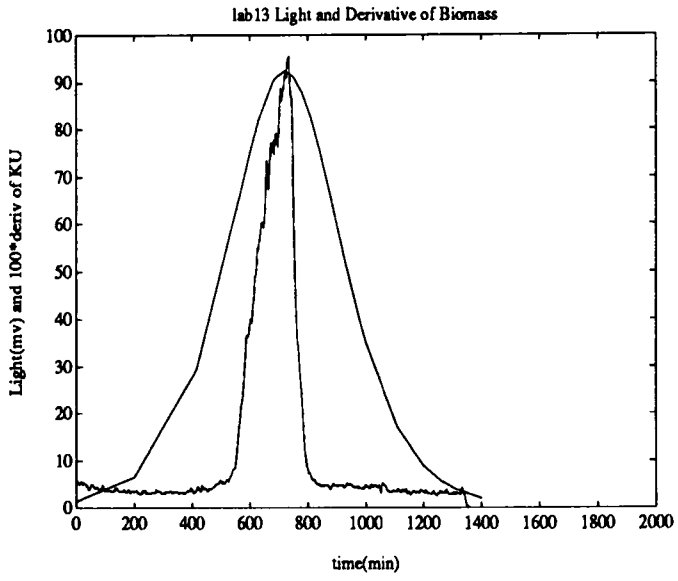


Figure 4.15: Comparison of the derivative of growth curve and bioluminescence curve. (The upper curve is the derivative of the growth curve, the lower is the bioluminescence curve).

Light emission data from experiments were normalized against the respective peak height such that the maximum is always 1, as shown in Figure 4.16. From a set of growth and light data, the relatively symmetrical growth curve appears to be characterized by a normal distribution function. A normal distribution function P(x) describes the probability density of obtaining the random variable x. It is characterized uniquely by two parameters: its mean μ (or a), and its variance σ, as shown in Figure 4.17. The two

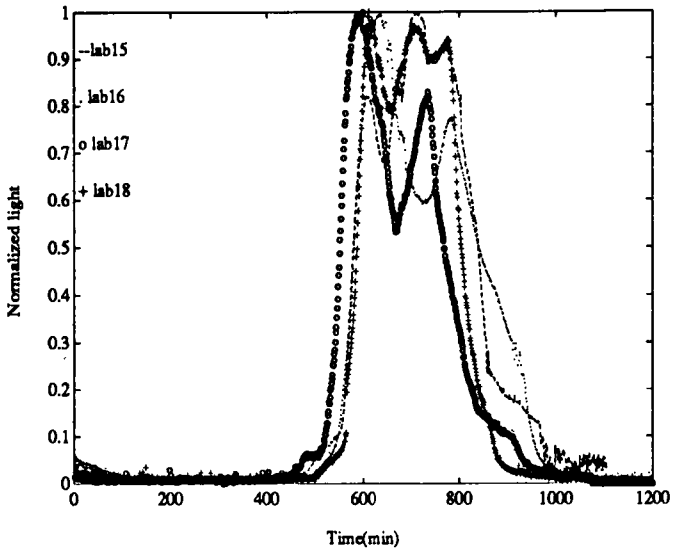


Figure 4.16: The comparison of normalized bioluminescent profiles in four batch runs.

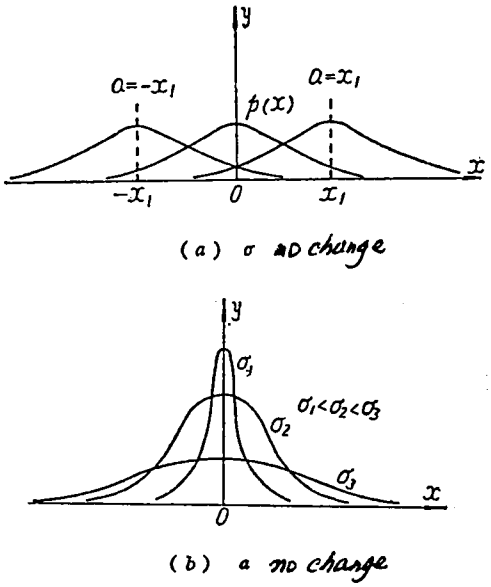


Figure 4.17: Normal distribution curve. σ denotes the standard deviation; a denotes the mean; p(x) denotes the normal distribution function; x denotes random variables.

parameters (the mean μ and the variance σ) that characterize either the biomass or the bioluminescence curve can be obtained from the normal distribution functions to fit the respective experimental data.(See Figures 4.18 and 4.19.) The curve fitting computer the programs and the derivative equations are documented in the Appendix.

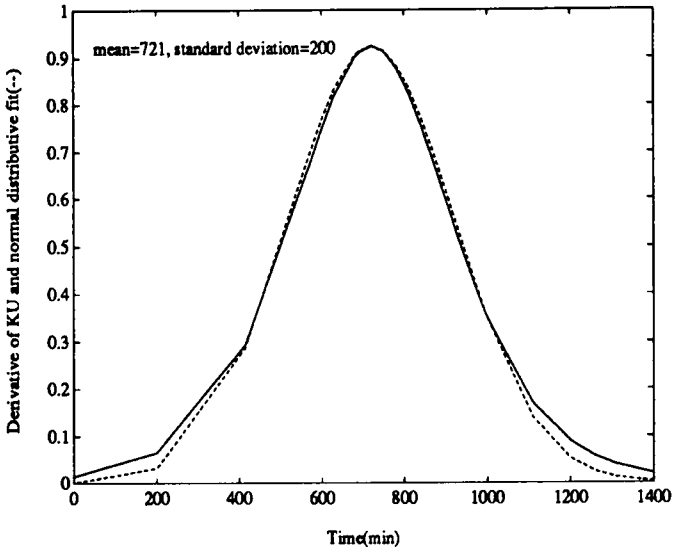


Figure 4.18: The derivative of growth curve fitted by a normal distributive function. Solid line denotes the derivative of growth curve from the experimental data, and dashed line denotes the fitted curve by a normal distributive function.

The strategy for correlation between the growth derivative curve and the bioluminescent curve is to attempt to get a consistent relationship between the respective variances σ for the two sets of curves. The two μ's will roughly be the same, since the two maximum points coincide. The normal distribution equation is described by [9]:

$$P(x) = \frac{1}{\sqrt{2\pi}\sigma} e^{-\frac{(x-\mu)^2}{2\sigma^2}} \tag{4.1}$$

$$(\sigma > 0),$$

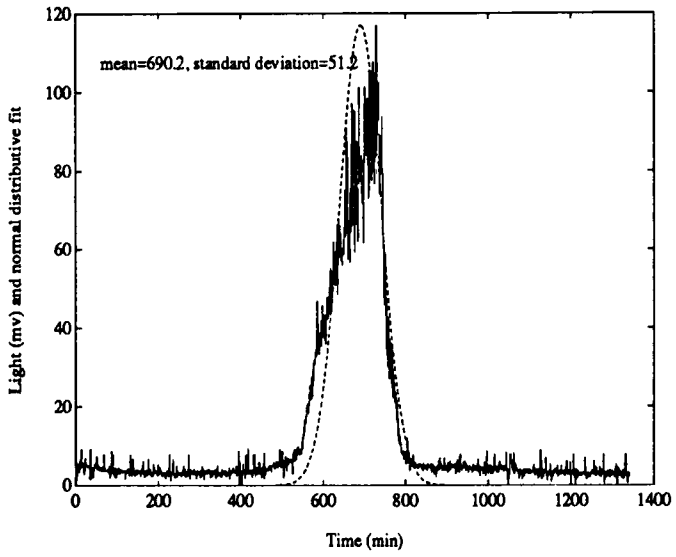


Figure 4.19: Light curve fitted by a normal distribution function. Solid line denotes the bioluminescence profile from the experimental data, and dashed line denotes the fitted curve by a normal distributive function.

where x is a random variable and P(x) is the probability density function of x, and the variables μ and σ represent the mean and variance respectively of the distribution of x. After fitting the derivative of growth and the light emission curves with a normal distribution curve, the parameters σ_l and μ_l (the subscript 'l' denotes light) can be determined by the bioluminescence curve , so can σ_g and μ_g (the subscript 'g' denotes growth) for the derivative of the growth curve. The jagged light peak was smoothed out with filled points, because we feel sure that the dip points were caused by the depletion of dissolved oxygen. If these parameters are found to be related as:

$$\sigma_g = K_2\sigma_l, and \tag{4.2}$$

$$\mu_g = K_3\mu_l, \tag{4.3}$$

with consistent K_2, and K_3 from batch to batch, then a consistent correlation between the biomass concentration and the normalized light intensity may be derived. Since the light emission peak corresponds roughly to the point of maximum biomass growth, μ_g is expected to be close to μ_l, and hence K_3 is expected to be close to 1. Empirical parameters σ_g and μ_g can be derived from experimental data, because the growth kinetics were controlled under identical conditions. The parameters σ_l, σ_g and μ_l, μ_g derived from the fitting of data from four batch runs are presented in Table 4.3.

More experiments need to be done in order to ascertain the correlation coefficient, K_2. However, from the first three sets of data shown in Table 4.3 (run15, run16 run17 data), the correlation coefficient K_2 between growth and bioluminescence has a specific range from 3.45 to 3.75, with a mean value of 3.52 and a standard deviation of 0.12. If a consistent K_2 can be derived and validated, then the following procedures can be used to on-line infer the biomass concentration in a batch operation from the light intensity

Table 4.3: The parameters of (σ_l, σ_g) and (μ_l, μ_g) from a fitted normal distributive model of four batch runs in *E.coli* RB1151

Experiment	μ_l, σ_l	μ_g, σ_g	K_2 (σ_g/ σ_l)
*Run*15	717,102	754,352	3.45
*Run*16	709,107	749,402	3.75
*Run*17	657,90	684,303	3.37
*Run*18	709,89	697,277	3.11

measurement:

1. Calculate from Eq. 4.4 the corresponding time $\bar{t}_i$ at which the light intensity $l(t_i)$, as measured at t_i would normally occur.

$$I = l(t_i) = \frac{1}{\sqrt{2\pi}\sigma_l} e^{-\frac{(\bar{t}_i - \mu_l)^2}{2\sigma_l^2}} \tag{4.4}$$

$$\Downarrow$$

$$\bar{t}_i = l^{-1}(I) \tag{4.5}$$

$$\Downarrow$$

2. Calculate, through the correlation coefficient K_2, the corresponding $\frac{db}{dt}$ (b=biomass) at this time point:

$$\frac{d(b(t_i))}{dt} \mid_{t_i=\bar{t}_i} = \frac{1}{\sqrt{2\pi}\sigma_g} e^{-\frac{(\bar{t}_i-\mu_g)^2}{2\sigma_g^2}}, \tag{4.6}$$

with $\sigma_g = K_2\sigma_l$.

$$\Downarrow$$

3. Numerically integrate b(t_i) to obtain b at t_i:

$$b(t_i) = b(t_{i-1}) + \frac{d(b(t)}{dt} \mid_{t=\bar{t}_i} (t_i - t_{i-1}) \tag{4.7}$$

with b=b_0(t=0).

In order to infer the biomass correlation using light intensity l(t_i) measurement at time t_i during a batch process, we utilize equations 4.4 and 4.5 to back calculate the time point $\bar{t}_i$ that would give a measurement of l(t_i) at time t_i. Then we substitute $\bar{t}_i$ into the biomass derivative equation 4.6. Finally, the biomass at time t_i is numerically calculated by discretely integrating equation 4.6 from t=0, with an initial biomass concentration b_o measured at $t = 0$, up to the time t_i.

4.2.2 Correlation Validation

In order to validate the parameter K_2 and the correlation model, we used the model derived from the first three sets of data in Table 4.3 to fit the last set of normalized experimental data (run 18). The simulation program is presented in the Appendix. The algorithm follows the procedures given above. The predicted biomass was computed

based on the on-line light intensity and then was compared with the experimental data. Figure 4.20 indicates the approximate agreement between the predicted and the experimental data for one batch culture of *E. coli.* The model appears to indicate the general trend of the growth kinetics. Availability of more experimental data would give a finer fitting, especially in the lag-phase region.

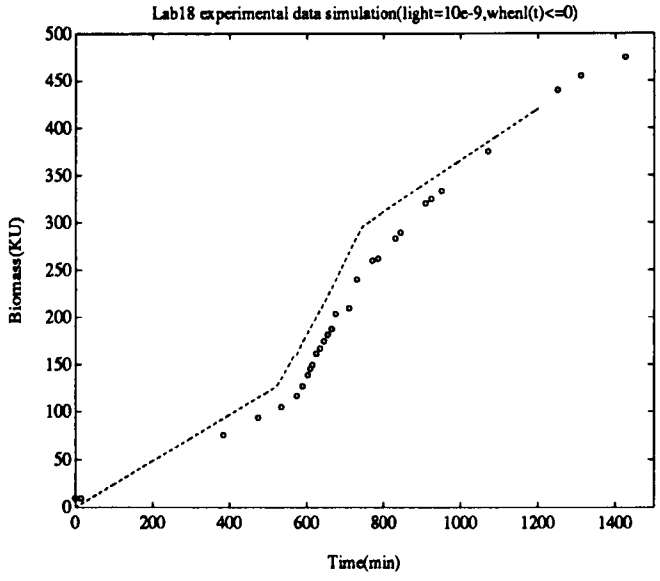


Figure 4.20: On-line prediction of biomass based on the measured light intensity during the batch culture of *E. coli.* (o) experimental biomass meausured off-line, (–) biomass predicted from the model.

4.2.3 Linear Correlation

The original objective was to develop an overall correlation between the intensity of emitted light and biomass concentration during the batch growth. An overall linear correlation was definitely not observed, and a limited period of linear correlation (3 hr) appears to exist during the exponential growth phase. A general linear relationship can be expressed as:

$$X(I) = m_0 + m_1 I, \tag{4.8}$$

where I is the light intensity during the exponential growth, X is the biomass concentration, and m_0, m_1 are the y-intercept and the slope respectively. Figure 4.21 shows the apparent correlation of biomass and light intensity during the exponential growth phase of about three hours duration, constructed from our early experimental data.

We performed linear regression analysis using data from more recent runs, where control of dissolved oxygen is consistently tighter. Controlled growth curves and bioluminescent profiles were shown in Figure 4.12 and Figure 4.13. The data are consistent with each other, possibly resulting from the more careful control of dissolved oxygen in later runs. Since the light is a reflection of the relative rate of growth (or the first derivative of the growth curve), we explored the linear correlation between the integral of the light kinetics and the growth kinetics itself during the exponential phase. (See Figure 4.22.) The linear correlation is presented in Figure 4.23. It appears to be consistent. However, the utility of linear correlation applicable to the exponential phase only is of rather limited use for a batch operation.

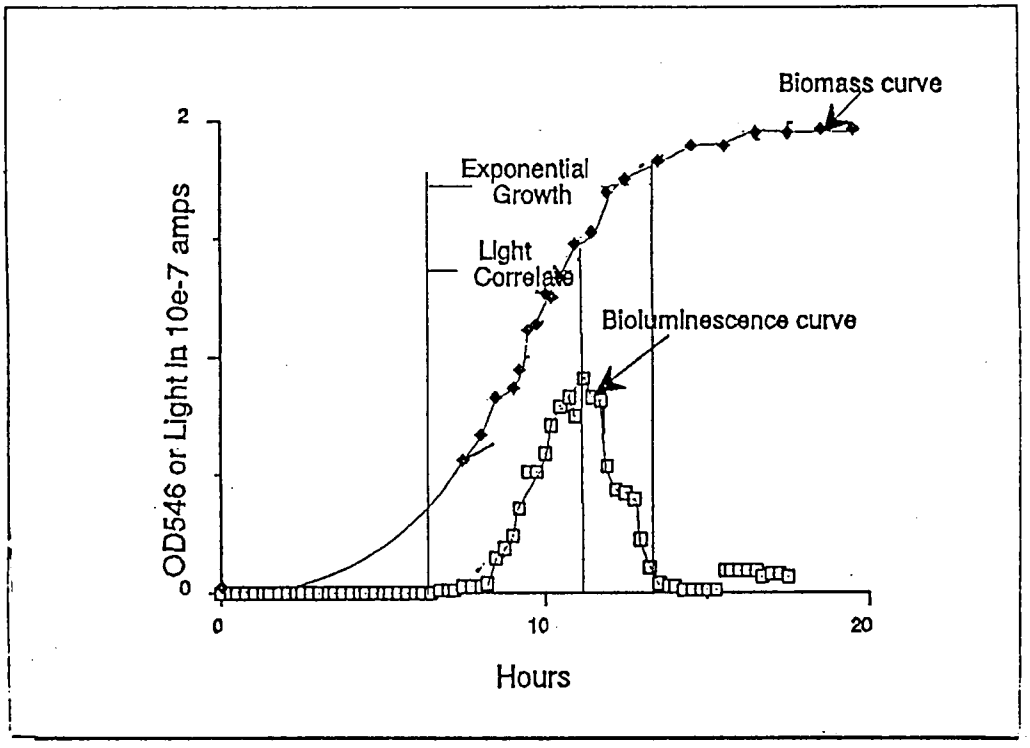


Figure 4.21: Apparent linear relationship of growth and light intensity during exponential phase for *E. coli* RB1151

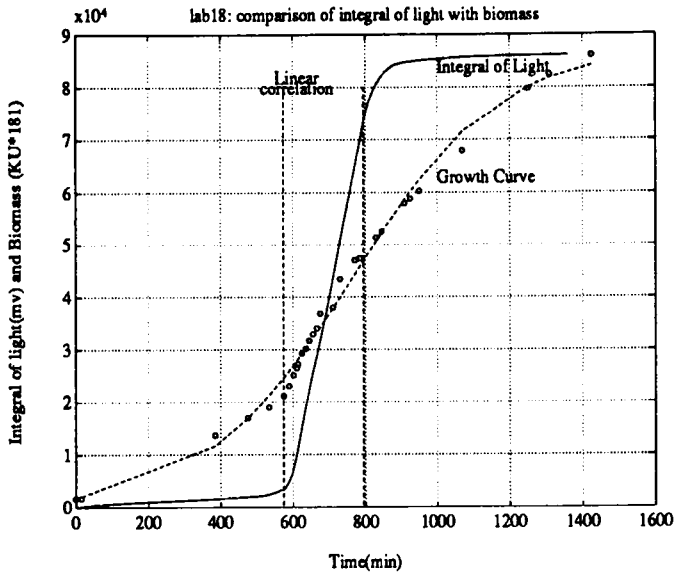


Figure 4.22: Comparison of integral of light curve with growth curve

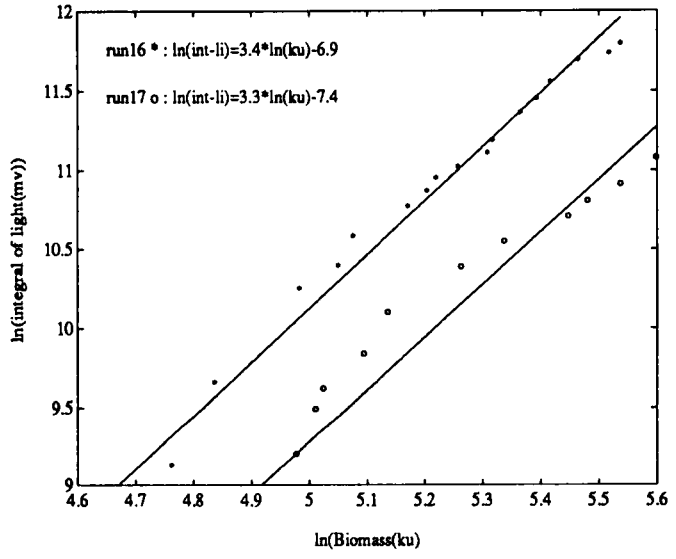


Figure 4.23: Log-log linear correlation curve between biomass and integral of light intensity during the exponential phase

CHAPTER 5

DISCUSSION

The original objective was to derive a correlation between the emitted light intensity and the biomass concentration of a bacterial culture during batch operation. From the analysis of eleven sets of experimental results, it was observed that the maximum region of the light emission curve almost always coincided with the point of the maximum **growth rate** of the bacterial growth curve (the point of inflection). This observation suggests that the light emission phenomenon is correlated with the process of growth, rather than with the total amount of biomass itself. This is physiologically plausible, taking into account the underlying mechanism of bioluminescence emission. Reactions involving the luciferase enzymes, oxygen, NADPH, and the flavin mononucleotide, $FMNH_2$, are responsible for producing the light emission. The intensity of emitted light is dependent upon 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 bacteria. Further, the greatest demand for oxygen occurs at the time of the maximum growth or the maximum light emission. If, during this time, the dissolved oxygen level is not adequate, one observes an immediate decline in the emitted light intensity. Figure 4.10 shows such an effect. As can be seen, the dips in the light emission peak region correspond to the time in which the O_2 level drops due to inadequate oxygen control.

The overall action of the *lux* gene product system is due to a number of factors. The energy available in the cell, the levels of luciferase, NADPH, and $FMNH_2$ are all

important contributers to the light emission. One of the most important factors is the consideration of the energy requirements for luminescence and the relative turnover rates of oxidative phosphorylation and oxidative-reduction pathways in generating ATP, an important cofactor in the luciferase-catalyzed bioluminescent reactions. Therefore, the availability of ATP during the growth process is the key factor for the light emission. The rate of oxidation-reduction of NADP and FMN molecules are all related to the availability of ATP. The level of the activity of the *lux* enzymes depends partially on the relative rates of the transcription and translation of the genes coding for the α and β subunits of luciferase, which in turn depends on the relative metabolic rate of the cells, therefore on the availability of substrates. Since the RB1151 strain harboring pUTK4 plasmid contains only the *lux* structural genes (*lux*CDABE), the bioluminescence reaction is aldehyde-independent, in that the regeneration of aldehyde is automatic. (See Figure 2.2 for the bioluminescence reaction.)

During the lag-growth phase, cellular metabolism starts to accelerate, resulting in the synthesis of the enzymes of reductase and luciferase, ATP, NADPH, and FMN_2. Initially, NADPH and FMN_2 will react slowly with O_2 in the absence of an abundant level of the reductase enzyme. After the pools of luciferae, reductase, and ATP pool are built up, the light emission starts to become significant. Since the *lux* system of RB1151 is constitutive, it does not require the accumulation of the autoinducer for the induction of bioluminescence to occur. However, our experimental results showed that the light emission remained almost at background level during the lag phase of cell growth. It is possible that our photomultipier system is not sensitive enough to measure the low light emission during the lag phase.

During the logarithmic growth phase, there is a rapid increase in the level of enzymes

and macromolecules. Bioluminescence increases concommitantly with the increase in cell mass. The specific rate of ATP production is reported to be linearly proportional to the rate of cell growth [43]. Therefore, more ATP is produced from the increasing turnover rate of the citric acid cycle and the oxidative phosphorylation pathway. With increasing growth rate, the turnover rates of transcription and translation also rapidly increase. All the above result in increased levels of enzymes and cofactors necessary for the bioluminescent reaction. An observation made several years ago is that when substrate is not limited, the initial rate of reaction is proportional to the product of the concentrations of reductase and luciferase [21]. Thus, it appears logical to conclude that the intensity of light emission is growth rate related, and not biomass related.

Bioluminescence of cells growing in liquid culture declines dramatically during the late log phase and continues to decline into the early stationary phase. In parallel with this deline, progressively less luciferase activity was observed. Baldwin et al [23] show that this decline does not appear to be due to the action of simple protease, which are known to inactivate luciferase. The rapid decline of the bioluminescent light after log phase can possibly be attributable to several factors. The slower rate of protein and RNA synthesis associated with an aging culture, presence of light-inhibiting metabolites in the culture broth, and the depletion of ATP required for the light emission process, are all possible causes for the decline in light emission. The cellular demand for ATP necessary to maintain the same level of bioluminescence should be at its greatest during this period, and the lack of an adequate supply of ATP due to the exhaustion of essential metabolites is probably the most influential factor in producing the decline of light emission.

Several reports [16] [29] [49] [60] have documented the observation of a correlation between biomass and light emission during the exponential phase of a bacterial growth

system. Therefore, we investigated this correlation by using our batch data and limiting the time interval to approximately three hours during the exponential growth phase. Figure 4.23 is a plot for two batch runs of the $\log_e$ of the biomass against the $\log_e$ of the integral of the emitted light intensity during this time interval. A linear relationship did appear to be evident. However, the utility of such linear correlation in batch runs would be limited only to the logarithmic growth phase, and thus not feasible for our purpose.

Although an apparent correlation cannot be derived between the light emission and the rate of growth due to the variation of light emission levels from batch to batch, a more consistent correlation was derived after the light emission profiles from four recent experiments were normalized against the respective light emission peak heights to remove the variation. The derived model is able to predict closely the growth kinetics of another (independent) experiment (see Figure 4.20). This suggests that perhaps a quantitative correlation does exist after all, and can be extracted if consistent light emission levels can be obtained from batch to batch . In the future, a different light detection system can be used to test this hypothesis.

It appears promising that a better correlation can be established for the exponential growth kinetics. Therefore, our plan for the next phase of research is to apply the study to a bioreactor operated in the continuous mode with substrate-limited growth. It is expected that such an operation, in which the biomass is always kept in exponential growth, would yield a more consistent and reproducible correlation.

CHAPTER 6

CONCLUSIONS

Based on current results, the following observations can be made:

1. It appears that bioluminescence is a reflection of the relative rate of growth rather than the absolute amount of biomass. This is deduced from the fact that the time of the light peak always corresponds closely to the time of maximum growth rate; and that no light is emitted after the biomass has reached a constant level during the late log phase.

2. The kinetics of light emission for batch runs depend heavily on the metabolic growth state, substrate concentration and the operating parameters (pH, dissolved oxygen, temperature, etc,). Light emission is especially sensitive to the level of dissolved oxygen.

3. With respect to the absolute value of light intensity, over the entire batch run period (24 hrs), a quantitative correlation cannot be derived mainly because of the inconsistency in the light intensity versus the time from batch to batch. However, with the inconsistency removed by normalization, it appears that a strong correlation does exist, suggesting that the light intensity variation from batch to batch is an artifact rather than inherent.

4. A linear correlation, limited to the exponential phase, appears to be evident, though its utility is rather limited (< 3 hrs).

5. We project that the correlations for the continuous-run mode will be considerably more precise and valid, since growth in a continuous culture would be maintained at the exponential phase and will overcome many of the problems encountered in the batch mode. In addition, the continuous-run mode prevents degradation of cells and accumulation of products to levels toxic to the host cells.

For a batch bioreactor operation, the bioluminescence emission profile can be used as an indicator for the metabolic state of cells because of its high sensitivity to environmental perturbations, such as a change in temperature, pH, dissolved oxygen level, and inhibitory factors. Among the eleven experiments, several qualitative correlation features were observed between the light emission and the biomass growth profiles. However, a consistent quantitative correlation could not be obtained, mainly because of the inconsistency of the level of emitted light from batch to batch. We plan to switch to the continuous mode of operation, thus controlling the growth at the exponential phase. With this method, we expect to get a much more consistent correlation pattern.

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APPENDIX

APPENDIX A

Matlab Simulation Programs

1.1 Model simulation for on-line prediction of biomass

This program uses on-line prediction of biomass from light measurements, as described in Section 4.2.1.

```
%
%=========================================================================
%
%         Normal distribution model + Integration
%
%=========================================================================
% The program calculated the time ttt in the batch run period using the
% on-line measured light intensity. Then tt was substitute into the
% derivative biomass equation which also was modeled by normal
% distributive equation. Hence, the biomass value at time ttt was
% determined by integration of derivative biomass equation.
%=========================================================================
%
%Here is the program for fitting lab17 light data
% sig_l: standard deviation of Guess Eq. for light data, sig_g
%        for biomass
% u_l:   mean of Guess Eq. for light data, sig_g for biomass
% nli or xlightv: original measured light intensity (mv)
% di :   real time scale
% bb :   biomass value
% db :   derivative of biomass value
%
%=========================================================================
global xlightv  nli nn di
clear
load lab17
sig_l=88.67;
u_l= 661.32;
K=3.52;
s_l=100;          % s_l is the average sig_l from three sets of data.
sig_g=s_l*K;  % sig_g, u_g, K=3.52 are determined by the previous
u_g=708;          % experimental data.
nn=max(size(nli));  % number of inputs
%di=2:nn;
%
% Get Maximum Normal Distributive Curve Value
for i=2:nn,
%  P(i)=(1/(sig_l*sqrt(2*pi)))*exp(-(di(i)'-u_l)^2/(2*sig_l^2))
   P(i)=(1/(sig_l*sqrt(2*pi)))*exp(-(di(i)-u_l)^2/(2*sig_l^2));
end
 lm=max(nli);
 pm=max(P);
```

```
%
%nli=nli/max(nli);
% Change The Light Intensity Scale In Order To Fit In The Model
nli=nli*pm/lm;
kk=max(ku);
%=====================================================================
% Calculate time points ttt based on the real light intensity
for i=2:nn,
       if nli(i) <= 0
          nli(i)  =0
       end
        ac=-2*sig_l^2*log(sqrt(2*pi)*nli(i)*sig_l);
        ac=abs(ac);
    if i<=u_l,
        ttt=u_l- sqrt(ac);
%       ttt=u_l- sqrt(-2*sig_l^2*log(sqrt(2*pi)*nli(i)*sig_l));
 %      ti1(i)=ttt;
    end
    if i>u_g,
        ttt=u_l+ sqrt(ac);
%       ti2(i)=ttt;
    end
    ti(i)=ttt;
%ti(i)=[ti1(i) ti2(i)]
%=====================================================================
% Calculate Derivative Biomass
     db(i)=(1/(sig_g*sqrt(2*pi)))*exp(-(ttt-u_g)^2/(2*sig_g^2));
% Integration
     bb(1)=db(1);
     bb(i)=bb(i-1)+db(i)
end
%=====================================================================
%
%   Plot Results
%
%=====================================================================
ft=kk/max(bb);
bb=bb*ft;
%ti=[ti ti+694.3]
%subplot(211)
axis([0,1500, 0,500])
 plot(di,bb,'--')
 hold
 plot(t, ku,'o')
 hold
%=====================================================================
%
%            THE END
%
%=====================================================================
```

1.2 Integral Program For The Bioluminescence Profile

This program is for integration of the bioluminescence profile in order to compare it with the corresponding growth curve.

```
%
%=========================================================================
% xlightv   = real experimental light intensity data
% int_light = integral value of the light intensity from 0 to i
% i         = measuring time point
%
%=========================================================================
 int_light(1)=xlightv(1);
 [nn nnf]=size(xlightv);
%=========================================================================
% Integration
%
 for i=2:nn
     int_light(i)=int_light(i-1)+xlightv(i);
 end,
%=========================================================================
% Adjust Biomass And Integral Of Light In The Same Scale for Comparison
%
fv1=max(int_light);
fv2=max(ku);
sc=fv1/fv2;
tt=1:nn;
%=========================================================================
%      Plot Results
%
%=========================================================================
% plot(t,ku*sc,'o',tt, int_light, t,ku*sc,'--',t, y*sc,'+',t,y*sc,':');
% grid
%
%
%=========================================================================
%
%      THE END
%
%=========================================================================
```

1.3 Program For Fitting Normal Distributive Curve

These two programs are used to fit the bioluminescence curve and derivative of growth curve, using fmins funtion in Matlab to minimize the normal distributive model with experimental data curve.

```
%=========================================================================
%
%          Light Curve Fitting
%
%=========================================================================
global xlightv nn nli di
clear
load lab16
nn=max(size(nli));
nli=nli/max(nli);
sig=60;
u= 700;
x=1:nn;
Io=[sig,u];
```

```
I=fmins('light', Io);
u=I(2);
sig=I(1);
for i=1:nn,
  P(i)=(1/(sig*sqrt(2*pi)))*exp(-(x(i)'-u)^2/(2*sig^2));
%   P(i)=(1/(sig*sqrt(2*pi)))*exp(-(di(i)'-u)^2/(2*sig^2));
end
 lm=max(nli);
 pm=max(P);
 if abs(pm) < 0.001;
    pm=1;
 end
 P=P*lm/pm;
 plot(di,P,'--')
 hold
 plot(di, nli)
 hold
%=========================================================================
%
%           THE END
%
%=========================================================================
%
  function fxx=light(xx)
   sig=xx(1);
   u=xx(2);
  x=1:nn;
  for i=1:nn,
     P(i)=(1/(sig*sqrt(2*pi)))*exp(-(x(i)-u)^2/(2*sig^2));
 end
 nnli=nnli/max(nnli);
 lm=max(nnli);
 pm=max(P);
 if abs(pm) < 0.001;
    pm=1;
 end
 P=P*lm/pm;
 fxx=nnli-P;
 fxx=norm(fxx),
%
%
%=========================================================================
%
%         Biomass Curve Fitting
%
%=========================================================================
global dy nn t
clear
load lab10
nn=max(size(dy));
sig=200;
u= 700;
%x=1:nn;
Io=[sig,u];
I=fmins('biom', Io);
u=I(2);
sig=I(1);
for i=1:nn,
```

```
  P(i)=(1/(sig*sqrt(2*pi)))*exp(-(t(i)-u)^2/(2*sig^2));
end
 lm=max(dy);
 pm=max(P);
 P=P*lm/pm;
 plot(t,P,'--')
 hold
 plot(t,dy)
 hold
%=========================================================================
%
%            THE END
%
%=========================================================================
%
  function byy=biom(yy)
   sig=yy(1);
   u=yy(2);
%  x=1:nn;
  for i=1:nn,
     P(i)=(1/(sig*sqrt(2*pi)))*exp(-(t(i)-u)^2/(2*sig^2));
 end
 lm=max(dy);
 pm=max(P);
 if abs(pm) < 0.001;
    pm=1;
 end
 P=P*lm/pm;
 byy=dy-P;
 byy=norm(byy),
%
%=========================================================================
```

VITA

Bo Huang was born on August 2, 1967 in China. She was attracted by the mysterious realm of molecular biology when she was in high school. She majored in biology and obtained her B.S. degree in June of 1989, when she still dreamed of entering the spherical kingdom of the double helix. Subsequently, She came to the United states with her dream on December of 1989.

In the spring of 1990, she started graduate studies in Microbiology at the University of Tennessee, Knoxville. After one year of theoretical studies, she started to hunt for something more practical. Finally, she found her interests in the field of biotechnology and started her research work in biosensors in the Department of Chemical Engineering. She continued her graduate studies in Bioengineering, and was awarded the Master of Science degree in Microbiology, in August, 1992.