# Chapter 4
# Continuous culture of Photobacterium

## 4.1 Introduction

Continuous culture devices, also referred to as bioreactors, are frequently employed in industry for the large-scale production of antibiotics and other fermentation products e.g., insulin form genetically engineered tissue cultures. The chemostat, the simplest type of continuous-culture device, was developed during the 1940s. It comprises a reaction vessel into which fresh culture medium can be pumped. The reaction vessel also has an outflow such as a side arm overflow or a pump so that it can be maintained as a constant-volume device. The reaction vessel is stirred so that the substrate and biomass concentrations are the same at each point in the reaction vessel. An important characteristic of the chemostat is its constant conditions or steady state. These devices have advanced understanding in areas such as prokaryotic and eukaryotic cell physiology and cell-cycle dynamics. In this chapter a miniaturized continuous-culture system is described optimized for the culture of Photobacteria.

The use of higher organisms, such as mice, has advantages and disadvantages. For example, mice closely represent humans in terms of biochemistry and physiology so the validity of any extrapolations will be greater than for bacteria. In a public-health programme a study may be required to address the biological effects of a fixed transmitting frequency at a particular power density. For example, in this type of study the behavioural response of mice or humans could be investigated in reasonable timescales and costs. However, the fundamental and contentious questions relating to mm-wave bioeffects apply to the nonthermal channelling of energy into vibrational modes. Experimentally, evidence of power or frequency dependence supports the non-thermal effects hypothesis. Microorganisms represent a more convenient system with which to search for these particular effects. Findings in these

systems would form the basis for further study in higher organisms and may have direct relevance to medical microbiology.

Potentially, there are many different assay systems available to study microbial responses to mm-wave radiation. Biosensors are analytical devices that incorporate a biological material and range in complexity from the very simple e.g. ionophores through to intact tissue organs. Some assays operate without disruption to the cell culture such as those measuring turbidity, whereas in other assays destruction of the cell is inherent. In addition, the specificity of an assay is an important consideration. Assays can integrate cell control and metabolism on several levels. The advantage is that the integrated response can reflect many activities simultaneously and therefore maximize the potential to detect a metabolic response.

*4.1.1 Whole cell biosensors: bacterial bioluminescence*

Bacterial bioluminescence was selected as the reporter system  The use of luminous marine bacteria for toxicity testing in reagent format is a product of more than 25 years (Bulich 1977) although its origins can be traced to the military requirements during the cold war (Freeman & Roberts 1982). The intensity of bioluminescent emission provides an instantaneous measure of the organism's metabolic state and, in this case, the knowledge of the actual toxicant and its main mode of action is unimportant. Commercial systems have been developed based on these principles, for example the Microtox™ assay system (Bulich & Isenberg 1981).

The preferred method for quasi-continuous toxicity testing is to use rehydrated lyophilised suspensions of bacteria. In some cases, such as water quality monitoring and measuring oxygen levels in fermentors, this can be laborious and inconvenient. Although the total period of light emission from cultures is about 20 hours (Wardley-Smith, White & Lowe 1975) in practice, the response of luminous bacteria to toxicants will not remain constant during this period (Middleton 1973). In addition,

sequential exposure to toxicants may also degrade performance of the biosensor (Middleton 1973). Therefore, where it is desirable to have a continuous supply of bacteria with constant properties, particularly constant luminosity, a continuous culture device is a potential solution. These have previously been described for the cultivation of luminous bacteria (Zavoruev & Mezhevikin 1982; Zavoruev & Mezhevikin 1983) but can be expensive due to their complexity and are unsuitable for autonomous operation.

*4.1.2 Bioluminescence as a oxygen indicator*

It has been recognized by the scientific community for over 90 years, that light-emitting bacteria may be used as $O_2$ indicators (Harvey 1957). Their unique sensitivity for quantitative measurement of low concentrations of $O_2$ became evident when Harvey and Morrison (Morrison & Harvey 1923) found a value for "just visible luminescence" of a pressure of 0.093 Pa $O_2$. Shapiro (Shapiro 1934) discovered that light intensity response was linear up to a saturation value and therefore could be used as a very sensitive bacterial biosensor. In fact, bacterial bioluminescent probes are more sensitive than other techniques for the measurement of oxygen (Lloyd 1990).

*4.1.3 Assay of toxic compounds by inhibition of bioluminescence*

As oxygen is an essential cofactor for bioluminescence, turning off the stirrer and air supply led to a sharp change in luminous intensity after about 4.5 min when dissolved oxygen was depleted by respiration to below the threshold at which emission is oxygen-limited. Bioluminescence then decreased rapidly, ($t_{½}$ = 0.34 min.) and oscillated above the "residual glow" intensity level (Schindler 1964) with a period of approximately 0.6 min. Restoration of stirring and air supply gave an overshoot, the "excess flash" phenomenon, which has been interpreted in terms of an accumulation of a luciferase complex under anaerobic conditions (Hastings & Nealson 1977). Oxygen measurements may be performed in either continuous flow or batch mode. *Photobacterium phosphoreum* cultured in the bioluminostat was deployed in a batch

system which combined oxygen electrode / light emission measurement capabilities (Lloyd et al. 1981)

Light emission is extremely sensitive to azide and cyanide, exquisitely so to an uncoupler of energy conservation, FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone) and to an inhibitor of ATP-synthase (venturicidin) hence the use of the test as a screen for poisons. Organisms were used from the continuous culture system to determine minimal detectable concentrations of a selection of known toxicants. The results are shown in appendix D. In Fig. 8 the differential effects of phenol at increasing levels of concentrations on the organism *Vibrio fischeri* are shown.

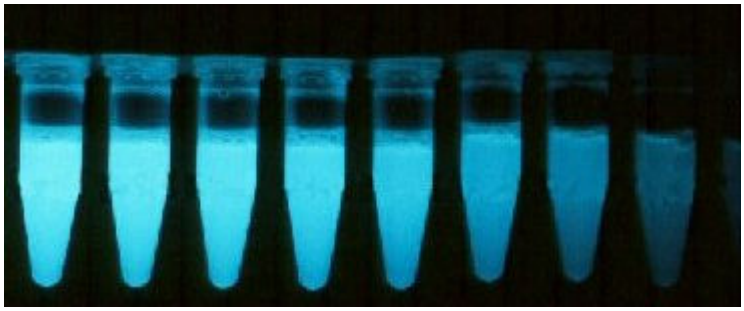


**Fig. 8 Inhibition of bioluminescence in *Vibrio fischeri* with different concentrations of phenol**

### 4.2 Design and construction of the continuous-culture device

Described is a continuous-culture device for cultivation of bioluminescent bacteria over extended periods. Its miniature design obviates some of the problems associated with running earlier devices over long time periods. The bacteria utilize very small volumes of medium and the instrumentation is based on an inexpensive photodiode light detection system which is time-division multiplexed, thus dispensing with the requirement for photomultipliers and high-voltage power supplies. As with other miniaturized continuous-culture devices (Gu et al. 2001) the "bioluminostat" does not require additional instrumentation such as pH and dissolved oxygen sensors. Because of the "complex" response of photobacterium to fresh growth medium, non-standard control methodology is employed.

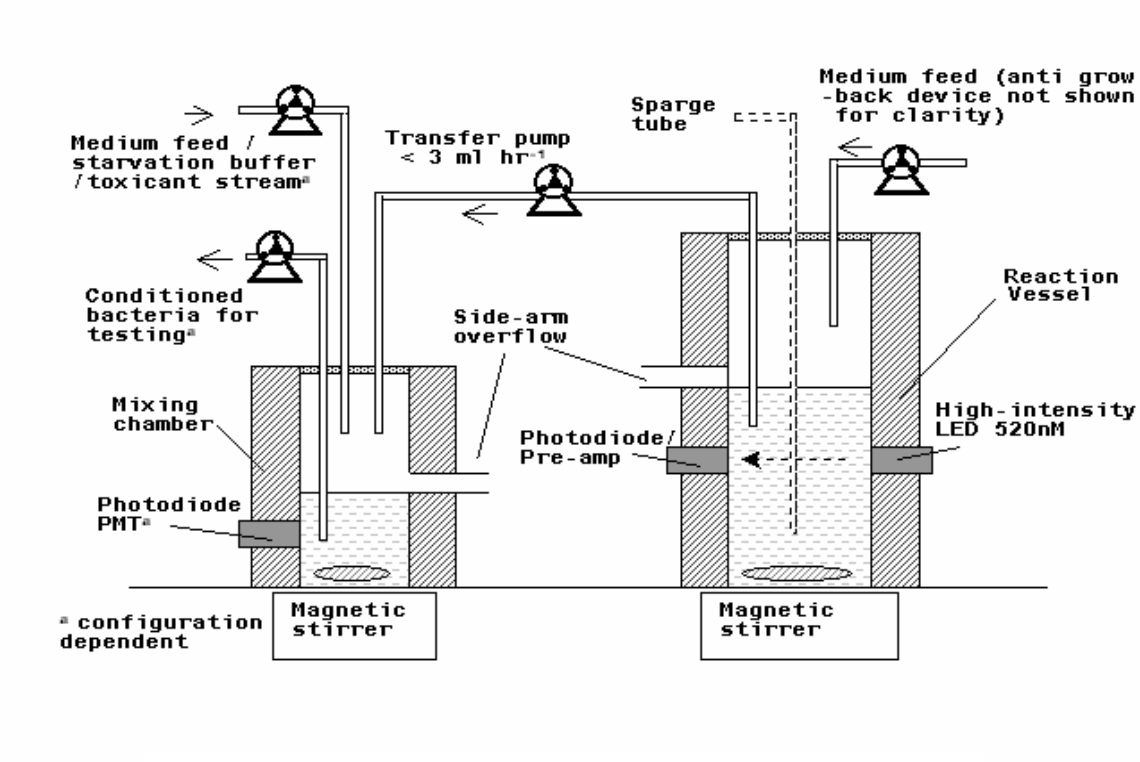



**Fig. 9 Schematic of the continuous-culture device**

*4.2.1 The culture vessel*

The culture vessel consists of a 50 ml "Quickfit" test tube that was modified to incorporate an overflow giving a 20 ml working volume. Attachments to the growth chamber, made via a three-way adapter, consist of a sparge tube, a drying tube acting as air outlet and two splash-heads connected in series to prevent "grow back" into the nutrient supply reservoir. Air was pumped through an in-line filter (HEPA-VENT, 99.97 % $\geq$ 0.3µm, Whatman, U.K.) at a rate of 130 ml $min^{-1}$ oxygenating the culture via the sparge tube. The drying tube was loosely packed with cotton wool to prevent contamination and maintain a small positive pressure difference between the reaction vessel and environment. The culture vessel was mounted on a small-volume magnetic stirrer (Variomag Mono, H+P Labortechnik, Munich, Germany) designed for continuous use and operated at 300 rev. $min^{-1}$. Silicone rubber tubing connections were used throughout. Storage of the media was in 10 l autoclavable vessels, sufficient for continuous operation for many weeks. The culture vessel, stirrers and mixer were housed in a light-tight incubator at 293 K $\pm$ 0.1 K. Temperature stability is crucial with photobacterium as large changes in luminosity can occur with relatively small temperature changes (Waters & Lloyd 1985). The medium reservoir, peristaltic feed pump (same model was used in all pumping applications, 101U/R Watson Marlow, Cornwall, U.K.) and computer were located outside the incubator.

A mixing chamber with a 3.2 ml working volume connected to the culture vessel added flexibility to the system. As the control system actuated media flow intermittently, it was undesirable to couple the culture vessel and mixing chamber directly. Rather, a peristaltic pump continuously transferred organisms from the culture vessel to the mixing chamber at a lower rate than that of the medium feed pump (averaged over 1 hour) so as not to deplete the culture vessel. The side arm overflow maintained constant volume in the culture vessel. The mixing vessel could be configured to dilute organisms with fresh medium or with a starvation

buffer. Starvation buffers have been shown in previous studies to make the biosensor more suitable for oxygen measurements. The dilution rate was typically 10-fold in this second stage. As in the culture vessel, a side arm overflow in the mixing chamber was used to maintain constant volume. The mixing chamber was stirred although owing to the favourable surface area no additional oxygenation was required.

*4.2.2 The light / turbidity monitoring system*

Instrumentation for the measurement of bacterial bioluminescence is usually based on photomultiplier tubes, owing to the high sensitivity they offer in the blue region. Silicon photodiodes are inexpensive, robust, compact, and have low power consumption although spectral response in the blue-green region, where bioluminescence occurs, is rather poor. If a bright strain is selected and the detector positioned carefully in relation to the culture vessel then its response is adequate for the control of the "bioluminostat".

A hybrid 5 $mm^2$ photodetector (OSI5-10M/10k, Centronic Ltd, Surrey, U.K.) combined a photodiode enhanced to operate in the blue spectral range with an integrated operational amplifier. Turbidity was measured optically by detecting the varying intensity of a beam of light (550 nm) passing through the culture vessel. Only one detector was required as the light emitting diode (LED) was driven with a 50% duty cycle. Since the photodetector receives a composite light signal when the LED is active, it is necessary to decode the signals for bioluminescence and turbidity at a later stage. The LED intensity was adjusted to approximately the same value as bioluminescence using a potentiometer. An adjustable gain stage was used to condition the photodetector signal prior to digitization using a differential-mode technique (PCI-6023E, National Instruments Corp, Austin Texas). The acquisition rate and timing were under software control (Labview 6.1, National Instruments). A noise component associated with aeration and stirring of the culture vessel was minimized using a digital low pass filter and circular buffer. The luminosity and

turbidity components of the signal were decoded and had the requisite stability for use in the control system.

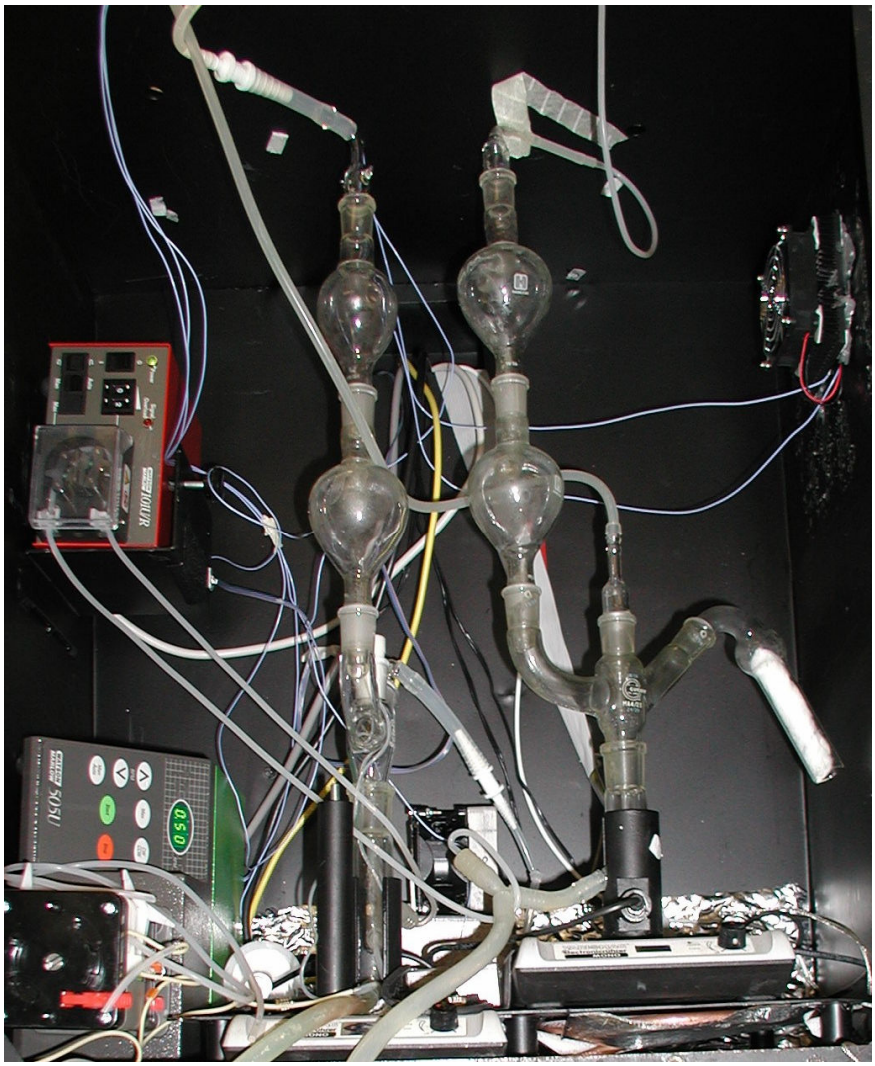


**Fig. 10 Continuous-culture device**

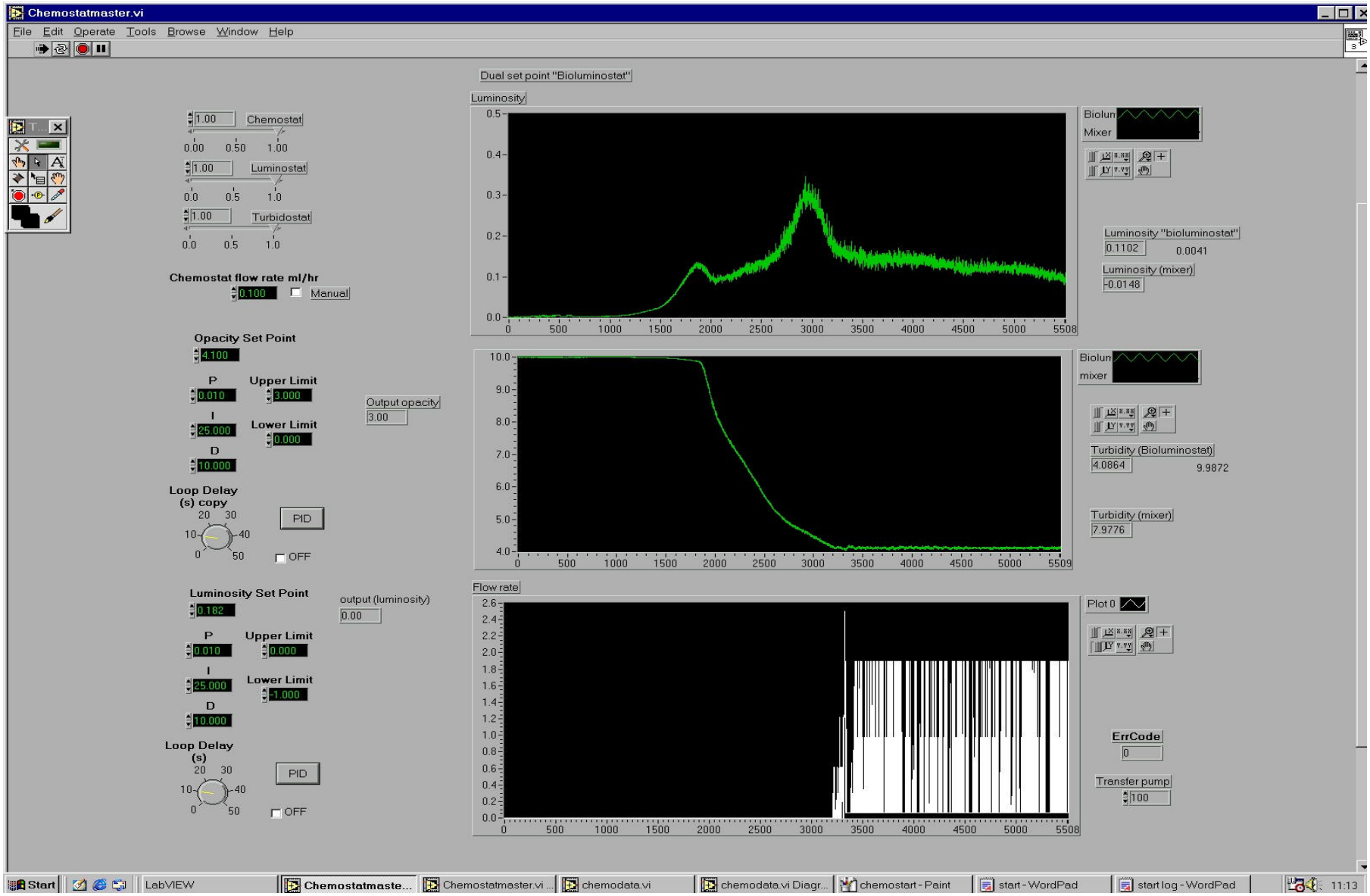
*4.2.3 Control system*

In initial experimentation, the system was run using a single control variable i.e. luminosity with a proportional-integral-derivative (**PID**) controller. Luminosity responded in a complex manner to the addition of fresh medium, falling initially and then recovering. This complexity results from the synthesis of luciferase system and the expression of bioluminescence in growing bacterial cultures being subject to control by many interacting factors. For example, growth rate, oxygen concentration,

N-acylhomoserine lactone autoinducers, temperature, salt and nutrient conditions and absence of catabolite repression, are some of the more clearly identified examples (Meighen 1999; Meighen 1994; Wilson & Hastings 1998). In another preliminary experiment, the continuous-culture system was run using turbidity control only. With the PID controller, it was possible to control turbidity to within ± 1% of the set point over an extended period. PID tuning parameters were determined empirically for each strain of photobacterium cultured. However, luminescence in the turbidostat was inconsistent and light intensity fell over a period of several hours. The bioluminostat ran with approximately $5x10^8$ cfu / ml.

The most successful long-term control strategy was based on a combination of both light emission and turbidity. The normalized output from the turbidostat/luminescence controller was converted into an analogue signal (CIO-DDA06/JR, 12-bit D/A conversion card, Measurement Computing, Mass, U.S.A) and used to drive the pump. The relative weighting of luminescence and turbidity as control components could be selected and optimized for different organisms or applications. During combined control, the mean medium flow rate was approximately 3.7 ml $h^{-1}$ (dilution rate 0.18 $h^{-1}$) for NRRL B-1117 with the transfer pump operating at a maximum of 1 ml $h^{-1}$. With one particular strain NRRL-B-1117 (National Agricultural Culture Collection, IL, U.S.A.), as an alternative to the dual set point control strategy, it was found that steady-state luminescence could be maintained by initiating medium flow intermittently using only turbidity control via a low integral coefficient in the PID system. This regime was maintained by selecting a low figure for the integral component of the controller and a maximum pump flow rate in excess of 15 ml $hr^{-1}$. However, the device still performed better in dual set point mode. Fig. 11 shows the bioluminostat and mixing chamber control panel. The two PID tuning loop control parameters are located in the lower left hand corner. The upper window relates to bioluminescence, the middle to opacity of the reaction vessel and the lower to medium feed pump activity.

**Fig. 11 Continuous-culture device and mixing chamber control panel**



### 4.3 Bioluminescence and the Fröhlich condensate: a conjecture

Speculatively, it may be appropriate to consider the function of bacterial bioluminescence in connection with the Fröhlich effect. Miller *(*Miller et al. 1996) showed experimentally that a continuum light source could act as a pump provided that the photons are absorbed in a system and it meets certain isolation criteria. The pump was driving a Fröhlich-type phonon-mode condensate although in the study monochromatic mm-wave sources performed this function more efficiently.

It is possible that bacterial bioluminescence is in fact a pump driving a Fröhlich condensate in a similar manner to the example above. In the bioluminescence model, light acts as the Fröhlich condensate pump and primary excitation source. Czyz *et al* showed that the *lux* gene expressed in *E coli* had DNA repair enhancing effect that was attributed to a photoreactivation-based repair system. This explanation is somewhat unsatisfactory because the photoreactivation enzyme system operates at a shorter wavelength than the emission spectrum of bacterial luciferase although there is a slight overlap. Rojavin (Rojavin 1995) describes a similar DNA enhancing effect but with mm-wave radiation which could not be mediated through photoreactivation. Light has been identified in important cofactor for bioelectromagnetic effects in a range of other organisms.

**Summary**

**The continuous culture device and mixing chamber are designed to supply Photobacterium to the exposure cell in a consistent physiological state. A dual set point control scheme is used, as growth and bioluminescence are not tightly coupled. This allows the continuous culture device to operate over extended periods. The Photobacterium which have been cultured can be diluted in the mixing chamber .**