RESEARCH OF MARINE ISOLATES IN DEVELOPMENT OF BIOSENSORS FOR ENVIRONMENTAL POLLUTANTS

Trupti GOKHALE - Almas WALI - Shailee PARIKH - Neeru SOOD

Abstract: *Bioluminescence is a phenomenon exhibited by various organisms like marine bacteria, glow worms, fireflies, anglerfish, jelly fish, etc. These organisms can produce and emit light. Bioluminescence is a natural process of energy release in the form of emitted light. The organisms produce chemicals, luciferin, which is a pigment and an enzyme luciferase. Luciferin reacts with oxygen to release energy in the form of light with the help of enzyme luciferase. The reaction requires energy in the form of ATP. In bacteria, the bioluminescence is controlled by a set of genes called Lux operon. In the present work Vibrio spps. have been isolated from the surface of fresh squids. The bioluminescence exhibited by these bacteria was quantitated using the luminescence mode of the spectrophotometer. The bioluminescence exhibited by the bacteria was studied during its growth and found to be a maximum of 30 hours. The circadian cycle exhibited by these bacteria was also studied. The bioluminescence was observed to decrease in the presence of water pollutants like heavy metal ions, complex aromatic hydrocarbons and pesticides. Since the genes (lux operon) controlling bioluminescence are sensitive to presence of pollutants, the construction of biosensors using these genes could have great application.*

Keywords:
- *Vibrio*
- bioluminescence
- circadian cycle
- water pollutants
- biosensors

1. INTRODUCTION

Bioluminescence is a type of luminescence that occurs among a variety of organisms ranging from bacteria, dinoflagellates, protozoa, sponges, mollusks, echinoderms, insects and fish [1]. The majority of bioluminescent species live in the sea, although there are also many terrestrial bioluminescent insects, especially the beetles. It has been estimated that 60-80% of the fishes in the deep sea are bioluminescent [2]. The bioluminescent bacteria mainly fall under three genera namely - *Photobacterium, Vibrio,* and *Photorhabdus.* Species within the genus *Photobacterium* and *Vibrio* generally exist in marine environment, whereas the terrestrial species belong to the genus *Photorhabdus* [3]. The bacteria form a symbiotic relationship with the host organism, where the host provides a nutrient rich environment for the growth of the bacterium and in return benefits from the luminescence such as camouflage or protection from its predator [3].
The emission of light by the luminescent bacteria arises from exothermic chemical reactions catalyzed by the key enzyme, luciferase present within the cytoplasm of the bacteria. Apart from the involvement of luciferase, there are other enzymes that supply and regenerate the substrates for luciferase. All of these enzymes related to bioluminescence are coded by a cluster of genes called *lux operon* [3]. These bioluminescent bacteria use quorum sensing to coordinate their gene expression according to the local density of their population [4-6]. The bacterium *V.fischeri* when present as a symbiont, exhibits bioluminescence, but while in the planktonic habitats as a single cellular form, production of light seems a waste and hence the bacterium does not emit light. The quorum sensing system of *V.fischeri* employs two genes, *luxI* and *luxR* which activate the expression of the *lux* structural genes [6].
Bioluminescent circadian cycles have been reported in various systems such as *Gonyaulax* [7], *Lampyris noctiluca* [8] and in cultured cells of *Arabidopsis* with Bioluminescence Reporters as transgenes [9]. But the presence of the circadian

cycle for the *Vibrio* species has not been reported till date.

In this paper we have isolated six bioluminescent bacteria from fresh squids. All of these belonged to the *Vibrio* spps. and hence only one isolate was selected for further studies. The quorum sensing system of the bacterium was studied in presence of various water pollutants such as, heavy metals, hydrocarbons and pesticide. We have also established the presence of circadian cycle in the *Vibrio* species.

2. MATERIALS AND METHODS

2.1. Isolation of the bioluminescent bacteria

A fresh catch of squid was procured from the local fish market of Sharjah, U.A.E. The squid was immersed in 3.0 % (w/v) NaCl so that approximately 80-90% of the squid was below the NaCl solution. The squid was incubated in dark at 20 °C and observed intermittently for bioluminescence up to 4 hours. The area of the squid exhibiting bioluminescence was scraped with a scalpel and streaked on BOSS agar plates (Peptone 10.0g, NaCl 30.0g, Beef extract 3.0g, Glycerol 1.0g (0.888ml), Bacto agar 15.0g, in 1L distilled water) [10]. The plates were then incubated in dark at 20°C and checked after every hour for the appearance of the colonies exhibiting bioluminescence. The colonies exhibiting luminescence were purified and maintained on the BOSS agar. The cultures were identified to generic level using routine microscopic, cultural and biochemical analysis, based on the Bergey's manual of systematic bacteriology [11, 12].

2.2. Growth and Luminescence

Three out of six cultures were selected for further studies. The isolates AW1, AW2 and AW3 were selected for studying the growth and luminescence of the bacteria in Luminescent broth (Peptone 10.0g, NaCl 30.0g, K_2HPO_4 2.0g, $MgSO_4$ 0.25g, Glycerol 2.0g (1.77ml), in 1L distilled water) (10). The growth of the cultures was determined in terms of their optical density at 660nm, while the luminescence was recorded photometrically using the luminescence mode of the Perkin Elmer Victor 2030-0030.

2.3. Circadian Cycle and Effect of water pollutants

The isolate AW1 was selected for the further studies. Luminescence exhibited by isolateAW1 grown in the Luminescent broth was recorded every 3 minutes for a period of 30 minutes using the Perkin Elmer Victor 320. The effect of pollutants such as 2.5mM sodium arsenate, 5mM lead nitrate, 0.05% Toluene, 0.05% n-hexane and 0.05% Dursban 4C pesticide on luminescence was monitored by adding the pollutants to 200µl of 24h grown cells of isolate AW1.

3. RESULTS AND DISCUSSIONS

3.1. Isolation of the bioluminescent bacteria

The squid placed in 3% (w/v) NaCl solution started exhibiting luminescence after 4 hours of incubation. The area exhibiting luminescence was scraped and used for isolating the bioluminescent bacteria. Six luminous bacterial colonies were observed on the BOSS agar after 7 hours of incubation. However, the plates were incubated for 24 hours for complete development of the bacterial colonies (Figure 1). The isolated bacterial colonies exhibited similar morphological and microscopic characters. They were found to be Gram negative, motile, non -sporulating, oxidase positive, short rods (Table 1). The cultures had a definite Na^+ requirement for growth, and possessed distinct polar flagella, resembling the *Vibrio* sps. [11, 12].

Table 1. Cultural and biochemical characteristics of the selected six isolates

Isolates	AW1	AW2	AW3	AW4	AW5	AW6
Gram's Staining	-	-	-	-	-	-
Morphology	Short Rods	Short Rods	Short Rods	Short Rods	Short Rods	Short Rods
Polar Flagella/ Motility	+	+	+	+	+	+
Spores	-	-	-	-	-	-
Oxidase Test	+	+	+	+	+	+
Na^+ requirement	+	+	+	+	+	+

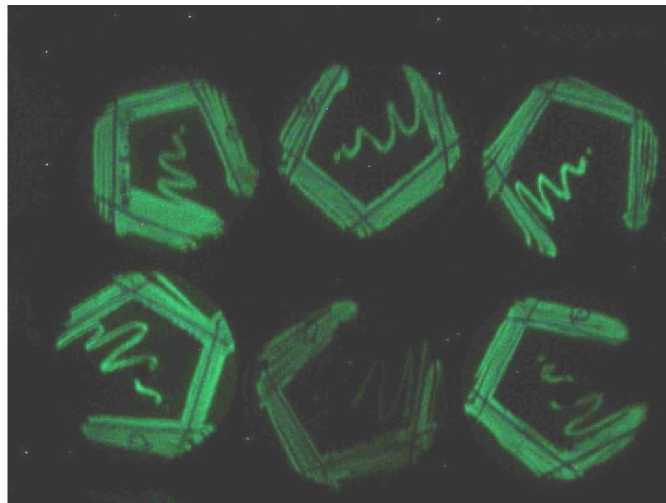

Figure 1. Bioluminescence exhibited by the six cultures isolated from the fresh squid using Boss Agar

3.2. Growth and Luminescence

Three isolates AW1, AW2and AW3 were selected to study their growth in Luminescent broth. All the three isolates entered the exponential phase without a distinct lag phase, however, bioluminescence was observed only after 24 hrs (Figure 2). This can be attributed to quorum sensing where bioluminescence is observed only when the cell density has reached a threshold value [4-6]. The luciferase gene which is responsible for the bioluminescence appears to be completely inactive in a freshly inoculated culture and a subsequent sharp rise in luminescence is due to the transcriptional regulation by autoinduction [13].

The bioluminescence by the isolate AW1 was monitored closely with a 3 minute interval for a period of 30 minutes. The bioluminescence showed a marked increase followed by a decrease in the intensity of luminescence (Figure 3). Over the period of 30 minutes, three such cycles were observed signifying the presence of a probable circadian rhythm in *Vibrio*. The circadian rhythm may occur due to the changing metabolic activities of the bacteria during growth. Increased luminescence is seen due to high metabolic activity which builds up the energy levels i.e. ATP in the cells. The excess of ATP is diverted to the emission of light in the luminescence. However, as the level of ATP in the cell decreases, the light reaction does not take place resulting in lowered luminescence observed as a sharp drop. As the ATP builds again, the light reaction takes place exhibiting increased luminescence.

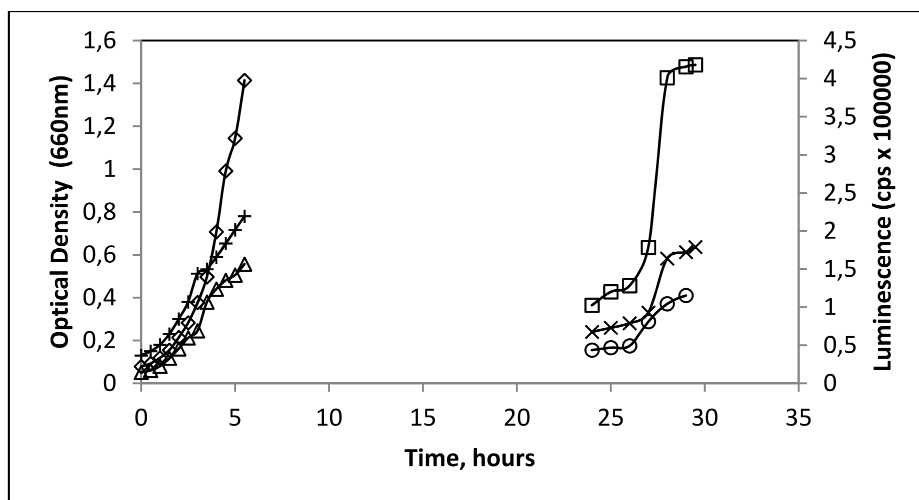

Figure 2. Bioluminescence by the selected three isolates along growth in the Luminescent broth.
◇———◇ *Growth of AW1,* □———□ *Luminescence exhibited by AW1,*
+———+ *Growth of AW2,* X———X *Luminescence exhibited by AW2,*
△———△ *Growth of AW3,* ○———○ *Luminescence exhibited by AW3.*

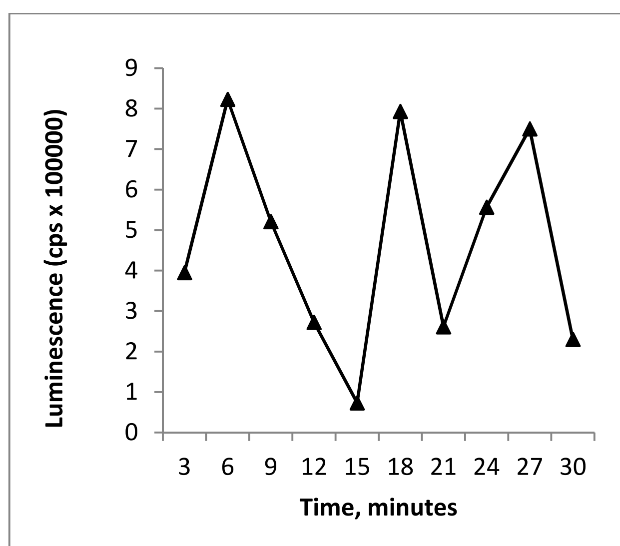

Figure 3. Bioluminescence by isolate AW1. The luminescence exhibited by isolate AW1 was recorded every 3 min for a period of 30 min in a spectrophotometer.

3.3. Effect of Pollutants on Bioluminescence

The bioluminescence by isolate AW1 showed a marked decrease in the presence of externally added water pollutants such as 2.5mM sodium arsenate, 5mM lead nitrate, 0.05% toluene, 0.05% n-hexane and 0.05% Dursban 4C pesticide. The bioluminescence was most affected in presence of Dursban 4C pesticide (82.09%) followed by n-hexane (78.3%), toluene (50.1%), lead nitrate (21.8%), and the least by sodium arsenate (16.9%) (Figure 4). The surprising and outstanding result was due to the known toxicity of heavy metal ions on bacterial cells as contrasted to hydrocarbons. This can be attributed to the fact that low concentrations of heavy metal ions induce bioluminescence [14]. However, the decrease in the bioluminescence due to presence of water pollutants is very significant and hence the isolate can be used to develop biosensors which can detect water pollution.

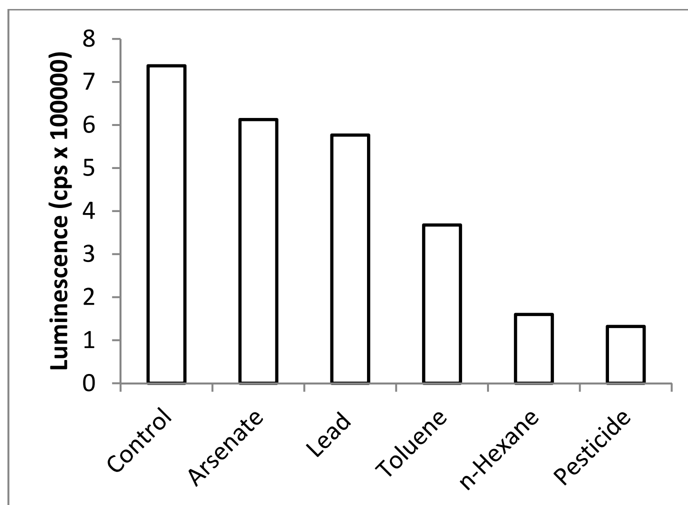

Figure 4. The effect of pollutants on bioluminescence exhibited by isolate AW1 when grown in Luminescence Broth. The pollutants used were 2.5mM sodium arsenate, 5mM lead nitrate, 0.05% toluene, 0.05% n-hexane and 0.05% Dursban 4C pesticide

Biosensors are analytical devices that are constituted of a biological element like an enzyme, an analyte, which is generally a substrate for the enzyme and a transducer that converts the chemical signal or light into an easily measurable signal. The *Vibrio* species isolated in this work can be efficiently used in developing the biosensor where the biological element can be the bacterial cells or the *lux* genes isolated from the bacterium. Since the cells of *Vibrio* species exhibit a decreased luminescence in presence of different pollutants (shown in Figure 4), they possess a high potential in developing biosensors.

4. SUMMARY

Six bacterial cultures exhibiting bioluminescence were isolated from a fresh squid. These isolates when studied for their morphological and biochemical characteristics were confirmed to belong to the *Vibrio* species. Three of these isolates were studied for their growth characteristics and luminescence. The presence of a circadian rhythm was confirmed in isolate AW1, which distinctly showed a rise and fall in the intensity of luminescence with respect to time. The intensity of the bioluminescence decreased significantly in the presence of water pollutants. Thus the isolate AW1 can be used as an indicator of water pollution as it can act as a biosensor to detect the presence of pollutants in the environment. The future work would be focused on isolation of the *lux* genes responsible for the bioluminescence to develop a biosensor.

REFERENCES

[1] Shimomura, O.: *Bioluminescence Chemical principles and Methods*, World Scientific Publishing Co. Pte. Ltd., Singapore, 2006.

[2] Lars Olof Björn: *Photobiology: The science of light and life*, Springer, New York, 2002.

[3] Lin, L.Y., Sulea, T., Szittner, R., Vassilyev, V., Purisima, E.O. and Meighan, E. A.: *Modeling of the bacterial luciferase-flavin mononucleotide complex combining flexible docking with structure-activity data*, Protein Science, Vol. 10 (2001), p. 1563-1571.

[4] Waters, C.M. and Bassler, B. L.: *Quorum sensing: cell-to-cell communication in bacteria,* Annual Review Cell Developmental Biology, Vol. 21 (2005), p. 319-346.

[5] Miller**,** M. B., Bassler**,** B. L.: *Quorum sensing in bacteria***,** Annual Review of Microbiology. Vol. 55 (2001)**,** p.165-199.

[6] Fuqua, C., Winans, S. C. and Greenberg, E. P.: *Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators,* Annual Review of Microbiology, Vol. 50 (1996), p.727-751.

[7] Kiessig, R. S., Herz, J. M. and Sweeney, B. M.: *Shifting the Phase of the Circadian Rhythm in Bioluminescence in Gonyaulax with*

Vanillic Acid, Plant Physiology, Vol. 63 (1979), p.324-327
[8] Dreisig, H.: *The circadian Rhythm of bioluminescence in the glow worm, Lampyris noctiluca L. (Coleoptera, Lampyridae)*, Behavior Ecology Sociobiology, Vol. 3 (1978), p.1-18.
[9] Nakamichi, N., Ito, S., Oyama, T., Yamashino1, T., Kondo, T. and Mizuno, T.: *Characterization of Plant Circadian Rhythms by Employing* Arabidopsis *Cultured Cells with Bioluminescence Reporters*, Plant and Cell Physiology, Vol. 45 (2004), p.57-67.
[10] Atlas, R. M.: *Handbook of microbiological media*, ASM Press, Washington, D.C., 2010.
[11] Kreig, N. R. and Holt, J. G.: *Bergey's manual of systemic bacteriology*, Vol. 1, William's and Wilkins, Baltimore, 1989.
[12] Sneath, P. H. A., Mair, N. S., Sharpe, M. S. and Holt, J. G.: *Bergey's manual of systemic bacteriology*, Vol. 2, Williams and Wilkins, Baltimore, 1989.
[13] Nealson, K. H., Platt, T. and Hastings, J.W.: *Cellular control of the synthesis and activity of the bacterial luminescent system*, Journal of Bacteriology., Vol.104 (1970), p.313-22.
[14] Stoyanov, J. V., Magnani, D. and Solioz, M.: *Measurement of cytoplasmic copper, silver and gold with lux biosensor shows copper and silver but not gold, efflux by copA ATPase of Escherichia coli.* FEBS letters, Vol. 546 (2003), p.391-394.

Received: 11. 11. 2011. Accepted: 30. 01. 2012.

Author's Address:

Dr. Trupti Gokhale
Almas Wali
Shailee Parikh
Dr. Neeru Sood
Department of Biotechnology,
BITS Pilani Dubai Campus,
PO BOX 345055,
Dubai International Academic City,
Dubai,
United Arab Emirates
E-mail addresses of the authors
trupti@bits-dubai.ac.ae
almas.wali@hotmail.com
shaileeparikh@gmail.com
sood@bits-dubai.ac.ae