Biochemical and molecular basis of bacterial bioluminescence and its utilization as a biosensor for the assessment of environmental toxicity

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***Abstract*: Biochemical and molecular basis of bacterial bioluminescence and its effect on pollutants is of great importance. As the ill-effects of environmental pollution increases, there is an utmost need for developing reliable tools for rapid assessment of the potential toxic and genotoxic effects of environmental samples. Bioluminescence can be used as a reliable reporter for the assessment or monitoring of various aquatic samples containing toxicants such as pesticides, PCBs, aromatic hydrocarbons, fuels, alkanes, alcohols and heavy metals because of its high degree of response and low cost of performance. This review intends to investigate the physiological, biochemical as well as genetic diversity and relationships among bioluminescent bacterial strains isolated from various beaches of India. Present analysis is also targeting to explain the potential use of newly isolated strains in construction of heavy metal bioassay or biosensor to monitor the heavy metal contaminants in water samples.**

***Keywords*: Bioluminescence, Bacteria, Luciferase, Lux Gene, Biosensor, Autoinducer, Environmental Toxicity.**

INTRODUCTION

Bioluminescence is a light production phenomenon performed by means of chemical reactions or cellular secretion existing in the cells of living organisms or in the cells and organs of the symbiotic organisms which live together with them. Luminescent bacteria are the most abundant and widely distributed amongst the light-emitting organisms. The mechanism of the bioluminescence reaction is catalysed by luciferase which produces light by the oxidation of a light-emitting molecule called the luciferin. The luciferase enzyme encoded by the luxAB genes catalyses the unique light producing bioluminescence reaction. For this light reaction, dissolved oxygen, reduced flavine mononucleotide and a long chain aldehyde coded by luxCDE genes are vital substrate requirements.[1] The DNA sequences which encode the proteins in the luminescent system are known as the 'lux genes'. The two polypeptide chains consisting of the luciferase enzyme are encoded by luxAB genes. The aldehyde required for the reaction is synthesized by an enzymatic complex. These enzyme complex are encoded by three different genes present in all bioluminescent bacteria, luxCDE. The three proteins that form the enzymatic complex consist of a reductase, a synthetase and a transferase. Other proteins that are involved in the reaction are only present in particular species of bacteria. Both are called accessory proteins because they are not crucial for the basic reaction completion.[2] Nowadays the most interesting object of research is the prospective use of bacterial bioluminescence trait for deciphering various basic and applied problems. The biochemical and molecular basis of bioluminescence and its effect on pollution is of great importance. As the ill-effects of environmental pollution increases, there is an utmost need for developing reliable tools for rapid assessment of the potential toxic and genotoxic effects of environmental samples.

Bioluminescence can be used as a reliable reporter for the assessment of various aquatic samples containing toxicants such as pesticides, PCBs, aromatic hydrocarbons, fuels, alkanes, alcohols and heavy metals because of its high degree of response and low cost of performance.[3] Since marine luminous bacteria are ecologically resourceful, utilize various nutrients, occupy many niches in the marine environment and their bioluminescence being extremely sensitive to the numerous toxicants. They are appropriate bioassay candidate for detecting nano or picomolar concentrations of impurities in water bodies, pharmaceuticals and in the food industries.**[4]** This review intends to investigate the physiological, biochemical as well as genetic diversity and relationships among bacterial bioluminescent strains isolated from various beaches of India. Present analysis is also targeting to explain the potential use of newly isolated strains in construction of heavy metal bioassay or biosensor to monitoring the heavy metal contaminants in water samples.

BACTERIAL BIOLUMINESCENT

Almost all luminous bacteria have been classified into the three genera *Vibrio, Photobacterium, and Xenorhabdus*, with most of the species being marine.**[2]** Among different isolates of each species, there exist significant differences between their lux operon systems. Other species of luminescent bacteria are *Vibrio logei, Vibrio splendidus, Vibrio cholerae* and *Altermonas haneda*, classified in the genus *Alteromonas.*[5]

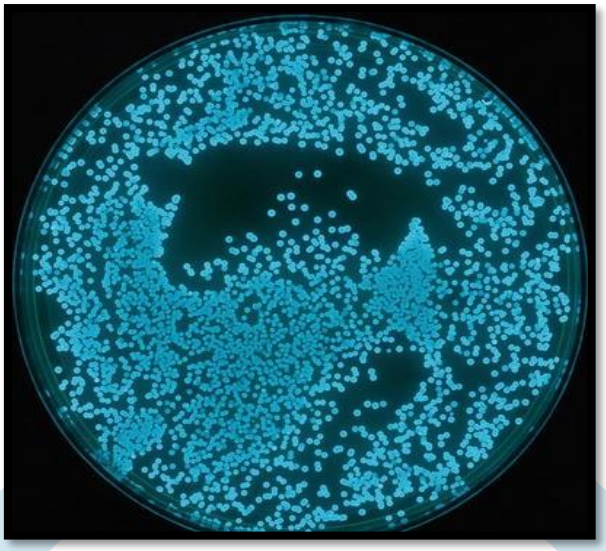

Fig 1 : Luminous Bacteria (The Prokaryotes – Prokaryotic Physiology and Biochemistry[6])

Because most strains classified in the genus *Photobacterium* have been isolated as specific light organ symbionts of fishes, it appears that this may be their natural habitat[7]. Luminescent bacteria found as symbionts in deep-water fish are primarily *Photobacterium phosphereum*, whereas symbiotic luminescent bacteria in fish in shallow and temperate waters are *Photobacterium leiognathi* or *Vibrio fischeri* (Fig.1*). Photorhabdus luminescens* has only been found infecting terrestrial organisms primarily acting in symbiosis with nematodes in a parasitic infection of caterpillars.[8]

***Vibrio fischeri,* the most studied luminescent bacterium**

The family Vibrionaceae are motile, Gram-negative rods that are natural inhabitants of sea water but can be found in fresh water. A bioluminescent bacterium *Vibrio fischeri* (Fig.2), is often found in symbiotic relationships with marine animals like the bobtail squid. Their bioluminescence stems from the expression of genes contained in their *lux* operon system. *Vibrio fischeri* is an oxidase-positive, Gram-negative bacterium, composed of a cell wall that consists of an outer membrane containing lipopolysaccharides, a periplasmic space with a peptidoglycan layer, and an inner, cytoplasmic membrane.[9] Bioluminescence of *Vibrio fischeri* is controlled by a small set of genes known as the *lux* operon.

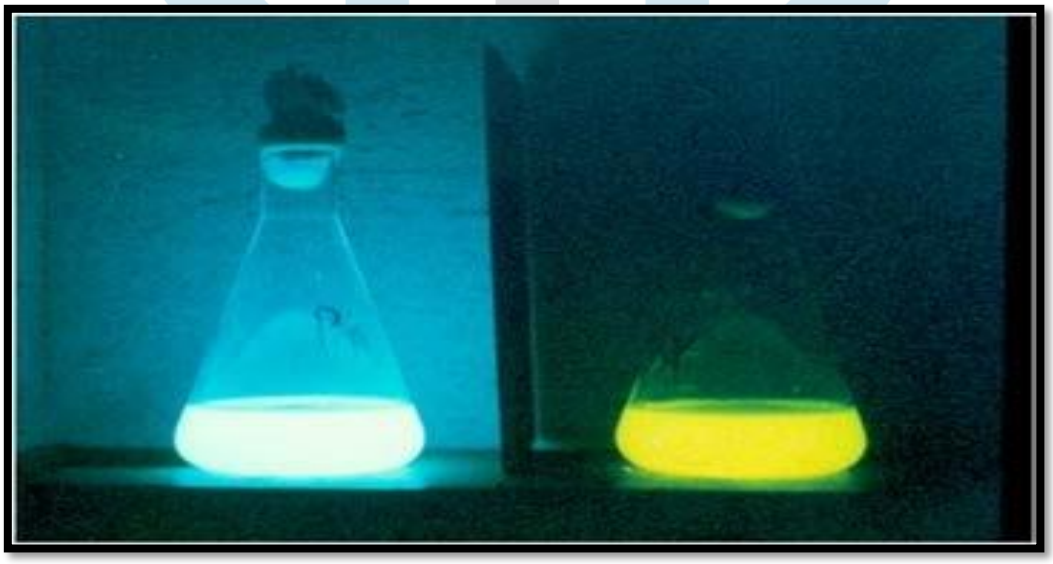

Fig 2 : Bacterial cultures of *Photobacterium phosphoreum* on the left and *Vibrio fischeri* Y-1 on the right. The light emission in blue and yellow, respectively[10]

Photobacterium phosphoreum

Photobacterium phosphoreum is another luminescent organisms, found in marine environment. *Photobacterium phosphoreum* is a light organ symbiont (Fig.2), living in the gut of the fish where metabolites are provided in exchange for bioluminescence, which is used for communication, prey attraction, and predator avoidance.[11] *Photobacterium phosphoreum* is typical of deep sea fishes, but can also be found in dim and dark versions. The gut of the fish, where *Photobacterium phosphoreum* is cultivated, is connected

to some light organ on the fish which can be controlled with shutter apparatuses. The gene products of *lux*A, α-luciferase, are necessary for the light-emitting reaction of all known luminous bacteria. Unlike the *Vibrio lux* system, *Photobacterium phosphoreum* has a new gene, *lux*F which is located in the *lux* operon between *lux*B and *lux*E. The functions of the *lux*A to *lux*E gene products are known to be involved in the luminescent pathway, therefore *lux*F likely has the same function. The organization of the *lux* operon in *Photobacterium phosphoreum* is as follows: *lux*C *lux*D *lux*A *lux*B *lux*F *lux*E.
In *Photobacterium phosphoreum*, *lux*A and *lux*B gene products are luciferase subunits and were shown to catalyze light emission in the presence of FMNH2, O_2, and aldehyde. The *lux*C, *lux*D, and *lux*E gene products are fatty acid reductase subunits and are responsible for aldehyde biosynthesis. The new gene, *lux*F, was found to code for a new polypeptide of 26kDa.[12]

ECOLOGICAL SIGNIFICANCE OF BIOLUMINESCENCE

The function of events of light emission in higher organisms generally falls under three (3) kinds: to help in predation (offense), to support in avoiding predators (defense) and for intraspecific communication such as courtship. Bacterial bioluminescence predominates in marine ecosystems, particularly among fish. Studies of marine bioluminescence have provided great understanding on symbiotic relationships particularly from the *Euprymna scolopes* and *Vibrio fischeri* mutualism.[13]
The ecological significance of bioluminescence is of great importance. Entire organism is utilized to assess the potential biological impact (toxicity) of a water or soil sample. These systems are based on the use of luminescent bacterium, *Vibrio fischeri*, to measure toxicity from environmental samples. Bacterial bioluminescence has proved to be a convenient measure of cellular metabolism and consequently, a reliable sensor for measuring the presence of toxic samples.[14] Bioluminescence has aided us to understand the intricacies of microbial ecology. It has led to significant discoveries on how bacteria interact with higher organisms and among themselves. This has permitted the understanding of symbiotic associations and the discovery of bacteria communication.

BIOCHEMISTRY AND GENETIC CONTROL OF BACTERIAL BIOLUMINESCENCE

Bioluminescence is chemiluminescence that requires an enzyme (luciferase). Early studies in the elucidation of the bacterial bioluminescence mechanism[15] suggested that a series of steps would be involved in bioluminescence. Initially, it was proposed that one molecule of reduced flavin mononucleotide (FMNH2) was utilized to reduce luciferase. These conclusions were modified a year later, when two reduced flavin molecules instead of one were found to be involved.[16] Another research group[17] suggested that during bioluminescence one molecule of FMNH2 combined with oxygen to form a highly reactive organic peroxide while the other combined with an aldehyde to form an aldehyde-FMNH2. These reactions were believed to account for the energetic, but it was difficult to reconcile this with its spectral requirements.
Bacterial luciferase catalyzes light emission at the heart of bacterial bioluminescence. Nevertheless, these catalytic machinery which involved in the continuous light production in luminous bacteria includes not only bacterial luciferase, but also the enzymes that supply and regenerate the substrates of bacterial luciferase. The DNA sequences which encodes the proteins in the luminescent system are known the lux genes. Bacterial luciferase is a heterodimer, consist of two different polypeptides, named alpha and beta (of molecular mass 40 kDa and 37 kDa), respectively, and encoded by the *lux*A and *lux*B genes, respectively (Fig.3A&B). The active site is located within the subunit.[18] Without beta subunit, the alpha subunit alone functions not efficiently and as a result, yields poor light.

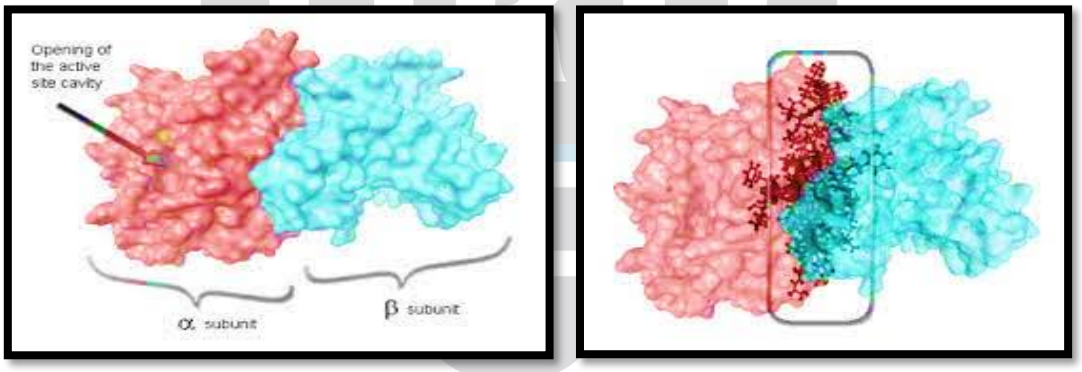

Fig. 3A : Bacterial luciferase structure.[19] Fig 3B: The network of extensive inter-subunit interactions, highlighted in the rectangular box, and involving ionic attractions, hydrogen bonds, and hydrophobic contacts, is responsible for the assembly of the functional bacterial luciferase.[19]

The substrates of bacterial luciferase are reduced flavin mononucleotide (FMNH2), molecular oxygen, and long chain fatty aldehyde (Fig.4). The excess energy, which is liberated from the oxidation of FMNH2 and aldehyde concomitant with the reduction of molecular oxygen, is released as blue/green light emission (λ MAX ~ 490 nm).

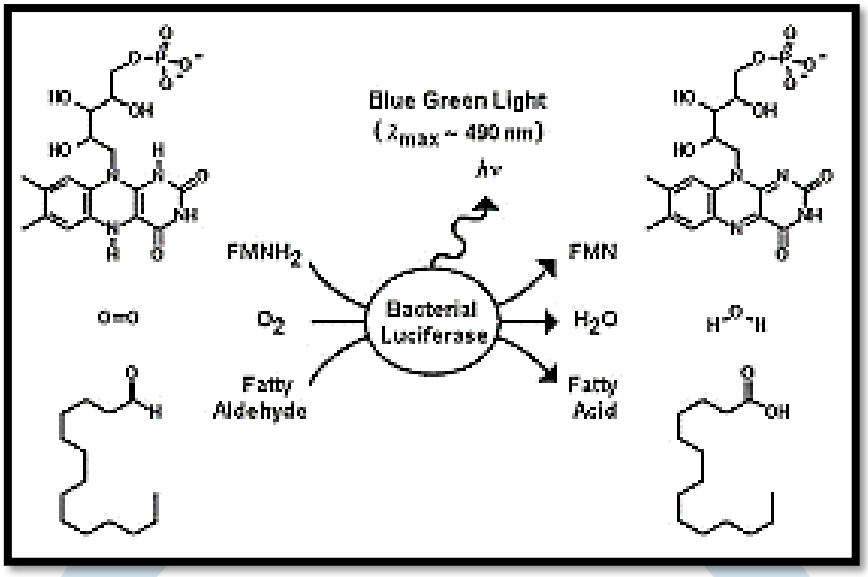

Fig 4: The net chemical equation of the bacterial luciferase catalyzed reaction[19]

The colour indicates the energy level of the photon. This photon was produced when the excited electron on the flavin chromophore returns to the ground state. Researchers have discovered that flavin analogs with substituted atoms in the chromophore moiety resulted in different luciferase emission colours.

In addition, it has also been shown that the colour emission spectrum of bacterial bioluminescence are distorted by point mutations at the flavin chromophore's binding site. This indicates distinctive emission colour depends not only on the chromophore that emits the photon, but also the electronic nature of the chromophore-binding microenvironment in luciferase. Apart from bacterial luciferase, some luminous bacteria carry fluorescent proteins to regulate the emission colour, isolating themselves from other strains. Interaction of blue fluorescent protein (also known as the lumazine protein) in *Photobacterium phosphoreum* and *Photobacterium leiognathi* with the respective luciferases yields photon emission at higher energy (λ MAX~478 nm) corresponding to a blue colour. The yellow fluorescence protein present in the strain Y-1 of *V. fischeri* resulting in yellow light emission (λ MAX ~545 nm). The participation of the fluorescence proteins in the luciferase catalyzed reaction also alters the reaction kinetics of bacterial luciferase.[20]

The molecular basis for the change in emission colours favours the ability of fluorescence protein to interact (e.g., stabilize or destabilize) with the high-energy luciferase reaction intermediate (e.g., excited chromophore) resulting in different colors of light emission. Nevertheless, the energy input is clearly provided by the oxidation of FMNH2, and aldehyde and bacterial luciferase. For continuous light emission for prolonged period, substrates must be supplied to bacterial luciferase continuously. As the time and the level of generation of product in enzymatic reactions are limited by the availability of the substrates, the constant light emission in luminous bacteria must be brought up by several different enzymes which continuously generates the substrates for the bioluminescence reaction. Those enzymes that replenish the aldehyde substrate are coded on the *lux* operon; in particular, the fatty acid reductase, a multienzyme complex, whose lux genes *(luxC, luxD, aud luxE)* immediately flank the *luxA* and *luxB* genes of luciferase. Other genes including *lux*F, *luxG*, and *luxH*, whose functions are neither clearly defined nor apparently necessary for bioluminescence are also found in some *lux* operons[20] (Fig.5).

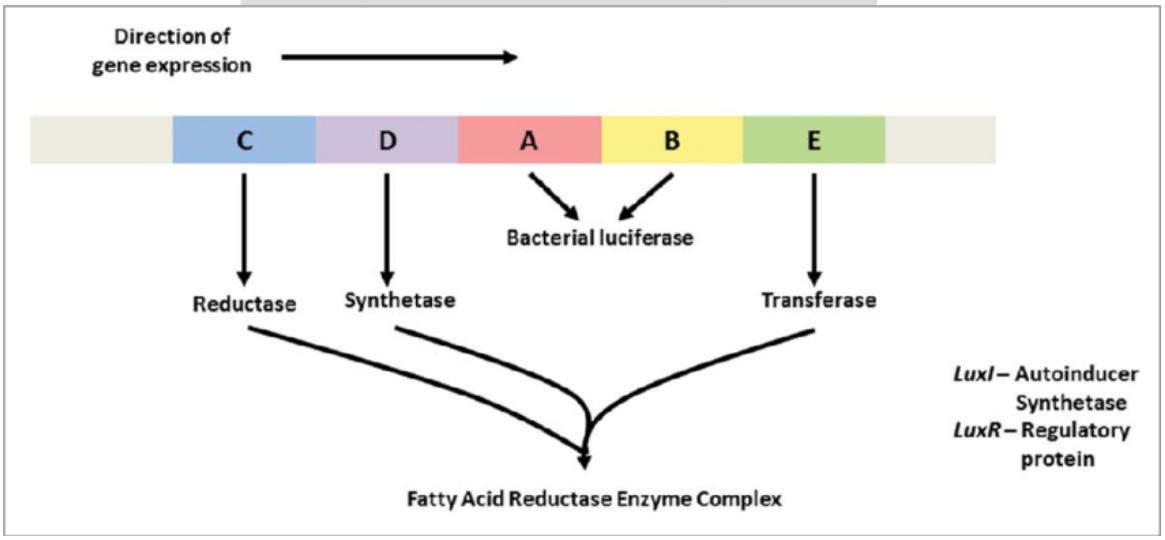

Fig 5: Fatty Acid Reductase enzyme Complex The arrangement of the *lux*CDABE open reading frames[20]

REGULATION OF BACTERIAL BIOLUMINESCENCE

Bioluminescence in bacteria can also be regulated through a phenomenon known as autoinduction.[21,22,23] Autoinduction or quorum sensing was first reported in *Vibrio fischeri*. It is cell-to-cell communication that regulates gene expression to bacterial cell density. Quorum sensing involves the self-production of a diffusible pheromone known as an autoinducer (AI). These AI serves as an extra cellular signal molecule that accumulates in the medium and stimulates a characteristic response from cells.[24] In bioluminescence, the concentration of the AI when touches its specific threshold (above 107 cells mL-1), it evokes the synthesis of luciferase and other enzymes involving in luminescence. The cells can sense the level of AI and therefore they are able to estimate their density and also ensure that the luminescent product will be sufficiently high to cause an impact in the environment [25], making the process cost effective. The AI for *V. fischeri*, N-acyl homoserine lactone (AHL), was once thought to be species-specific,[22] however recent studies have established that AHL can serve as a signalling molecule for more than 16 genera of gram-negative bacteria. This suggests that the AI protein can facilitate interspecies communication [26,27], allowing quorum-sensing bacteria to monitor the population of other species as well as their own. Quorum sensing is now known to be a widespread regulatory mechanism in bacteria, particularly among a number of pathogens [28] ,influencing their ecology and associations with eukaryotic organisms.

The a and b subunits of luciferase are encoded by the *luxA* and *luxB* genes, respectively. In *Vibrio fischeri*, these genes are adjacent in the *lux* operon. The lux operon consist of the genes which encodes the proteins that make up the fatty acid reductase complex for aldehyde synthesis (*luxC*, *D*, and *E*).[19] In most luminescent bacteria, the luciferase genes *luxAB* are flanked upstream by *luxCD* and downstream by *luxE* with transcription from left to right (Meighen, 1993). However, Mancini and coworkers[12] found that in *P. phosphoreum*, the gene *luxF* is situated between *luxB* and *luxE*. Also, in *P. phosphoreum*, the nucleotide sequences of *luxA* and *luxB* genes are considerably different from other bacterial luciferases.[29]

The autoinduction in the *lux* system of *V. fischeri* [30, 26] is regulated by two genes (*luxR* and *luxI*) (Fig.6). The genes are present in two divergent operons. In the rightward side of operon, the *luxI* gene is situated together with the *luxCDABE* genes while the *luxR* is in the leftward operon. The *luxI en*codes an autoinducer synthase. This autoinducer synthase is responsible for the production of the AI. The *luxR* gene encodes the LuxR protein, which serves as both a receptor for the AI and a transcriptional activator of the *lux* operon.[26, 21] Binding of the AI to the LuxR protein forms a complex that acts as a transcriptional regulator, activating transcription from the *lux* operon promoter.[31] Once induction begins, the AI level increases instantaneously. This happens due to the gene for AI synthase is part of the *lux* operon. In this way, the AI controls its own synthesis through a positive feedback circuit.[32]

With the advancement of molecular biology, researchers have made possible the cloning and expression of the *luxAB* genes in bacteria that are normally non-luminescent, transforming them into bioluminescent bacteria. The resulting transformations provide enormous applications in industry [33,34], medicine [35,28] , microbial ecology [36,37], and environmental fields.[38]

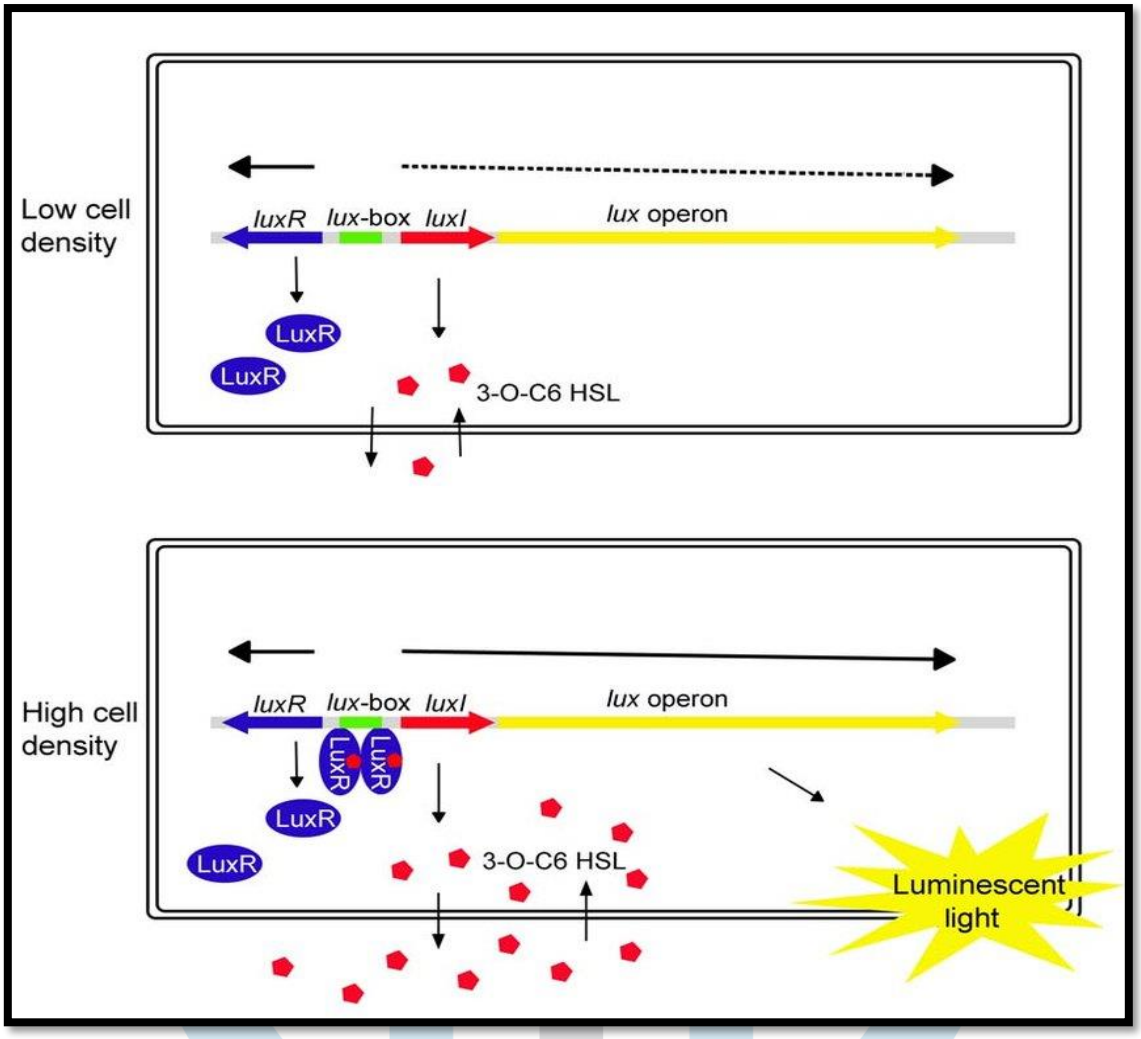

Fig 6: Lux pathway controlling bioluminescence in *Vibrio fischeri* [39]

UTILIZATION OF BACTERIAL BIOLUMINESCENCE AS A BIOSENSOR

A biosensor is a kind of sensor that can track and identify a compound within a cell or tissue.[40] The main issues regarding the performance of a whole cell-based biosensor are: (i) the selection of reporter gene, as well as (ii) the selectivity and sensitivity of the molecular recognition that occurs when regulator proteins bind to their target analytes.[41] Whole cell-based biosensors can detect a wider range of substances and thus are more sensitive to change in the electrochemical state of a tissue sample, other cells or in the environment.[42] Because of the advantages of whole cell-based biosensors, they have been used successfully to fields such as environmental monitoring, food analysis, pharmacology and drug screening.[41] Pollutants, such as heavy metals and biocides, are now commonly found in places where there is rapid industrial transformation and these pollutants are considered a global threat.[43] Toxins in the environment typically are associated with detrimental health outcomes and loss of ecological diversity.[44] Rapidly and accurately detection of pollutants is essential to reduce these threats.

The main function of bacterial luciferase is to catalyze the emission of light. This feature together with the aldehyde substrate generation by fatty acid reductase can be successfully produced in other bacteria, by the transfer of *lux*CDABE genes, which convert non-luminescent bacteria into light emitters. The light emitting property of the *lux*CDABE genes has been utilized as a reporter of gene expression. The study of this regulatory control involves in affecting the efficiency of RNA polymerase in initiation and transcription at different promoters. The control of *lux*CDABE genes are under the environmentally regulated promoter. The promoters whose efficiency is highly sensitive to the level of mercury, arsenic, or other pollutants. The structural lux genes play an important role as a biosensor, whose expression will assess the presence of toxic waste in the environment.

Therefore the *lux* gene organization has stimulated the use of bioluminescence genes for the development of whole cell biosensors that have a broad range of environmental applications.[33,38,45] These applications include construction of biosensors for detection of contaminants, measurement of pollutant toxicity [46] and monitoring of genetically engineered bacteria released into the environment.[47,48,49] Biosensors have also been used as indicators of cellular metabolic activity[50] and for detection of pathogens.[33,34] Till date, many bioluminescent reporter bacteria have been genetically engineered by placing a *lux* gene construct under the control of an inducible promoter.[33] These biosensors can be exceptionally useful in bioremediation studies. The utilization of these biosensors determine the presence and concentrations of specific pollutants. A biosensor is developed [51] which uses the fusions of Tn21 mercury resistance operon (*mer*) with promoter less *luxCDABE* from *V. fischeri*. This *mer-lux* biosensor exhibited the

semiquantitative detection of inorganic Hg (II) in natural water in the 0.1 to 200 ppb range and was a proper system for characterizing bioavailable from unavailable forms of mercury [51] (Fig.7).

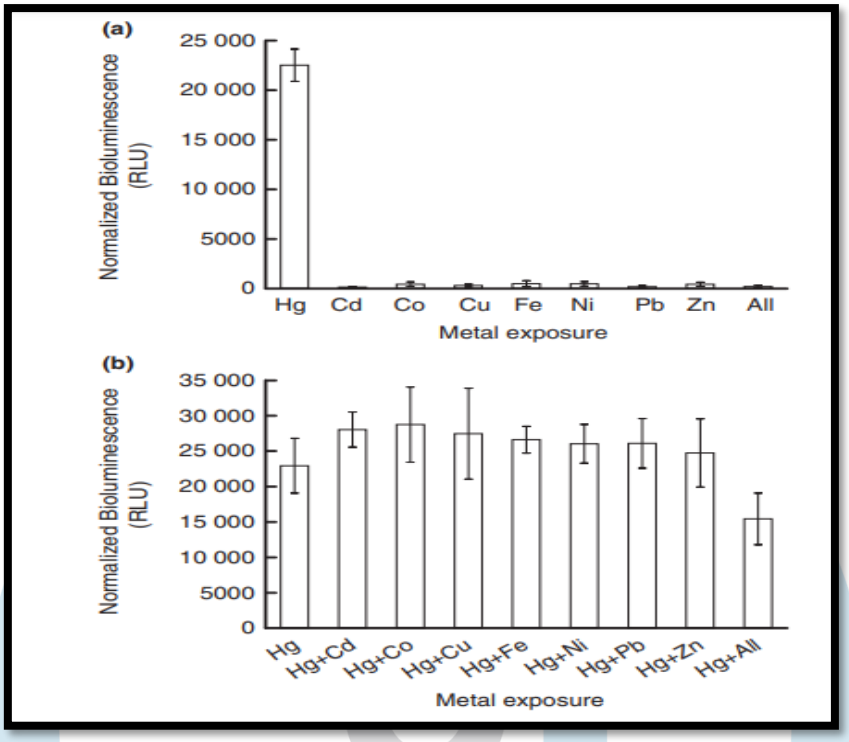

Fig 7: Response of Luminescent bacteria to the exposure of heavy metal toxicity[52]

CONCLUSION

In nature, the occurrence of bioluminescence have always been a focus of human attention. Prior to the identification of luminous organisms, the presence of even a dim luminous glow in the dark frightened countless numbers of individuals, who perceived the glimmer as ghosts or supernatural spirits. Nevertheless, the inquisitiveness of scientists to resolve the mysteries of nature led to the identification of many luminous organisms which are responsible for the emission of light in different environmental condition. FMNH2, O2, and long fatty aldehyde are utilized by all luminescent bacteria as substrates for the bioluminescence reaction. The reaction is mainly catalyzed by luciferase (LuxAB), with the fatty acid reductase complex (LuxCDE) synthesizing the long chain aldehyde substrate of tetradecanal. A NAD(P)H-dependent FMN reductase found in most if not all bacteria generates the FMNH2 substrate. Therefore, in the lux operon only the five genes, *lux*CDABE, are required to produce the emission of light, even in bacteria that normally do not emit light. Thus providing the opportunity to use the bacterial *lux* system as a light emitting sensor in many bacteria. In luminescent bacteria, the regulation of induction *lux*CDABE genes expression at high cell density has led to the development of molecular prototypes for quorum sensing, an important new regulatory mechanism signaling bacterial crowding, and now found controlling key secondary metabolic pathways in many nonluminescent bacteria. At present, several discoveries in understanding the molecular mechanisms, structures and regulation of the *lux* enzymes and genes of luminescent bacteria has answered many of the different questions.

Over the last 30 years, there has been great development on physiology, biochemistry and genetic control of bioluminescence in bacteria. These fabulous discoveries, not only have revolutionized the area of Environmental Microbiology but have also shed light into areas of industrial, ecological and medical significance. The explanation of luciferase genes regulation accepted the discovery of intercellular communication among bacteria. This, in turn, has led to a better understanding the pathogencity of bacteria and the associations of microorganisms in the environment. With the advent of molecular biology, it has been possible to demonstrate that the light emitting property of the *lux*CDABE genes has been employed as a reporter of gene expression. The regulation of this gene expression is essential for studying the regulatory controls involved in affecting the efficacy of RNA polymerase in initiation and transcription at different promoters. The control of *lux*CDABE genes are under the environmentally regulated promoter. The efficiency of these promoters is highly sensitive to the level of mercury, arsenic, or other pollutants. Today these pollutants are considered as a global threat to our civilization.[43] Therefore the structural lux genes play an important role as a biosensor, whose expression will monitor the presence of toxicity in the environment.

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