Chapter 7

Studies on bacterial colony growth dynamics
by laser induced fluorescence

Abstract

The growth kinetics of an aerial bacterial colony on solid agar media is studied using laser induced fluorescence technique. Fluorescence quenching of Rhodamine B by the bacterial colony is utilized for the study. The lag phase, log phase, and stationary phase of growth curve of bacterial colony are identified by measuring peak fluorescence intensity of dye doped bacterial colony.

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7.1. INTRODUCTION

Microbial growth can be defined as an increase in cellular components. It leads to an increase in cell number when microorganisms reproduce by processes like budding or binary fission. Growth also results when cells simply become longer or larger [1]. Investigating colonial growth of microorganism is of considerable importance both in theoretical as well as applied research. There are many ways to measure microbial growth. The most obvious way to determine microbial number is through direct counting using different techniques such as microscopic counts, membrane filter technique, plate count etc. Microbial growth can also be determined by measuring cell mass. The most direct approach for measuring cell mass is the determination of microbial dry weight. However, no single technique is always the best; the most appropriate approach will depend on the experimental situation [1].

The present chapter deals with Laser induced fluorescence (LIF) technique to study the growth kinetics of an aerial bacterial colony in a closed system. Laser Induced Fluorescence has proven to be a versatile tool for a myriad of applications [2-9]. It is a powerful technique for studying molecular interactions in analytical chemistry, biochemistry, cell biology, physiology, nephrology, cardiology, photochemistry, and environmental science. The first gastrointestinal laser-Induced fluorescence spectroscopy (LIFS) study was performed by Kapadia et al. in 1990 [2]. In an ex vivo study the authors were able to discriminate 16 colon adenomas from hyper plastic polyps with a sensitivity and specificity of 100% and 94% respectively [3]. Rex et al. [4] utilized laser induced fluorescence to determine NADH in experimental neuroscience using an optical fiber probe. Giorgadze et al. [5] measured degree of abnormality of tissue with the help of LIF. Shomacker [6] confirmed the ability of LIFS to differentiate between neoplastic and non-neoplastic tissue with a sensitivity and specificity of 80 and 92%. There are many examples in
biological applications where LIF technique is applied. In bacteriological studies, the LIF has been shown to be a very sensitive analytical tool to distinguish between the two species of bacteria [7].

Study on growth kinetics of bacterial colony using LIF was carried out by doping Rhodamine B dye in culture medium. Rhodamine B is an appropriate dye for doping because of its high fluorescence quantum efficiency [10, 11]. It was found that the concentration of Rhodamine B at 0.3x10^{-4} M was appropriate to give sufficient fluorescence intensity. Higher concentration of the dye effects bacterial growth negatively.

7.2. EXPERIMENTAL DETAILS

Nutrient agar medium containing 0.3x10^{-4} M Rhodamine B dye was used for the study. The nutrient agar was exposed to air for a short duration and then incubated at 25^\circ C at room temperature. In general terms, bacterial colonies grown from single cells on nutrient-poor media show ramified structures, whilst on nutrient-rich media the compact colonies have an overall circular shape with a rough edge. The colonies developed on the surface were observed and a circular colony with regular margin was selected for studying the growth kinetics. Fig.7.1 shows digital photographic images of the dye doped aerial bacterial colony formed on agar plate.

Figure: 7.1. *Aerial bacterial colony formed on dye doped nutrient agar medium*
The selected pure culture was streaked on to fresh nutrient agar plate containing Rhodamine B and the intensity of fluorescent emission was measured. Diode pumped solid state laser of 532 nm (5 mW) with a spot size of 3 mm was used as an excitation source. The power of the laser source was reduced using neutral density filters. The laser was irradiated on to the colony in such a way that it excites the entire colony. A multimode plastic fiber having a core diameter of 980 µm was used to collect the fluorescent emission, which was placed at an angle of 42° with excitation source. The other end of the fiber was coupled to the slit of 0.25 m monochromator-PMT (Mc Pherson) assembly [Fig.7.2]. The size variation of the growing colonies were measured with the help of an eyepiece micrometer of a binocular magnifier.

Figure: 7.2. Experimental setup to study the growth kinetics of bacterial colony by using Laser Induced Fluorescence technique
7.3. RESULTS AND DISCUSSIONS

7.3.1. Effect of Growth of Bacterial Colony on Fluorescence.

The fluorescence spectra of bacterial colony sample marked by Rhodamine B was studied at different days (Fig. 7.3). As seen in this figure the intensity of emission was reduced with number of days due to fluorescence quenching by bacterial colony. So it is clear that fluorescence of dye is strongly quenched by bacterial colony. This indicates that the cultured bacterium was a gram positive bacterium and it was confirmed with gram staining method. The quenching effect of dye by gram positive bacterium is due to its thick cell wall. It consists of a thick layer of peptidoglycan embedded with techoic acids. The peptidoglycan is the binding site of dye and the thick layer blocks the dye from further penetrating into the cell. The quenching effect of the dye is due to the effect of techoic acid [7, 12-14].

![Fluorescence spectra of bacterial colony marked by RhodamineB showing Quenching Effect. (a,b,c represent fluorescence spectra corresponding 4th, 5th, 6th day)](image)
7.3.2. Growth Kinetics of bacterial colony as inferred from fluorescent emission intensity

7.3.2.1. Radial growth of bacterial colony as a function of time.

To study the growth dynamics of bacterial colony, radius of the colony vs time is plotted (Fig.7.4). Radius measurement started after 12 hrs of incubation. From Fig.7.4, it is clear that after 25 hrs of growth, the colony radius R appeared to increase linearly with time. Radial growth rate of the colony (µ_r) is found to be 0.083 mm/hr. The value of µ_r remains constant as long as the growth condition in the peripheral zone do not change. A plot of area of bacterial colony against time is shown in Fig. 7.5. Here area increases exponentially after 25hrs of growth. Rate of exponential growth is calculated to be 0.039 mm^2/hr by theoretical fit. This indicates the exponential growth of bacterial population associated during this period to exponential phase of colony growth. After 85 hrs, the growth was terminated. At initial stage also the value of µ_r is found to be very small. The shape of the bacterial growth curves depends on medium composition and inoculums density [15].

![Figure: 7.4. Radial growth of Bacterial Colony Vs Time](image_url)
Fig. 7.6 shows the typical growth curve of microbes grown in a batch culture or closed system. Here the growth of microorganism is plotted as the logarithm of the number of viable cells versus the incubation time. Since the area of the bacterial colony is proportional to the number of cells in colony it is possible to compare Fig. 7.6 and Fig. 7.5. By comparing these two plots it is clear that the lag phase represents initial points in Fig. 7.5. During the period of 25 to 85 hrs of growth, the area increases exponentially representing exponential or log phase of growth curve. It is clear from Figs. 7.5 and 7.6 that bacterial colony enters into stationary phase after 85 hrs of growth.

7.3.2.2. Peak fluorescence intensity vs time plot

Fig. 7.7 shows peak fluorescence intensity vs time plot of growth of growing bacterial colony. From the figure it is clear that the fluorescence intensity decreases as bacteria grows. The rate of reduction in fluorescence intensity was negligible at initial stage (up to 25hr). By comparing with Figs. 7.5
and 7.6 it is clear that, this stage represents lag phase of bacterial growth curve. Although cell division does not take place right away and there is no net increase in mass, the cell is synthesizing new components. The lag phase varies considerably in length with the condition of the micro organism and the nature of the medium. After 25 hrs of growth the fluorescence intensity decreases exponentially. The rate of decay of fluorescence is found to be 0.034 V/hr by theoretical fit. During this period area of the colony increases exponentially with a rate of 0.039 mm²/hr. It is clear that quenching of the fluorescence of dye by bacterial colony is proportional to the increase in growth rate of the bacterial colony. This can be identified as the log phase or exponential phase of growth curve. In this phase micro organisms are growing and dividing at the maximal rate. That is microorganism are dividing and doubling in number at regular intervals and their rate of growth is constant. The intensity of the fluorescence is constant after 85 hrs of growth. This stage represents the stationary phase of growth curve. Bacterial cells enter into stationary phase mainly because of nutrient depletion and accumulation of toxic waste products. The reason for quenching of fluorescence is due to thick layer of bacterial cell wall as mentioned earlier. Quenching effect of dye is a measure of growth of gram positive bacterial cells.

Figure: 7.6. Microbial growth curve in a closed system
7.4. THEORY OF GROWTH KINETICS OF BACTERIAL COLONY

The cultivation of bacteria on the surface of a solid nutrient agar medium is a general experimental technique. But no satisfactory quantitative theory was so far proposed to describe the development of bacterial colonies. The growth of bacterial colonies on the surface of a solid medium is a common process and by appropriate variation of the environmental conditions a wide variety of colony morphologies can be observed. To study the growth dynamics of bacteria, it is necessary to know the rate of development of microbial growth and size of the colony. A few workers have described simplified model profiles for particular bacterial species [16-20]. Pirt (1975) developed a model that describes the growth of a bacterial colony on solid homogeneous surface [16,21] In a pioneering study of the growth kinetics of surface colonies of bacteria, a virtual constant rate of radial growth for colonies of Escherichia coli, Klebsiella...
aerogenes and Streptococcus faecalis is observed [17]. Julian W et. al described simple method for measuring the profile of bacterial colonies[18].

In the present study, the growth dynamics of the bacterial colony was determined by analyzing fluorescence intensity of dye which is doped in culture medium. From the study it is found that quenching of the fluorescence is in exponential manner in log phase.

Let I be the intensity of fluorescence of dye doped bacterial colony at time t. By assuming that the specific growth rate $\mu$ remains constant, growth equation can be written as

$$\frac{dI}{dt} = -\mu I$$

which gives fluorescence intensity at given time t, which describes growth dynamics of bacterial colony during exponential phase.

7.5. CONCLUSIONS

Growth dynamics of an aerial gram positive bacterial colony on nutrient agar medium was studied using laser induced fluorescence technique. This technique has been shown to be a useful technique for obtaining information about the different growth phase of the bacterial colony. Quenching effect of dye by bacterial colony can be effectively used to analyze growth kinetics of bacterial colony. Quenching effect of fluorescence indicates that cultured bacteria were gram positive. The rate of quenching of fluorescence of dye from bacterial colony was proportional to the rate of increase in area of the bacterial colony which in turn indicates that the rate of quenching of the fluorescence was proportional to rate of growth of bacteria.
REFERENCES


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