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BIOLUMINESCENT BACTERIA: THE SPARKLING HOPE FOR POLLUTION DETECTION

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ABSTRACT

Air and water pollutants have hazardous effect on human health and can cause death of living beings. In this research project, an attempt was made to show the possible use of naturally bioluminescent bacteria in detection of environmental pollutants. The bioluminescent bacterial isolate obtained from marine fish was immobilized in Ca alginate beads and exposed to common water and air pollutants i.e. Arsenic, Mercury, Cadmium, lead and SO_2, H_2S, CO_2 and NH_3 respectively. The results of our study revealed that the bioluminescent beads when in contact with these pollutants showed a decrease in the luminance and the reduction in luminance was proportional to concentration of most pollutants. Arsenic trioxide and mercuric chloride showed up to 99% decrease in the luminance but less effectively for Lead acetate and ammonia. Our study also demonstrated a simple yet innovative and effective way of measuring bioluminescence (without using luminometers) i.e. with a camera phone and free open source software. Positive results of such studies may in future form the basis of developing a (real time) user friendly, portable sensor for detecting the environmental pollutants.

KEYWORDS : Bioluminescent bacteria, Air pollution, Biomarkers

Air pollution causes severe damage to the human body, causing transient changes in the respiratory tract and impaired pulmonary functions. There is also increasing evidence for adverse effects of air pollution on cardiovascular system. In India, air pollution is caused by three main ways; through vehicles, industries or domestic wastes. The Central pollution control board introduced the National Air Quality Monitoring Programme (NAMP) to regularly check pollution across 127 cities but the major limitations of the methods used for monitoring the air pollution is that they need trained man power, 24hrs electricity, regular calibration of equipment, excessive cost etc. Therefore, a biological system for the cost effective detection of the pollutants will be of great use in solving these issues. Key to the development of efficient detection systems is sensitivity of test organisms to even lowest of concentrations of pollutants.

Bioluminescence is a visible light produced by living organisms. Bioluminescent species are found in large numbers across the animal phyla but majority of them dwell in marine habitat (Haddock et al, 1994; Hastings et al., 1983; Shimomura O, 2006). Species within the *Photobacterium* genus are generally light organ symbionts of marine animals, whereas the *Vibrio* species exist as symbionts as well as free-living forms in the sea (Cheng Lin et al., 2009). Bacterial population emit light through a population dependent mechanism called as quorum sensing i.e. they show luminescence upon reaching a certain cell density. The bacterial luminescence reaction, catalysed by luciferase, involves the oxidation of a long chain aliphatic aldehyde and a reduced flavin mononucleotide ($FMNH_2$), generating luciferin (FMN), the oxidized form of the aldehyde, and water, with the liberation of excess free energy in the form of a blue-green light at 490nm (Lin & Meighen, 1991).

The bioluminescent organisms are being studied for varied applications including cancer diagnostics, biomarkers and reporter systems, biosensor etc. One of the suggested applications is using bioluminescent organism for the detection of toxic pollutants. It is said that the intensity of bioluminescence reflects overall health of the organisms (Lin & Meighen, 1991). Previous researches showed that when these organisms comes in contact with any kind of pollutant or toxic organic matter, the intensity of light produced by them decreases (Boynton, 2009 ; Gokhale, et al, 2012). Most of the on-going research work is centred on non-bioluminescent bacteria genetically modified to produce luminescence and used as a tool for detection of water pollutants (Sharma, et al., 2013; Zhao et al. 2013; Charrier et al., 2011). Almost all the environmental researches with bioluminescent organisms are either focused on water or soil pollution monitoring. There is almost no data available where these organisms are used for detection of air pollutants. Most of these studies also require expensive instruments like HPLC/GLC and sophisticated lab set up.

In our study, an attempt was made to evaluate the sensitivity of immobilised bioluminescent organisms to

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toxic heavy metals and gases using a simple method. Thus, this work provides the basis for the creation of biosensors using bioluminescent bacteria for monitoring of pollutants.

MATERIALS AND METHODS

Sample collection and Isolation of bioluminescent bacteria

The sea water samples used for isolation of bioluminescent bacteria was acquired from Bhayandar creek (Mumbai) during winter season and stored in dark at 4°C till further use. Two different fresh marine fish samples *Stolephorus indicus* (Mandeli Fish) and *Harpadon nehereus* (Bombay duck) were acquired from local fish market. These fish were dissected and the area near their gills, scales, eyes and intestines were scraped out with sterile scalpel and suspended a sterile sea water saline. Loopful of these saline suspensions were streaked on sterile Luminescent Agar Plate in duplicates and incubated at 32°C for 24hrs. Plates were inspected in dark and colonies emitting brightest light were selected. The procedure for isolation was repeated until pure culture was obtained.

Selection of best medium for bioluminescence

Three different media; Seawater Complete Medium (SCM), Seawater Medium (SM), and the Luminescent Medium (LM) were streaked with the suspension of selected culture and the medium on which colonies showed maximum bioluminescence was used for further experiments. (Eberhard et al., 1986. Lee et al., 2001)

Immobilization of luminescent isolate

Active bioluminescent isolates were grown in the LM at 32°C for 24 hours and 200 ml of LM broth was mixed with 8g of Sodium alginate. Beads were prepared by dropping dropping this LMSA mixture into a chilled 6% $CaCl_2$ solution using a syringe at a constant rate. After 1 hour, these beads achieved required hardness.

Optimization of luminance in immobilized state

4 grams of prepared beads were incubated in petriplates at 3 different temperatures RT, 32°C and 40°C. The luminescence produced by beads was captured at every 30 minutes interval for a period of over 6 hours.

Preparation and Exposure to air pollutants

Four air pollutants used in this study, included Sulphur dioxide (SO_2), Ammonia (NH_3), Hydrogen sulphide (H_2S) and Carbon dioxide (CO_2) prepared and used in gaseous form (Gupta, 2012).

Three concentrations of individual gases were tested on the beads. Beads were exposed to gases for 15, 30, 60, and 120 seconds at 32 °C in an enclosed chamber. After every 15 sec of exposure, the image of the luminescent beads was captured along with a control set which was unexposed to pollutants.

Exposure to water pollutants

The water pollutants used for this study included 0.01M solution of AS_2O_3, $HgCl_2$, $CdCl_2$, $Pb(CH_3COOH)_2$. A Range of dilutions of these solutions (up to 10^3 dilution) were prepared. 4gms of the luminescent beads were exposed to 3ml dilution of each pollutant individually in a petriplate. The control set had three ml of D/W added. The liquid pollutant was expected to diffuse during the exposure time and penetrate into the bead matrix thereby reaching the immobilized bacteria. After 15 sec, the intensity of light emitted was captured.

Device for capturing luminance and quantitative measurement of luminance intensity: In all of the above mentioned experiments, the luminance was captured (in triplicates) on a high quality camera, Nokia Lumia1020 (42MP) from a constant distance of 20 cm. The shutter speed of the camera was adjusted to 4 seconds with 4000 ISO. Three images of luminescent beads were captured sequentially and analyzed later quantitatively for brightness intensity using image processing software Image-J-fiji (an image processing open source package of ImageJ, which facilitates scientific image analysis) (Schindelin et al. 2012).

RESULTS AND DISCUSSION

The bacterial culture isolated from the gills of *Mandeli* fish showed intense blue bioluminescence which remained visible for two days (Figure 1, a). No other sample showed growth of such intense bioluminescent bacteria. It has been observed by several scientists that most marine bioluminescence is expressed in the blue-green part of the visible light spectrum, since these colours are more easily visible in the deep ocean. Also, most marine organisms are sensitive only to blue-green colours and are physically unable to process yellow, red, or violet colours. There are three major genera, into which most luminous bacteria are classified: *Photobacterium*, *Vibrio*, and *Photorhabdus*.

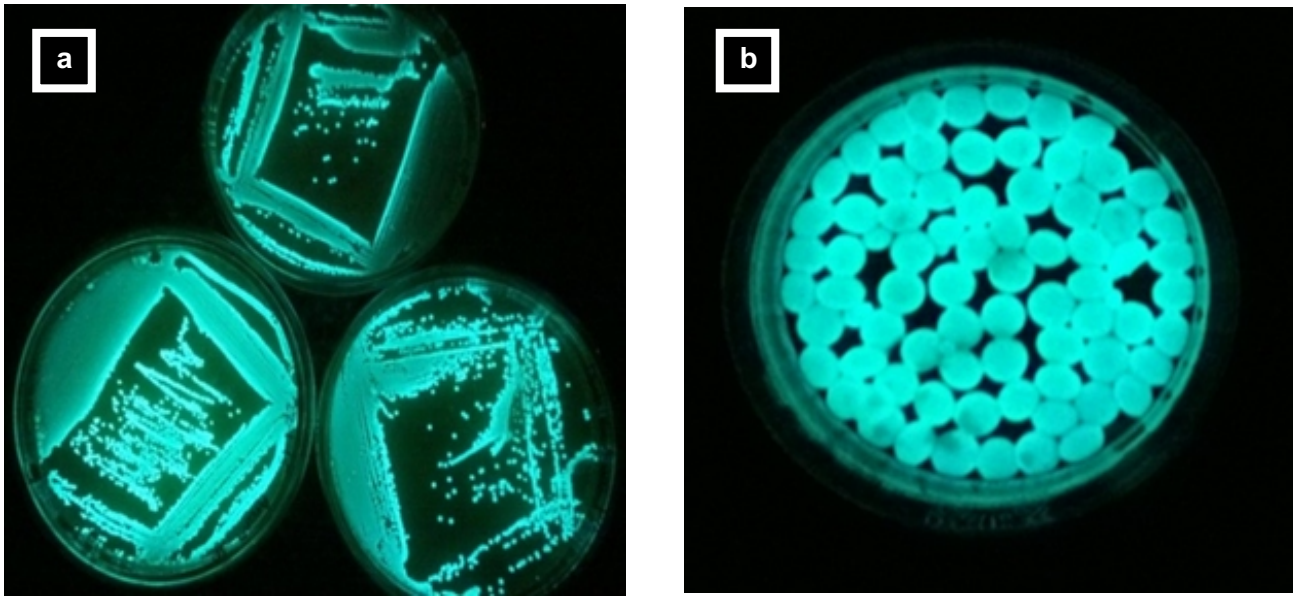


Figure 1: (a) Pure culture of bacterial isolate from fish on LMSA captured in dark
(b) Bioluminescent bacteria trapped in Ca alginate beads

Identification of bioluminescent bacteria

The Gram staining of the isolate showed Gram negative plump rods. Since the isolated bacterium is not curved shaped, it may not be *Vibrio* and the light intensity seemed good enough, for it to be a Photobacterium species, but it is yet to be identified. 16s RNA sequencing or other techniques are needed to confirm the genus and species of the isolated bacteria. Bioluminescent isolate showed its best luminance when grown on Luminescent medium (LM) as compared to the other media.

Optimization of luminescence in an immobilized state

The whole-cell immobilization method offers advantages like high sensitivity, low cost, large test populations, and rapid responses. For this, the pure culture of bioluminescent isolate grown in Luminescent medium (LM) was trapped in Ca alginate matrix which is a very commonly used class of hydrogel immobilization matrices and provides higher surface area, porosity and diffusion of O_2 which leads to better luminance production (Figure 1,b). Increasing temperature is a powerful way of accelerating diffusion and increasing the concentration of the chemicals inside the beads, but high temperatures may inhibit luciferase activity. In our study, the highest luminance intensity was observed at the temperature of 32ºC after incubation time of 120 minutes (Figure 2). The light intensity didn't decrease further till 2 hours or more and this time period is the best for study of effect of pollutants on luminance intensity of the immobilized bacteria.

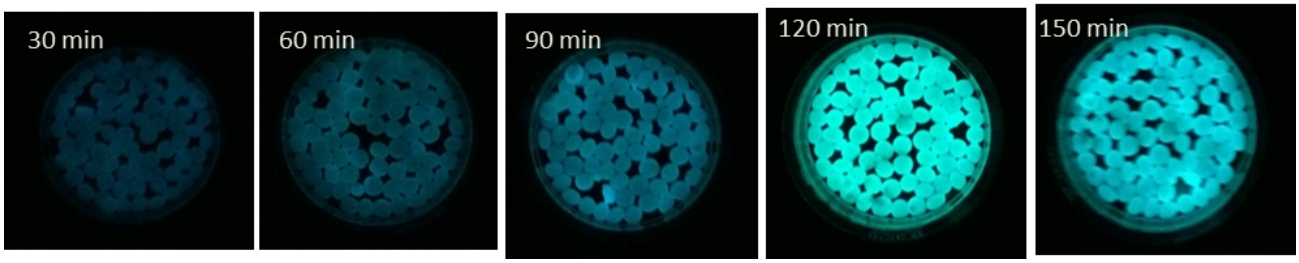


Figure 2: Determination of optimum time for maximum luminance of Ca alginate beads
Maximum luminance was seen at 32 ºC, 120 min after preparation

Hence the beads used for taking test readings were always freshly prepared and incubated for 120 min, for maximum luminance. One reason for the beads to produce higher luminance after incubation could be the fact that marine luminescent symbionts living in a confined localized nutrition-rich environment emit a high level of light. In order for the light emission to occur, luminous bacteria have to grow in environments in which the autoinducer can be accumulated. Therefore when the concentration of the autoinducer increases to a certain level in the extracellular environment, it will activate the luminous system of these bacteria (Lin et al., 2009). Another reason for the beads to produce higher luminance after incubation could be the circadian cycle which is observed in some bioluminescent organism.

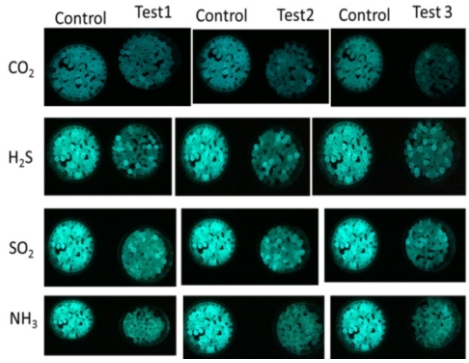
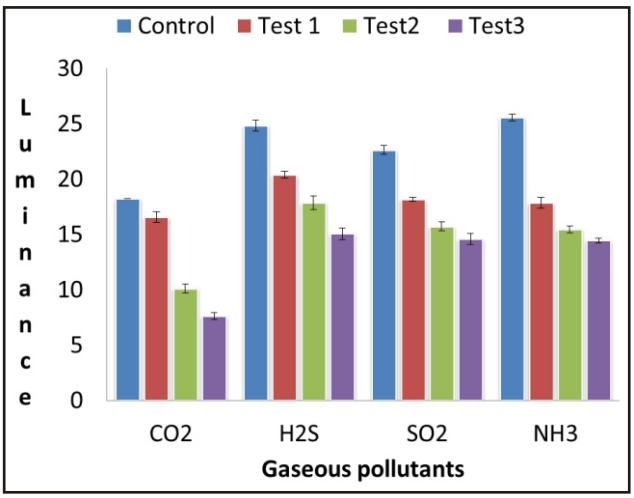


Figure 3: (a) Effect of different air pollutants on the luminance of immobilized bioluminescent bacteria (b) Graph showing Effect of gaseous pollutants on alginate immobilized bioluminescent bacteria after 15 sec exposure. Control showed brighter luminance compared to test (three different concentrations of pollutant gases: test1-low, test2 intermediate, test 3-high concentration)

The results obtained by exposing luminescent beads to three different concentrations of air pollutants (with test 1 having lowest and test 3 having highest concentration) showed that the luminance of the alginate immobilized culture was substantially affected (Figure 3, a). At high concentration, the reduction in luminance was 35%, 39%, 43% and 58% when exposed to So_2, H_2S, NH_3 and Co_2 respectively. In case of Co_2, cells were less sensitive at low concentration (only 9% reduction) while most sensitive to low concentration of NH_3 (Figure 3, b). In general, the luminance intensity decreased proportionately as concentration of air pollutants increased. However, the beads which were exposed to ammonia gas showed less variation in reduction of luminance at different test concentrations compared to other gases.

These beads were also used to study the effect of water pollutants on the light produced by the bacteria (Figure 4). Arsenic trioxide and Mercuric chloride could decrease the bacterial bioluminescence (up to 99%) as compared to unexposed control. There was a substantial reduction (26-35%) even at lowest concentrations of these metals (i.e. 0.197 ppm and 0.271 ppm respectively). The undiluted (0.01M) Cadmium chloride solution was also able to inhibit the luminance of bacteria as in case of other heavy metals. The luminance of the beads however, didn't decrease as much when exposed to the solution of lead acetate (only 45% reduction).



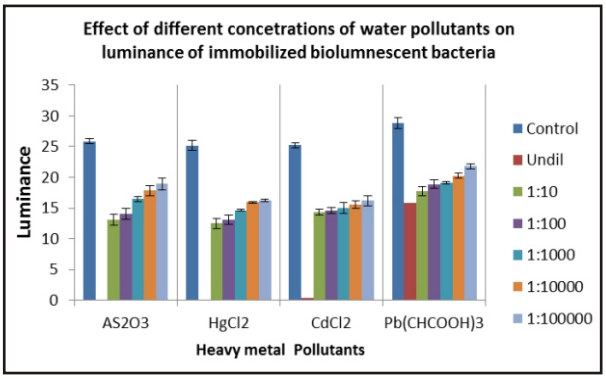


Figure 4: heavy metal pollutants As, Hg, Cd (0.01 M-undiluted) completely inhibited bioluminescence of immobilized bacteria while lead (Pb) resulted in less reduction in luminance compared to control. Sensitivity was seen even at very low concentration (0.00001M) of these heavy metals

In general, increasing the concentration of all pollutants tested, decreased the luminance of the immobilized cells in a dose-dependent manner. Previous researches have shown that when bioluminescent organisms get affected by toxic elements, their intensity of light emitted decreases (Li Boynton, 2009, Gokhale et al., 2012, Zhao, 2013). Most of the research on pollution monitoring done till date, however, is focussed on genetically engineered bioluminescent organisms and unlike our studies no other work revealed lowest concentration of the water pollutants which decreases in the luminance of naturally bioluminescent bacteria. Conventional methods allow for highly accurate and sensitive determination of tested air samples; but exhibit limitations such as the need to collect air samples on-site and thereafter transport them for analysis to a sophisticated laboratory with expensive facilities like gas chromatography (GC), or high-performance liquid chromatography (HPLC) and fluorometry. All these techniques require skilled personnel and are time-consuming. They provide detection of only single or a group of structurally related compounds at any given time. Our study demonstrated a simple but innovative way of measuring bioluminescence using easily available and portable device, i.e. camera phone and free open source software. Such system won't need, trained people or electricity to function and regular calibration of instruments. It will be much cheaper, hence, easily affordable.

In conclusion, immobilised bioluminescent bacteria can be of great use in air and water pollution monitoring. However, specific research is needed on stabilizing the luminescence of the beads or reviving the luminescence of the stored beads. This will form the basis of developing a (real time) biosensor for rapid monitoring of air and water pollution in different environments.

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