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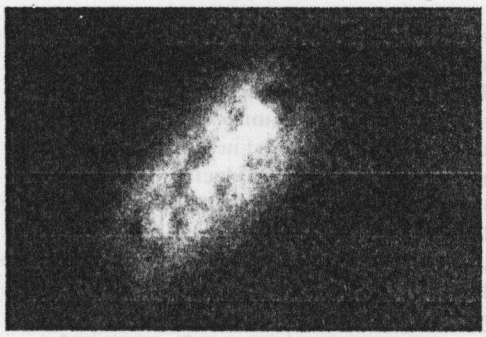
Abstract: Optical fiber based laser induced fluorescence (LIF) measurements were carried out using Rhodamine B to analyze two different species of bacteria, a Gram-positive bacteria namely *Bacillus smithii*, and *Vibrio alginolyticus*, a Gram-negative bacteria. The fiber sensor was clearly able to distinguish between the two species of bacteria. Quenching effect of the dye Rhodamine B by *Bacillus smithii* was observed. The effect of dye on the samples was also studied in detail.



Phase contrast image of *Bacillus smithii*

Laser induced fluorescence based optical fiber probe for analyzing bacteria

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1. Introduction

Laser induced fluorescence (LIF) has been shown to be a very sensitive analytical tool for biological studies. There are lots of applications using LIF technique. Measurement of the degree of abnormality of tissue with the help of LIF was studied by G.K. Giorgadze et al. [1]. A.K. Sankarankutty et al. [2] and O. Minet et al. [3] utilized laser induced fluorescence for analyzing liver under different experimental parameters. These are some examples of LIF technique. The quantum efficiency of fluorophores can change as a function of variations in the local environment of the fluorophore molecule like viscosity, temperature, refractive index, pH, calcium and oxygen concentration, electric field, etc. Measuring fluorescence quantum efficiency is one of the experimental techniques to characterize biological samples. However, this measurement is difficult in the case of biological medium due to the presence of various experimental parameters. Usually, fluorescing dyes are also chosen for LIF based studies apart from the fluorophores present in the cells or tissues. Knowledge of exact spatial distribution of the fluorescing dye makes it optically possible to see different levels of physiological processes in cells at the same time without any repetitive measurements [4].

The availability of coherent optical sources coupled with optical fiber has enabled the bacteriological studies using various optical processes. Optical fiber has been utilized effectively in various LIF based studies. A Rex et al. used optical fiber probes for the determination of NADH in experimental neuroscience by laser induced fluorescence [5].

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The present paper deals with optical fiber based LIF techniques to analyze and differentiate two different species of bacteria namely *Bacillus smithii* and *Vibrio alginolyticus*. The *Bacillii* is a Gram-positive, spore producing bacteria, while the *Vibrio* used in this study is a Gram-negative marine fish pathogen producing exopolysaccharides [6]. Bacteriological studies based on fluorescence are carried out by doping appropriate dyes in the medium. Rhodamine B was chosen for doping because of its high quantum efficiency, strong fluorescence yield and short relaxation time of about 4 ns [7,8]. Concentrations of Rhodamine B ranging from 10^{-4} M to 0.5×10^{-3} M were used. Minimum inhibitory concentration test confirmed the two bacteria were surviving in the dye solution which was used for the study.

The present work contains two parts (1) Distinguish between the two bacterial samples using fluorescence (2) Quenching of fluorescence of Rhodamine B under varying experimental parameters.

2. Optical fiber based laser induced fluorescence (LIF) measurements to differentiate between *Vibrio alginolyticus* and *Bacillus smithii*

2.1. Sample preparation

The bacterial strains used in this study represent the Gram-positive and Gram-negative bacteria, which show characteristic differences in their cell-wall composition. This difference can be visualized by a differential staining procedure called as Gram staining. *Bacillus smithii*, and *Vibrio alginolyticus*, was studied to understand whether this basic difference between them could be observed using optical fiber based laser induced fluorescence (LIF).

The two bacterial samples were each inoculated to Luria Bertani (LB) broth supplemented with 0.5% NaCl. Additional 0.5% of NaCl was added because both *Bacillus smithii* and *Vibrio alginolyticus* are marined in origin. The *Bacillus smithii* culture was kept on shaker, at room temperature for the growth, while the *Vibrio alginolyticus* was incubated in static condition. After 18 hours, 2 ml of culture was drawn from each tube, was centrifuged at 5000 rpm for 6 minutes and the supernatant media is decanted off. Cell pellet was washed with 1ml of sterile de-ionized water by suspending the pellet in water and centrifuged again to settle the pellet. The water was decanted and 20 μl of Rhodamine B (0.5×10^{-3} M) was diluted in water and is added to the cell pellet, and re-suspended the pellet in dye. Then the sample was drawn with a sterile syringe and placed on a clean glass slide and was analyzed in LASER.

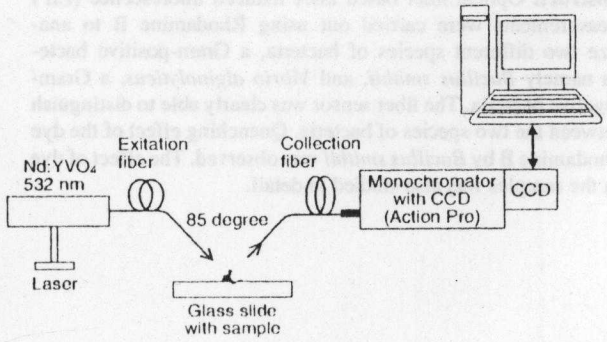

Figure 1 Experimental setup of optical fiber sensor comprising separate excitation and collection fiber arranged at 85° to differentiate bacteria

2.2. Experimental setup

An optical fiber based arrangement is used as a sensor element for the present studies as shown in Fig. 1. Optical lay out is chosen in such a way that excitation and collection fiber were arranged at an angle of 85° [9]. Emission at 532 nm wavelength from a diode pumped solid state laser ($NdYVO_4$, continuous, 532 nm, 5 mW) with a spot size of $\sim$ 3 mm was coupled to a single multi mode plastic fiber having a core diameter of $\sim$ 980 μm. The fiber is positioned in such a way that it excites the entire volume of sample. Another fiber having the same dimension as that of the excitation fiber was used to feed the output to the CCD (Princeton Instruments NTE/CCD detector) assembly. The laser was blocked using a shutter, except during the short intervals required for acquiring spectra. This is important because prolonged exposure of bacteria to light results in photo bleaching of dye [10].

3. Quenching effect of dye by *Bacillus Smithii*

3.1. Sample preparation

Bacillus smithii was inoculated to Luria Bertani (LB) broth supplemented with 0.5% NaCl and incubated, at room temperature on a shaker for the growth, while the *Vibrio alginolyticus* was incubated at room temperature in static condition. After 18 hours, 2 ml aliquot was drawn from each tube, centrifuged at 5000 rpm for 6 minutes, the supernatant media decanted off and the cell pellet obtained, was washed with 1ml of sterile de-ionized water, by suspending the pellet in water and centrifuged again to settle the pellet. The water was decanted and 50 μl of Rhodamine B (0.5×10^{-3} M) was diluted in water and added to the cell pellet, and pellet was re-suspended in the dye. The sample was then drawn with a clean syringe and placed in a 500 μl Durham test tube for further analysis.

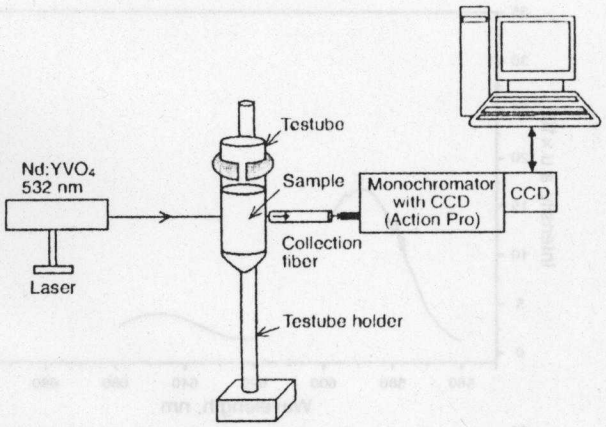

Figure 2 Experimental setup for confirming the Quenching effect of dye by *Bacillus smithii*

3.2. Experimental setup

The sample to be analyzed was taken in a 500 μl Durham test tube. Diode pumped laser of 532 nm was used as the source for excitation. The laser was focused on to the center portion of test tube. The fluorescence from the test tube was collected by a plastic fiber (core diameter of $\sim$ 980 μm and cladding of $\sim$ 20 μm) placed as shown in the Fig. 2, whose other end was fed to CCD (Princeton Instruments NTE/CCD detector) for further measurement. Here also the laser was blocked using a shutter, except during the short intervals required for acquiring spectra. In all the experiments, bacterial cells were grown to late log phase ($OD_{600} = 1$), so that the cell number of the bacteria under experimental conditions were the same.

4. Results and discussion

4.1. Laser induced fluorescence (LIF) based optical fiber configuration setup to differentiate the bacteria

In Fig. 3 the wavelength vs intensity plot of *Bacillus smithii* and *vibrio* are given, the reduction in the rate of fluorescence intensity of *Bacillii* and *Vibrio alginolyticus* samples marked by Rhodamine B was studied. Fig. 3a shows the initial intensity of fluorescence and Fig. 3b shows the fluorescence intensity of *Bacillus smithii* sample after three hours. It is clear from the figure that intensity of *Bacillus* has drastically decreased. Fig. 3c shows the initial intensity of fluorescence of *Vibrio alginolyticus* sample. It is clear from Fig. 3a and Fig. 3c that both *Bacillus* and *Vibrio* had the same fluorescence intensity at the initial time of measurement. Fig. 3d shows the fluorescence intensity of *Vibrio* taken after three hours and from this it is observed

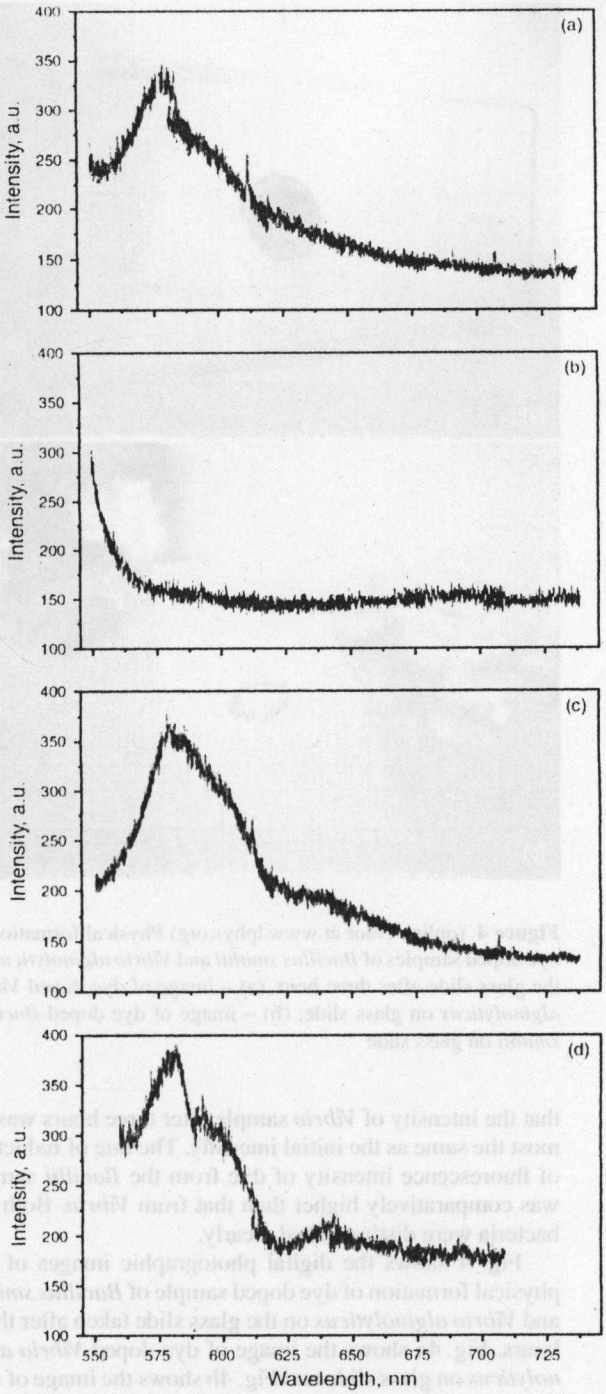

Figure 3 Wavelength vs intensity plot showing the rate of reduction in fluorescence intensity of *Bacillus smithii* and *Vibrio alginolyticus* samples marked by Rhodamine B. (a) – the initial fluorescence of *Bacillus smithii* sample; (b) – the fluorescence intensity of same *Bacillus smithii* sample after three hours; (c) – the initial fluorescence of *Vibrio alginolyticus* sample; (d) – the fluorescence intensity of same *Vibrio alginolyticus* sample after three hours

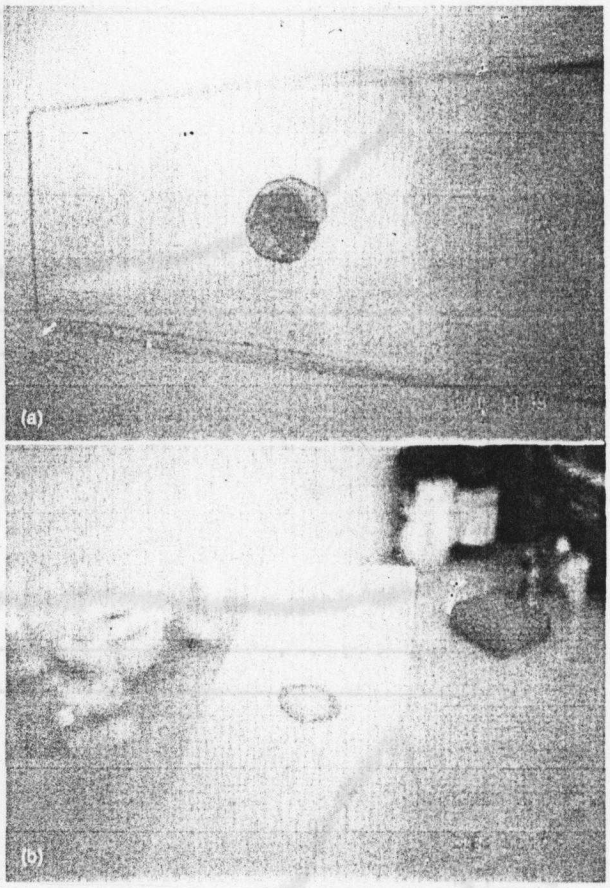

Figure 4 (online color at www.lphys.org) Physical formation of dye doped samples of *Bacillus smithii* and *Vibrio alginolyticus* on the glass slide after three hour. (a) – image of dye doped *Vibrio alginolyticus* on glass slide; (b) – image of dye doped *Bacillus smithii* on glass slide

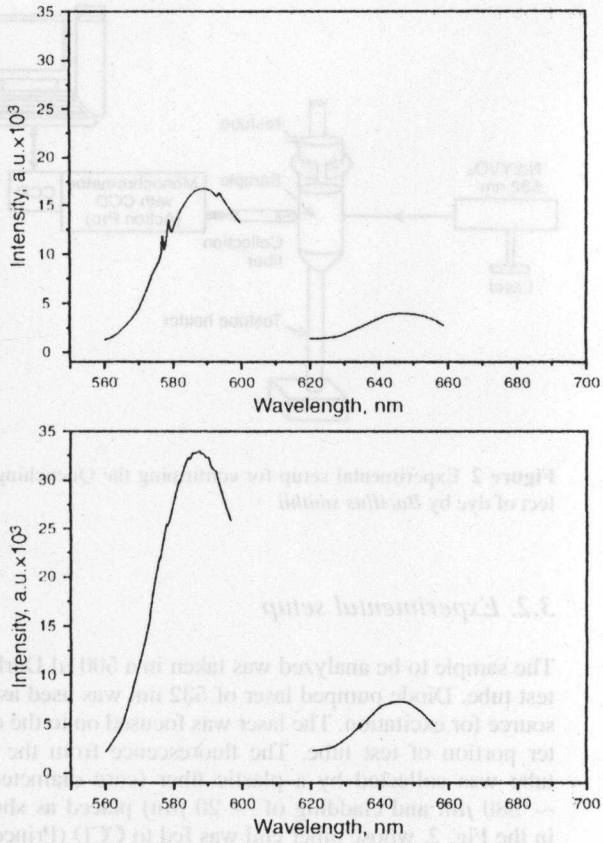

Figure 5 Wavelength vs intensity plot given for both *Bacillus smithii* and *Vibrio alginolyticus*, showing the quenching effect of dye by *Bacillus*. (a) – fluorescence intensity of *Bacillus smithii* taken from test tube; (b) – fluorescence intensity of *Vibrio alginolyticus* taken from test tube

that the intensity of *Vibrio* sample after three hours was almost the same as the initial intensity. The rate of reduction of fluorescence intensity of dye from the *Bacillii* sample was comparatively higher than that from *Vibrio*. Both the bacteria were distinguished clearly.

Fig. 4 shows the digital photographic images of the physical formation of dye doped sample of *Bacillus smithii* and *Vibrio alginolyticus* on the glass slide taken after three hours. Fig. 4a shows the image of dye doped *Vibrio alginolyticus* on glass slide and Fig. 4b shows the image of dye doped *Bacillus smithii* on glass slide. It is very clear from the images that the dye in the *Bacillus smithii* sample was drying off faster.

4.2. Quenching effect of dye by Bacillus Smithii

Fig. 5a shows wavelength vs intensity plot of *Bacillus smithii* and *Vibrio alginolyticus*. Fig. 5a shows the initial fluorescence intensity of *Bacillus smithii* and Fig. 5b shows the initial fluorescence intensity of *Vibrio alginolyticus* measured from the Durhams test tube. The measurement of the *Vibrio* sample was taken two minutes after that of the *Bacillus* measurement. In both the cases, the quantity of sample was the same, which meant that there is not much absorption or evaporation at the initial point, but the intensity of fluorescence of *Bacillus* was very less compared to *Vibrio*, which clearly proves the quenching effect on the dye by *Bacillus*.

It is well known that *Bacillus smithii* belongs to Gram positive bacteria. The Gram-positive cell wall consists of a thick layer of peptidoglycan embedded with techoic acids and a plasma membrane comprising phospholipids with integral membrane proteins traversing the lipid bilayer. Techoic acids could be one of the possible binding sites for dyes. It has been already studied that techoic acids do not bind to cationic Rhodamine B because Rhodamine B has some degree of dipolar ionic character; although over-

all it is predominantly cationic [10]. It seems as though the peptidoglycan in the cell wall of *Bacillus smithii* is been bound by the dye, but the thick layer blocks the dye from further penetrating into the cell. *Vibrio alginolyticus*, a Gram-negative bacteria on the other hand held the dye for a longer time and showed higher intensity of fluorescence than *Bacillus smithii*. Unlike Gram positive bacteria, the Gram-negative bacteria do not have very thick peptidoglycan layer and neither so they have any techoic acid. Thus effect due to techoic acid will not be observed in Gram negative species.

Small parts of zoomed portion of the phase contrast microscopic images of samples are shown in Fig. 6. Fig. 6a shows the *Bacillus smithii* sample, with the cell wall showing the fluorescence which suggests a probability of the peptidoglycan layer having prevented the dye from penetrating inside.

Figure 6b shows the *Vibrio alginolyticus* sample and it is clear that the dye has penetrated inside the cell wall of the bacteria so much so that even the morphology of *Vibrio* has changed. Gram-negative bacteria usually have higher lipid content than positive bacteria. Rhodamine B is lipophilic [11], so there is a greater affinity of the dye towards the lipid layer. This can also be one of the possible reasons why the *Vibrio* held the dye for a longer time as compared to the *Bacillus*.

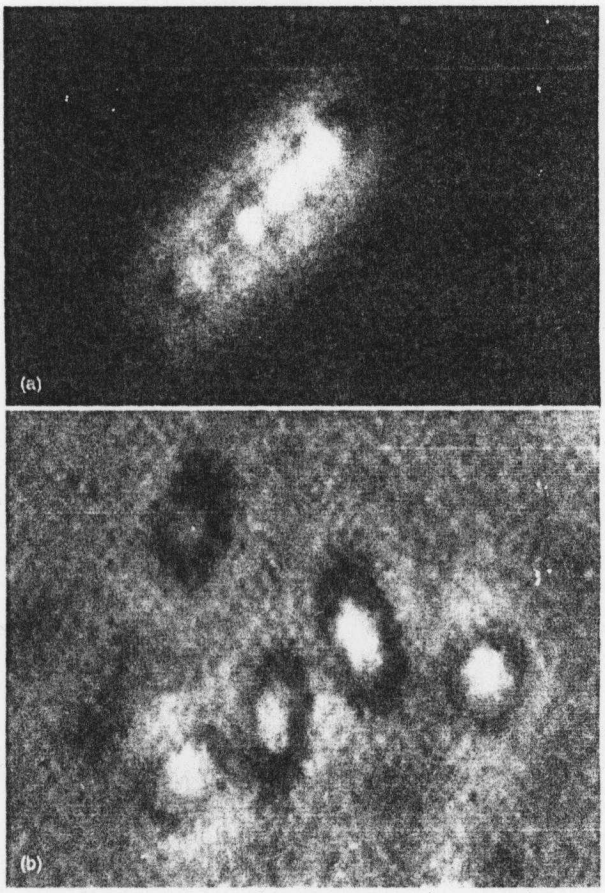

Figure 6 (online color at www.lphys.org) (a) – phase contrast image of *Bacillus smithii*; (b) – phase contrast image of *Vibrio alginolyticus*. These images show that the dye has penetrated inside the cell wall

5. Conclusion

A highly sensitive optical fiber based laser induced fluorescence (LIF) measurements setup was made, the bacteria *Vibrio alginolyticus* and *Bacillus smithii* were distinguished clearly. Quenching effect of dye by *Bacillii* was also observed. *Vibrio algyinolyticus* was holding the dye for a longer period compared to the *Bacillii* bacteria. It was understood that the peptidoglycan layer in *Bacillii* prevented the dye to an extent from penetrating into the cell, whereas in the *Vibrio* it seems the dye has penetrated inside the cell wall. Larger lipid content in Gram negative bacteria is an important factor in holding up the dye for a longer time. *Bacillii* morphology has not been changed noticeably compared with *Vibrio*. MIC test confirmed that both bacteria survived in the dye (concentration 0.5×10^{-3} M and below) used for the analysis, giving us some information about the behavior of dyes on bacteria.

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