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On-line monitoring of growth of *Escherichia coli* in batch cultures by bioluminescence

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Abstract Bioluminescence was used to monitor growth of *Escherichia coli* in batch cultures on-line. Light emission of a strain engineered for constitutive bioluminescence was monitored with a simple set-up consisting of a photodiode, a photodetector amplifier and a recorder. Bioluminescence and colony forming units (CFU) of the cultures increased and decreased proportionally and were correlated during every growth phase at temperatures between 28 °C and 40 °C. Up to the late log (deceleration) phase, both light emission and CFU increased rapidly. Beyond the stationary phase these characteristics decreased very slowly at lower temperatures, while at higher ones they declined more rapidly. Towards the end of the cultivation, light emission of the cultures dropped to undetectable levels, even though CFU were recovered. This was particularly marked at lower temperatures where non-luminescent cultures retained very high CFU. This indicates that the actual metabolism of cells in a culture can be at a very low level or completely shut down, yet cells retain their capability to be culturable. The on-line technology described here has a number of potential uses in the laboratory and industry.

Introduction

Escherichia coli is one of the bacterial species used in laboratories and biotechnology to produce a wide range of molecules, such as hormones, enzymes, antibodies, amino acids, plasmid DNAs, etc. Since production and yield of such products depend on the growth, viability and cellular metabolism of the microorganism, monitoring these characters is essential for both small-scale experiments and industrial-scale fermentations (Raju and Cooney 1992; Sonnleiter et al. 1992; Scheper and Lammers 1994). Various off- and on-line methods have been described to monitor growth, viability and metabolism of bacterial cultures. On-line methods are more desirable because, in contrast to off-line detection, they can provide almost immediate process control.

Growth (biomass accumulation) can be monitored by: (1) measuring the wet or dry weight of the cell mass after separation by centrifugation or filtration, and (2) determining either the total cell number by microscopy or colony forming units (CFU) by plate counting (Sonnleiter et al. 1992). However, these methods cannot be applied on-line; they need sampling and are both labour intensive and time consuming. Measuring optical characters (density or turbidity) of bacterial cultures by different approaches is also employed to monitor growth (Scheper et al. 1993). Optical techniques can be easily applied on-line, but their drawback is that not only living cells, but also dead cells and cell components, such as ruptured membrane fragments, increase the optical density or turbidity of the culture. Other approaches, such as acoustic (Blake-Coleman et al. 1986), electrical (Harris et al. 1987; Ding and Schmid 1990) and calorimetric (van Kleeff et al. 1993) measurements have also been described to monitor biomass accumulation, but these methods can be easily interfered with by extracellular factors. Furthermore, these methods require specialised instrumentation. In general, the major disadvantage of the above-mentioned methods of growth determination is that none of them reflects the viability and/or metabolic status of the culture.

Two major methods are known to determine viability of bacterial cells in cultures. One approach uses fluorescent dyes for differential staining of viable and dead cells that can be observed by microscopy or separated and detected by flow-cytometry (Lloyd and Hayes 1995). The other approach detects enzymatic activities of cells by either activity staining or biochemical reactions and by microscopy or spectroscopy (Sonnleiter et al. 1992). The

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drawback of both methods is that they are laborious and require sampling. On-line applications are not easy and cell detection requires relatively expensive equipment.

A number of methods have been described to monitor cellular metabolism in bacterial cultures. The most developed one is measurement of NADH-dependent fluorescence of the culture (Kunz et al. 1992). This method can be easily applied on-line, but it needs an expensive technology, and non-related fluorophores interfere with the measurement. NMR spectroscopy has been used to measure phosphate in bacterial cultures as an indicator of cellular metabolism (Chen and Bailey 1993), but extracellular phosphate interferes with the detection and a sophisticated device is required. Specialised techniques such as short wavelength near infrared spectroscopy (Sonnleiter et al. 1992) and Fourier transform infrared–photoacoustic spectroscopy (Gordon et al. 1990) have also been described to monitor the metabolic state of microorganisms, but these techniques are not widely used.

The methods described above monitor singly either growth, or viability, or metabolism of bacterial cultures. An approach which could be used to measure all of these characters simultaneously would be advantageous and desirable. For this purpose, bioluminescence may provide a solution.

Lasko and Wang (1993) used bioluminescence to determine viable cell number in bacterial fermentation. The authors cloned the firefly luciferase gene in *E. coli* and monitored bioluminescence in relation to growth of the culture. This method, although an accurate measure of viability and growth, has two drawbacks. Firstly, the system requires addition of luciferin, the substrate of the firefly luciferase enzyme, which is very expensive. Hence its total cost can be substantial, especially at the industrial scale. Secondly, after addition of the substrate, light is emitted by the firefly luciferase reaction as a rapid pulse, thus detection of the emitted light requires sophisticated and therefore expensive instrumentation. In regard to these difficulties associated with the firefly system, bacterial bioluminescence (Meighen 1993) can be a more easily applicable alternative.

In bioluminescent bacteria, five structural genes (*luxCDABE*) that form an operon are responsible for light emission. The *luxCDE* genes encode an enzyme complex that synthesises the substrate of luciferase, a fatty aldehyde (RCHO), using precursors from the fatty acid cycle (Meighen 1988). The *luxAB* genes encode the luciferase enzyme (Meighen 1988), which catalyses the light emitting reaction by the following formula:

$$RCHO + O_2 + FMNH_2 = RCOOH + H_2O + FMN + light_{490\ nm}$$

The *luxCDABE* operon of naturally bioluminescent species was cloned into other bacteria, which then displayed a luminescent phenotype (Schauer et al. 1988; Wolk et al. 1991; Eaton et al. 1993; Phillips-Jones 1993; Mackey et al. 1994). This indicates that precursors of the fatty aldehyde substrate are present not only in the original host but also in other species and that both the LuxCDE enzyme-complex and the LuxAB luciferase have catalytic activity in non-host bacteria. Consequently, the five genes are sufficient to produce a bioluminescent phenotype in other species.

In this paper I describe a simple on-line system to monitor growth of *E. coli* using the Lux phenotype as an indicator. This method has potential to be applied in areas where information about bacterial growth is essential.

Materials and methods

Strain and media

Construction of the constitutive bioluminescent *Escherichia coli* DH5(pLITE27) strain used in this study has been described previously (Marincs and White 1994). Plasmid pLITE27, harboured by the host DH5, is a derivative of plasmid pUC18 carrying the *luxCDABE* operon of *Xenorhabdus luminescens*. This operon was chosen for two reasons. Firstly, the *Xenorhabdus* LuxAB luciferase enzyme is more thermostable than other bacterial luciferases, for example the *Vibrio harveyi* luciferase (Szittner and Meighen 1990), and can be exposed to a relatively wide range of temperatures without losing its activity. Secondly, isolation, cloning and introduction of the *X. luminescens luxCDABE* operon into non-host bacterial species are technically easy because it is localised on a single *Eco*RI fragment (Marincs and White 1994).

The strain was maintained on LB (10 g trypton, 5 g yeast extract and 5 g NaCl per litre) agar plates supplemented with 100 μg ampicillin/ml for selection of pLITE27. For the growth experiments, either LB or ATCC271 (10 g trypton, 1 g yeast extract, 8 g NaCl, 10 ml 10% glucose, 2 ml 1 M $CaCl_2$ and 1 ml 10 mg thiamine/ml per litre) broth supplemented with 100 μg ampicillin/ml were used. For all experiments, a 4-ml fresh overnight culture of strain DH5(pLITE27) grown in LB with 100 μg ampicillin/ml was used as a starter. This was inoculated into either a 1-l Erlenmeyer flask or a stainless steel culturing vessel containing 500 ml broth supplemented with 100 μg ampicillin/ml and the bacterium was grown at the appropriate temperature either by shaking or in the apparatus described below.

CFU of bacterial cultures were determined by plating appropriately diluted samples on LB + 100 μg ampicillin/ml agar plates and colonies were counted after 16 h incubation at 37 °C.

Measurements

Optical density of cultures was measured at 600 nm with a Cary 1E spectrophotometer (Varian, Melbourne, Vic., Australia). Bioluminescence of bacteria was measured with either a BioOrbit 1253 luminometer (Bio-Orbit, Turku, Finland) or with a silicon planar photodiode (model VISD, World Precision Instruments, Mount Waverley, Vic., Australia). The VISD photodiode has a broad range spectral response (400–1100 nm) which covers the 490 nm wavelength light emitted by bioluminescent bacteria. Using this photodiode an apparatus was assembled as follows. A 2-l stainless steel container with lid (Cole-Parmer, Vernon Hills, Ill., USA) was used as the growing vessel to exclude external light, which would interfere with detection of the bioluminescence of the culture. A home made adapter with a glass window was fixed into a hole at the centre of the lid of this vessel. The VISD photodiode, from which the protecting cap was removed, was screwed into the adapter. The photodiode was connected to the input port of a photodetector amplifier, (model PDA1, World Precision Instruments), using a standard BNC cable. The output port of the amplifier was then connected to a chart recorder. The stainless steel vessel was

equipped with a magnetic bar and immersed into a magnetic-stirrer water bath (model HBR4, IKA Werke, Staufen, Germany) to ensure constant temperature and aeration of the bacterial culture. The set-up is shown in Fig. 1.

Results

Initially I investigated the correlation between growth and bioluminescence of strain DH5(pLITE27), grown at 37 °C in LB broth in a shaken flask. Samples were taken at regular intervals and their optical density and bioluminescence were measured. Both optical density and bioluminescence of the culture increased exponentially with time (Fig. 2). Plotting bioluminescence against optical density revealed a positive linear correlation between the two factors (Fig. 3). This suggested that measuring light emission of strain DH5(pLITE27) grown in batch cultures reflects the growth status of the culture directly and can replace other methods of growth determination.

In everyday laboratory practice, luminometers are commonly used to measure bioluminescence of living organisms and in the initial experiment described above such a device was employed. However, bioluminescence of strain DH5(pLITE27) is so intense that light emission from just a few millilitres of broth culture can be seen by eye in a darkroom. Because of this strong bioluminescent phenotype, it seemed possible that a simpler device, such as a photodiode, may be sensitive enough to detect the light emitted by the strain. Using a photodiode, a set-up as described in Materials and methods was assembled to monitor light emission of bacterial cultures on-line. With this apparatus, strain DH5(pLITE27) was grown under different conditions, light emission of cultures was recorded and CFU was concurrently determined. The data obtained using LB broth at different temperatures are shown in Fig. 4. In general, bioluminescence and culture growth were proportional, although lower temperatures resulted in higher CFU/

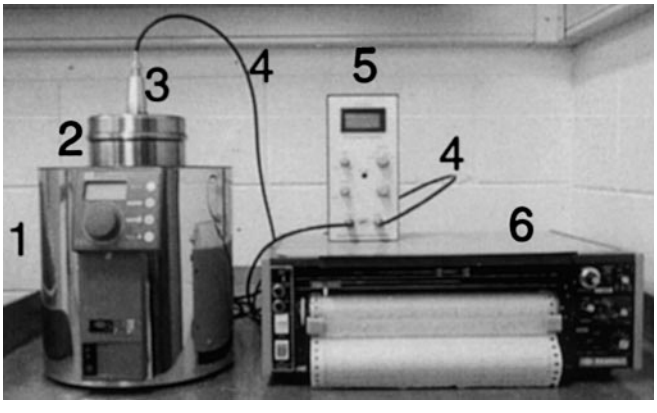

Fig. 1 The instrumentation used to detect light emission in stirred cultures on-line: *1* magnetic-stirrer water bath, *2* stainless steel growing vessel, *3* photodiode, *4* BNC cables, *5* photodetector amplifier, *6* chart recorder

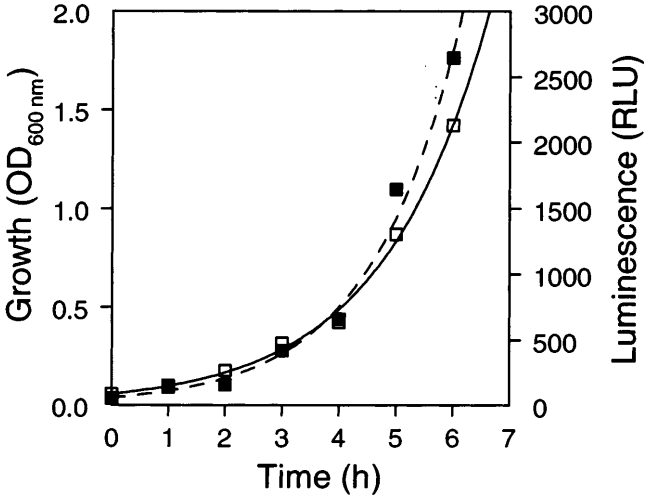

Fig. 2 Growth (□) and luminescence (■) of strain DH5(pLITE27) of a shaken culture in LB broth at 37 °C. Growth was monitored by measuring optical density of 1-ml samples spectrophotometrically at 600 nm. Luminescence of the same samples was measured for 1 s with a luminometer and is expressed as relative light units (*RLU*). *Markers* represent the mean values of three parallel samples (for clarity, error bars are not shown). *Continuous* and *dashed lines* represent the best-fitted curves for optical density ($r^2 = 0.9956$) and luminescence ($r^2 = 0.9899$), respectively

weaker bioluminescence and higher temperatures gave lower CFU/higher bioluminescence. CFU of cultures at 28 °C and 32 °C reached values of 1.17×10^9 and 5.97×10^9, respectively, which were about one order of magnitude higher than the maximum values of 1.83×10^8 and 1.06×10^8 at 36 °C and 40 °C, respectively (Fig. 4). The higher CFU values were relatively stable beyond the stationary phase at both 28 °C and 32 °C (Fig. 4A, B) unlike those at 36 °C and 40 °C where CFU diminished more quickly after reaching the maximum (Fig. 4C, D). Bioluminescence of the cultures

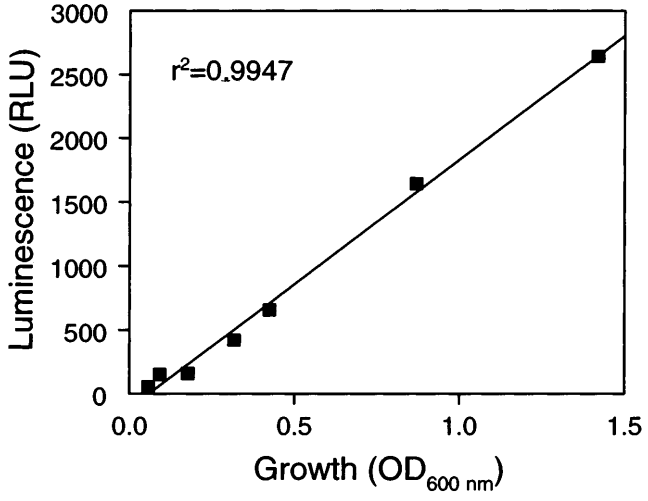

Fig. 3 Correlation between growth and luminescence of strain DH5(pLITE27). Luminescence data from Fig. 2 were plotted against optical density data from the same figure

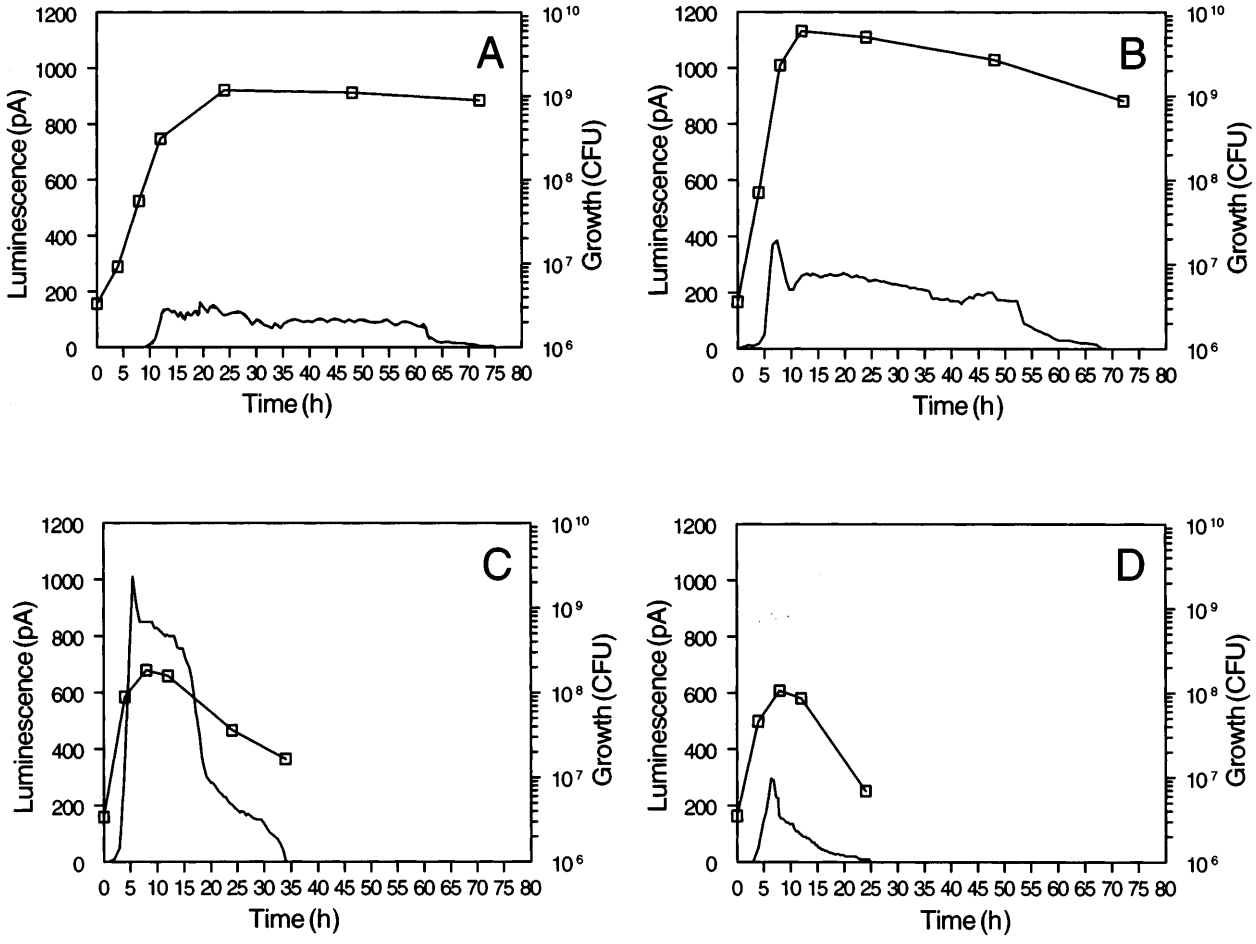

Fig. 4A–D Representative data for growth (□) and luminescence (*solid line*) of stirred cultures of strain DH5(pLITE27) in LB broth, at **A** 28 °C, **B** 32 °C, **C** 36 °C and **D** 40 °C. Growth of the cultures was monitored by determining colony forming units (*CFU*). Luminescence was measured on-line as described in the text and is expressed as the current (*pA*) generated by the emitted light in the photodiode. *Markers* represent the mean values of three parallel samples. Error bars are not shown, because their extent is smaller than the size of the markers

was relatively weak at 28 °C and 32 °C, while it was noticeably higher at 36 °C at least in the first 15–17 h growth (Fig. 4). At 28 °C, bioluminescence reached a value of 135 pA in the late log-phase. That level was quite stable in and beyond the stationary-phase and decreased only very slowly to 90–100 pA in the next 60 h. At 32 °C, light emission of the culture reached a peak at 390 pA, dropped rapidly by about 150 pA and than decreased relatively slowly up to 52 h. In contrast, bioluminescence of the culture at 36 °C peaked at around 1000 pA in the late log-phase, but dropped by about 200 pA immediately; and another huge decrease occurred at about 16 h. At 40 °C, the maximum value of light emission was around 300 pA, which than decreased evenly to zero at 25 h. Growth and light emission of the cultures displayed two distinct segments at every growing temperature. Up to the stationary phase (segment 1) both were increasing, while beyond the stationary phase (segment 2) both were decreasing. Therefore the correlation between them was analysed in these two segments. In both segments, light emission and growth were linearly correlated. The r^2 values for the best fitted curves for segment 1 and 2 were 1 and 0.99, 0.99 and 0.97, 0.98 and 0.98, 0.98 and 1 at 28, 32, 36 and 40 °C, respectively.

Duration of light emission of the cultures was much longer, 75 h and 68 h, at 28 °C and 32 °C respectively, than at 36 °C and 40 °C where bioluminescence of the cultures dropped to zero (undetectable level) after 34 h and 25 h, respectively (Fig. 4). Specific luminescence (the quotient of luminescence and growth) was calculated from the CFU and the corresponding luminescence data of Fig. 4. At 28 °C and 32 °C it varied between 2.4×10^{-7} pA/CFU and 9.8×10^{-8} pA/CFU, depending on the age of the culture. In contrast, at 36 °C and 40 °C specific luminescences were $3.6–5.5 \times 10^{-6}$ pA/CFU and $1–1.6 \times 10^{-6}$ pA/CFU, respectively. These data revealed a difference of about one or two orders of magnitude in specific luminescences at lower and higher temperatures, reflecting the different metabolic activity of cells at lower and higher temperatures.

Using ATCC271 media (a glucose-supplemented trypton–yeast extract broth), similar results were obtained to those with LB broth at all of the investigated temperatures (data not shown). Different carbon sources (glycerol, maltose, and galactose) instead of glucose in

ATCC271 media also had no significant effect on either growth or light emission of the strain (data not shown).

Discussion

In this paper I describe how bioluminescence can be used to monitor growth of *E. coli*. Strain DH5 (pLITE27) used in these experiments was constructed previously, carries the entire *lux* structural operon of *X. luminescens* and displays a constitutive bioluminescent phenotype (Marincs and White 1994). In shaken culture, both light emission and optical density of strain DH5(pLITE27) increased exponentially and were correlated (Fig. 2). This result agrees with the observations of others (Frackman et al. 1990; Meighen and Szittner 1992). The main aim of the present study, however, was to monitor growth of cultures on-line using the Lux phenotype as an indicator. It was possible to monitor light emission of the strain in real time using the apparatus shown in Fig. 1. Although lower (28 °C and 32 °C) temperatures resulted in weaker bioluminescence than higher (36 °C and 40 °C) ones, the instrumentation was appropriate to detect light emission from cultures at all temperatures. It is interesting to note that in strain DH5(pLITE27), expressing the *luxCDABE* operon of *X. luminescens*, the temperature optima for growth and bioluminescence were similar to those of *X. luminescens*. Strain DH5(pLITE27) displayed its strongest growth and light emission at 32 °C and 36 °C respectively, whilst growth and bioluminescence of *X. luminescens* was optimal at 33 °C and 37 °C, respectively (Colepicolo et al. 1989). In the on-line experiments, bioluminescence and CFU number of the cultures were similarly correlated as in the shaken culture experiment (see above). Different media and sugar supplements (energy sources) had no significant effect on growth and bioluminescence of the strain in the on-line apparatus (data not shown).

The on-line method described here has the potential to replace other methods for monitoring bacterial culture growth in both the laboratory and industry. It has several benefits compared to other techniques. For example, determination of CFU requires sampling and results are usually not available in less than 12 h. In contrast, the method described here provides real-time information about the culture. The emitted light can be detected in situ, without sampling, addition of any substances or further manipulations, which are prerequisites for a number of the methods described in the Introduction of this paper. Consequently, this method is labour and time effective. Furthermore, the instrumentation is inexpensive compared to that required for other methods, such as NMR, fluorometry and flow-cytometry. Compared to the firefly bioluminescence approach of Lasko and Wang (1993), the bacterial bioluminescence method described here does not require addition of a substrate. This can be produced endogenously by bacteria expressing the *luxCDE* genes.

There are further benefits from the method described in this paper. Firstly, bioluminescence genes of bacterial origin have been introduced and expressed in different non-host species (Schauer et al. 1988; Wolk et al. 1991; Eaton et al. 1993; Phillips-Jones 1993; Mackey et al. 1994). This method, therefore, could be easily applied to a range of industrially important bacteria. Secondly, light emission of a culture is not only correlated with growth but also reflects the metabolic status of the cells. Bacterial bioluminescence depends on complex cellular biochemistry in which a large number of genes and proteins are involved in a highly controlled manner (Meighen 1994). Consequently, light production indicates that the genetic and biochemical apparatus of the cell is very likely intact. Furthermore bioluminescence, like other cellular metabolic pathways, requires energy. Thus, light emission of bacterial cultures can indicate an active, energy-dependent metabolism. *E. coli* cells with reduced metabolism also have a reduced level of bioluminescence (Unge et al. 1999). The synthesis of $FMNH_2$, a cofactor required for bioluminescence, is also interconnected with the respiratory chain. Consequently dead bacteria, in which the respiratory chain is inactive, do not produce light (Hastings et al. 1985). Cultures of strain DH5(pLITE27) killed by antibiotics or heat have a dark phenotype (data not shown). It was also shown that starving bioluminescent *E. coli* populations with a constant number of viable (or active) cells can became dark, independent of the number of culturable cells (Duncan et al. 1994). In good agreement with this, my own results show that cultures with high CFU became dark after 60 h culturing (Fig. 4). In conclusion, measuring bioluminescence of a bacterial culture may provide more valid data about growth, viability and metabolic activity of the culture than other methods, such as CFU counting, activity staining or direct viable counting (Duncan et al. 1994).

There are some implications of this bioluminescence method for research and industry. Firstly, immediate process control in fermentation is a very important issue (Locher et al. 1992) and this method could provide one possible tool for this purpose. As it arises from the growth or metabolism of the bacterial culture, the intensity of the emitted light could be used through a feedback circuit to regulate the fermentation process. Secondly, as biomass accumulation in a bacterial culture is the result of growth and bioluminescence reflects growth, monitoring light emission could provide indirect data about biomass accumulation. Thirdly, experiments described in this paper indicate that high cell numbers in a culture are not necessarily accompanied by high biochemical activity and vice versa (Fig. 4). Thus, by measuring metabolic activity of a bacterial culture by the bioluminescent technique, a common problem of fermentation (i.e. obtaining a large biomass without a high amount of the desired product) might be avoided. Finally, different promoters are widely used to express foreign genes in bacteria for research and industrial purposes (Weickert et al. 1996; Hannig and Makrides

1998). Fusion between these promoters and the bacterial *lux* genes, coupled with the light detecting technology described here, may provide a rapid and simple technique to optimise conditions for gene expression from those promoters.

Because of the above-discussed aspects of the method, it has the potential to be a useful tool in both laboratory and industry for research and manufacturing.

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