



Original Research Article

Isolation of Bioluminescent Bacteria and Their Application in Toxicity Testing of Chromium in Water

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A B S T R A C T

Nature has its own beauty and phenomenon called bioluminescence proves it. Marine fishes like Japanese Threadfin brin (*Nemipterus japonicas*), Indian Mackerel (*Restrelliger kanagurta*) and Anchovy (*Engraulis japonicas*) were chosen as source of bioluminescent bacteria. Scales, gills and eye portion along with gut region of Indian Mackerel were suspended overnight in sterile sea salt saline and from this loopful suspension were streak inoculated on sterile Photobacterium agar, LA, Sea water agar and Boss Agar. By subsequent subculturing two luminescent isolates named as C1, C2 were obtaining on LA plate from the gut portion of the Indian Mackerel. They were used for further study. Morphological and biochemical characterization along with observation of PHB granules under light microscope suggests that the isolates may belong to Genus *Vibrio*. To reconfirm these results the isolates were inoculated on TCBS agar which is the highly specific media for the *Vibrio* identification and confirmation; which confirmed that the isolate C1 may be late sucrose fermenter *Vibrio* species. This isolate showed green coloured colonies after 36hrs of incubation. The TSI slant examination stated that the organisms were Gram negative enteric pathogen that ferments glucose, lactose and sucrose by acid production without accumulation of gas. After examination of growth pattern of both isolates; C1 and C2 were used for the checking toxicity of the chemical pollutant chromium in water. According to the United States Environmental Protection Agency for primary drinking water the standard Maximum Contaminant Level is 0.1 mg/L for total chromium. Standard protocols were used to determine total chromium level in water. Performance of the TVC of the both isolates lead to the results that the pollutant chromium inhibits the growth of bioluminescent bacteria and also affects the luminescence intensity. This whole study provides eco friendly and cost effective examination of pollutant chromium. The method can also be used to measure other chemicals, metal salts like mercuric chloride, zinc chloride and lead acetate etc.

Keywords

Bioluminescence,
Luciferase,
Auto-induction,
Pollutant chromium,
Toxicity testing
cultivation

Introduction

Bioluminescence a Greek word (“Bios” for “living” and Latin word “lumen” for “light”) which literally means a living light.

This type of chemiluminescent phenomenon have been seen in many types of organisms (bacteria, fungi, fishes, squid,

dinoflagellates) and have always gained an attention of scientists because of their beauty, physiology, biochemistry, genetics and ease of detection (Lee 2008; Vedprakash *et al.*, 2014). In late 19th century Raphael Dubois experimentally extracted the two key components, enzyme “luciferase” and “luciferine” of bioluminescence reaction which are able to generate light (Makemson, 1986; Lee, 2008). Many bacteria regulate their set of lux genes by the mechanism of quorum sensing or autoinduction in which autoinducers (AI) evoke the characteristic response from the cell (Rees *et al.*, 1998; Nyholm *et al.*, 2004; Walker *et al.*, 2006). In bioluminescence after achieving specific

threshold value (conc. of AI 10^7 cell mL⁻¹ in

culture media) it triggers the synthesis of enzyme luciferase and other enzymes involved in luminescence (Cao and Meighen

1989; Rees *et al.*, 1998; Walker *et al.*, 2006;

Berleman, 2009). This sensing (AI level)

help to ensure that the luminescence production is enough high to cause an impact in environment and making the process cost effective (Nealson *et al.*, 1970; Visick *et al.*, 2000). As the production of the luminous enzyme increases ultimately light generation also increases (Nealson *et al.*, 1970; Cao and Meighen, 1989; Berleman, 2009).

Due to the issues raised by animal rights and human rights, the use of microbes like

bacteria and algae gaining the attention for a toxicity evaluation as a model organisms (Roda *et al.*, 2004; Boynton, 2009). As well known; general metabolism, operon expression, bacterial growth, cultural condition affects the luminescence activity; from which one can articulate that the bioluminescent organisms are very much sensitive to any change (e.g. physical, chemical, radiological, etc.) in environment. Bioluminescent bacterial tests have been successfully used previously in determining the toxicity of aquatic samples, sediments, and soils (Lapota *et al.*, 1998; Kurvet *et al.*, 2005).

Materials and Methods

Anchovy (local name: Mandeli); Indian Mackerel (local name: Bangda); and Threadfin brin (local name: Raja Rani) these three fresh marine water fishes were selected as a source of bioluminescent bacteria and were obtained from fish market near Alpna Talkies, Camp, Pune. They were kept on ice and soon after brought to the laboratory for further processes.

Following fish parts were used as a source for luminescent bacteria (Table 1) (Walker *et al.*, 2006, web links).

Table.1 Fish parts used for luminescent bacteria

	Eye portion	Gill	Scales	Fin	Gut region
Anchovy	-	Used	Used	-	-
Mackerel	-	Used	Used	-	Used
Firm bream	Used	Used	Used	Dorsal fin	-

These different parts were suspended in sterile sea saline that is 3.5g of sea salt in 100ml distilled water for 24hrs and from this loopful suspension was streaked for isolation (Web link) on sterile

Photobacterium agar (PBA) casein enzyme hydrolysate 5g/L, yeast extract 2g/L, ammonium chloride 0.3g/L, magnesium sulfate 0.3g/L, ferric chloride 0.01g/L, calcium chloride 1g/L, potassium phosphate (monobasic) 3g/L, sodium glycerophosphate 23.5g/L, sodium chloride 30g/L, agar 15g/L (web link), sea water agar Part A: yeast extract 5g/L, peptic digests of animal tissue 5g/L, beef extract 3g/L, agar 15g/L, Part B: sodium chloride 24g/L, potassium chloride 0.7g/L, magnesium chloride. 6H₂O 5.3g/L, magnesium sulfate. 7H₂O 0.7g/L, calcium chloride 0.1g/L, pH 7.3 +/- 0.2 (web link), Luminus agar (LA) sodium chloride 10g/L, yeast extract 5g/L, peptone 10g/L, agar 15g/L, pH 7.3 (web link) and bioluminescent agar (Boss agar) sodium chloride 30g/L, glycerol 1g/L, peptone 10g/L, meat extract 3g/L, pH 7.3 plates (web link). Plates were incubated at RT for 48–72 hrs and observed for bioluminescence (web link).

Identification of isolated bioluminescent species

Two isolates were obtained by subsequent sub-culturing on sterile LA plates. The isolated were named as C1 and C2. After morphological colony characterization, Gram staining, motility and Poly-β-hydroxybutyrate staining (by Sudan Black B method) were performed (Bergey's manual 9th edition, web link). Bergey's Manual of determinative Bacteriology 9th edition was referred and accordingly biochemical tests like IMViC, amylase, oxidase, nitrate reduction were performed and fermentation of sugars were also checked with 12 different sugars (Table 2).

Growth on Thiosulfate Citrate Bile Sucrose Agar (TCBS)

TCBS Agar is both differential and highly

selective for *Vibrios* and differential due to presence of sucrose and the dyes. TCBS agar (proteose peptone 10g/L, yeast extract 5g/L, sodium thiosulfate 10g/L, sodium citrate 10g/L, oxagall 8g/L, sucrose 20g/L, NaCl 10g/L, ferric citrate 1g/L, bromothymol blue 0.40g/L, thymol blue 0.40g/L, agar 15g/L, pH 8.6±0.2 at 25°C, formula adjusted, standardized) is highly selective, meets the nutritional requirements of *Vibrio spp.*, and allows *Vibrios* to compete with intestinal flora (Engebretsch *et al.*, 1983; Musa *et al.*, 2008; Ransanga *et al.*, 2012; Kannahi and Shivshankari, 2014). TCBS Agar is recommended by APHA (American Public Health Association) for the selective isolation of *V. cholerae* and *V. parahaemolyticus*. Enrichment in alkaline peptone water (M618), followed by isolation on TCBS Agar is routinely used for isolation of *V. cholerae*. *Vibrios* are natural inhabitants of sea water (Kumar, 2010). It is possible that a few sucrose-positive *Proteus* strains can grow to form yellow colonies. *Vibrio parahaemolyticus* is a sucrose non-fermenting organism and produces blue-green colonies, as does *Vibrio vulnificus* (Engebretsch *et al.*, 1983; Musa *et al.*, 2008; Orengo *et al.*, 2010; Ransanga *et al.*, 2012; Kannahi and Shivshankari, 2014).

Triple sugar iron agar (TSI)

TSI is recommended in the identification of gram-negative enteric pathogens in routine examination of faeces. This medium indicates the ability of an organism to ferment lactose, sucrose and glucose with formation of acid and gas and also its ability to produce hydrogen sulfide gas (Musa *et al.*, 2008; Kumar, 2010; Ransanga *et al.*, 2012; Kannahi and Shivshankari, 2014) (Table 3 and 4).

Growth curve

Loopful of 24 hr old culture of isolate C1 and isolate C2 were inoculated in 50 ml Sterile Luminous Broth(LB) and were kept overnight till absorbance reached 0.1 at 600 nm. After 0.1 absorbance was reached, growth curve experiment was performed by taking absorbance at 600nm at 30 minutes interval time period (Eberhard, 1972; Engebretsch *et al.*, 1983; Cao and Meighen, 1989; Kumar, 2010; Gokhale *et al.*, 2012) (Graph 1 and 2).

Application of bioluminescent bacteria in toxicity testing of chromium

The bacterial bioluminescence test (BBT) is metabolic inhibition test that uses a standardized suspension of luminescent bacteria as test organisms under standardized conditions. This test method provides a rapid, reliable and convenient means of determining the toxicity of waste material. We have performed this test for checking out the chromium (heavy metal) toxicity on one of our bioluminescent bacterium. 0.1 mg of potassium dichromate was added in 100ml distilled water (APHA, 2012, Tchobanoglou *et al.*, 2004). Following protocol was used to convert total chromium to the hexavalent state chromium by oxidation with potassium permanganate. The oxidation process may not provide total conversion of all chromium species to Cr⁶⁺. For total chromium determination, acid-digestion of the sample was done. The hexavalent chromium is determined colorimetrically by reaction with diphenylcarbazide in acid solution. A red-violet colures complex of unknown composition is produced. The reaction is very sensitive, the molar absorbtivity based on chromium being about 40,000 L /g/cm at 540nm (APHA, 2012; Tchobanoglou *et al.*, 2004).

Chemicals Required: Conc. Nitric Acid, Conc. Ortho-phosphoric acid, Methyl

Orange Indicator solution, Conc. Ammonium hydroxide, Potassium permanganate solution: Dissolve 4 g KMnO₄ in 100ml water, Diphenylcarbazide solution: Dissolve 250 mg in 50ml acetone and Store in brown bottle (APHA, 2012). Protocol: (APHA, 2012; Tchobanoglou *et al.*, 2004). Pipette a portion of digested sample into 125 ml conical flasks, Add few drops of methyl orange indicator and then add conc. Ammonium Hydroxide until solution just begins to turn yellow, Add 1+11 ml of sulphuric acid until it turns acidic+1ml sulphuric acid in excess, Adjust the volume to 40ml, Add porcelain pieces and boil, Add 2 drops of potassium permanganate solution to give a dark red colour, if it fades add more potassium permanganate, Boil for 2mins, Add 1ml sodium azide solution, If red colour doesn't fade completely after boiling for approximately 30 seconds, add another 1ml sodium azide solution and continue boiling for 1 min, after color has faded completely, then cool, Add 0.25 ml ortho-phosphoric acid and using 0.2N sulphuric acid adjust pH to 1 for yellow colour development and take O.D. at 540 nm. A 24 hour old culture was inoculated in sterile Luminous Broth to adjust the O.D of 0.1 at 600nm absorbance. C2 was used for this testing. 4ml of such absorbance adjusted broth culture was exposed 1ml of different metal salt solution that is potassium dichromate (K₂Cr₂O₇) at different concentration up to 60 minutes. Samples were withdrawn at different time intervals of 10, 20, 30, 40, 50, 60 minutes and serially diluted up to 10⁻⁵. Dilutions 10⁻³and 10⁻⁴ were plated out on sterile LA plates. Sample of unexposed culture was plated out as control (Eberhard, 1972; Engebretsch *et al.*, 1983, Cao and Meighen, 1989; Kumar, 2010, Gokhale *et al.*, 2012; APHA, 2012; Tchobanoglou *et al.*, 2004).

Results and Discussion

Observing plates in dark revealed that the colonies obtain from the gills and scales of Fim Brim inoculums and gut of Indian Mackerel inoculums were glowing beautifully also in presence of other bacterial population. After subsequent subculturing on LA it had been observed that the colonies were obtain from the gut flora of Indian Mackerel were glowing with more intensity than the colonies obtain from gills and scales of Fim Brim. And because of this these colonies were chosen for the experimental purpose (Engebrech *et al.*, 1983; Musa *et al.*, 2008; Orengo *et al.*, 2010; Ransanga *et al.*, 2012; Kannahi and Shivshankari, 2014; web link). Two different bioluminescent isolates were observed on the LA plates and were examine for further studies (Photo 1). Microscopic examination of these two isolates states that the circular, creamy white colonies having convex elevation with smooth (C1) and Mucoid (C2) consistency were Gram negative motile short rods (Berger's Manual, 9th edition) Both of the isolates C1 and C2 found to be poly-β-hydroxy butyrate granules non producers; as no accumulation of PHB were seen in the cells after staining by Sudan Black B method (Berger's Manual, 9th edition; Ransanga *et al.*, 2012). By referring Berger's manual of determinative bacteriology (9th edition), biochemical test were performed on both the isolates. Both of the isolates were able to produce enzyme gelatinase, catalase and cytochrome oxidase and showed negative results for IMViC and enzyme amylase production. All 12 chosen sugars were fermented by C1 and C2 with acid production in absence of gas (Berger's Manual, 9th edition; Musa *et al.*, 2008; Ransanga *et al.*, 2012). These results indicates that the both isolates belong to the genus *Vibrio* (Berger's Manual, 9th edition; Engebrech *et al.*, 1983; Musa *et al.*, 2008;

Orengo *et al.*, 2010; Ransanga *et al.*, 2012; Kannahi and Shivshankari, 2014) (Table 2). To reconfirm the present results both of the isolates were streak inoculated on TCBS agar which is specific for *Vibrio* identification (Musa *et al.*, 2008; Kumar, 2010, Ransanga *et al.*, 2012). Our finding indicates that the isolate C2 may be late sucrose fermenters as it showed green coloured colonies after 36hrs which was showing yellow colonies after 24hrs of incubation while C1 showed green colonies after 24hrs of incubation. Hydrogen sulfide gas production was absent for both C1 and C2 (Musa *et al.*, 2008; Ransanga *et al.*, 2012). These results confirm that both the isolates were from Genus *Vibrio* (Engebrech *et al.*, 1983; Musa *et al.*, 2008; Orengo *et al.*, 2010; Kumar, 2010, Ransanga *et al.*, 2012; Kannahi and Shishankari, 2014). Inoculation of C1 and C2 on TSI slants identify that organisms were Gram negative enteric pathogens (Musa *et al.*, 2008; Ransanga *et al.*, 2012) C1 fermented the lactose or sucrose or both the sugars in absence of H₂S in acidic condition while C2 fermented glucose by producing acid in absence of gas (Engebrech *et al.*, 1983; Musa *et al.*, 2008; Orengo *et al.*, 2010; Kumar, 2010, Ransanga *et al.*, 2012; Kannahi and shishankari, 2014) (Table 3 and 4). The important growth curve experiment of both the isolates indicates that there was rapid increase in absorbance showed by C1 and C2 isolates were showing steady increase in cell number. This information gave an idea regarding when cells are going to be in log phase. In log phase bioluminescent activity is more prominent (Eberhard, 1972; Cao and Meighen, 1989; Gokhale *et al.*, 2012) and all because of this reason this experiment was the key for the whole toxicity experiment (Eberhard, 1972; Engebrech *et al.*, 1983; Cao and Meighen, 1989; Kumar, 2010; Gokhale *et al.*, 2012) (Graph 1 and 2).

Heavy metal toxicity testing

These bioluminescent bacteria were used to check toxicity of heavy metal chromium. Chromium may exist in water supplies in both the hexavalent and trivalent form although trivalent form rarely occurs in potable water. The U.S. EPA (United States Environmental Protection Agency) primary drinking water standard MCL (Maximum Contaminant Levels) is 0.1 mg/L for total chromium (APHA, 2012; Tchobanoglous, 2004). So serially diluted sample was checked for the total viable count between

10^{-3} and 10^{-4} dilution. Comparison with

control plates which was free from chromium ions showed presence of $396\text{CFU/ml} \times 10^7$ but it was found to be gradually decreasing as found in table 5. These results conclude that the presence of chromium affect the bioluminescent bacterial growth. So the use of this bioluminescent bacteria for the toxicity testing was successfully accomplished with easy detection (APHA, 2012; Tchobanoglous, 2004).

Table.2 Characteristic and biochemical test results

Isolate	C-1	C-2
Morphology		
Gram character	-/S	-/S
Motility	+	+
Consistency	Smooth	Mucoid
PHB Production	-	-
Luminescence	+/blue colour	+/green colour
Fermentation of sugar		
Arabinose	A	A
Fructose	A	A
Galactose	A	A
Glucose	A	A
Lactose	A	A
Maltose	A	A
Manitol	A	A
Mannose	A	A
Rhamnose	A	A
Ribose	A	A
Sucrose	A	A
Xylose	A	A
Biochemical Tests		
Indole test	-	-
Methyl Red test	-	-
Voges-Prosker test	-	-
Citrate	-	-
Nitrate Reduction test	-	-
Catalase test	+	+
Amylase test	-	-

Gelatinase test	+	+
Oxidase test	+	+

Key -/S= Gram negative short rods, A= acid production, no gas production was observed

Table.3 Reference table for interpretation of result TSI
(Bergery's Manual, 9th edition; web link)

REACTION	EXPLANATION
Acid butt(yellow), alkaline slant (red)	Glucose fermented
Acid throughout medium, butt and slant yellow	Lactose or sucrose or both fermented
Gas bubbles in butt, medium sometimes split	Aerogenic culture
Blackening in the butt	Hydrogen Sulfide produced
Alkaline slant and butt (medium entirely red)	None of the three sugars fermented

Table.4 TSI result

Isolate number	Slant	Butt	Gas	Result
1	Yellow	Yellow	No gas production	Lactose or sucrose or both fermented
2	Red	Yellow	No gas production	Glucose fermented
Control (<i>E.coli</i>)	Yellow	Yellow	Gas production	Lactose or sucrose or both fermented and Aerogenic culture.

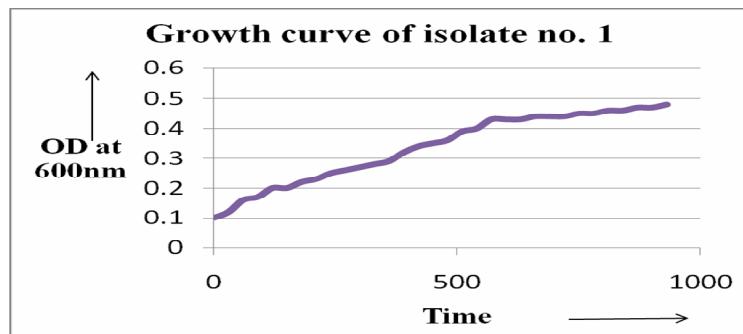
Table.5 Effect of chromium on growth of C2

Time (mins)	CFU/ml	
	10^{-3}	10^{-4}
10	Lawn growth	56
20	Lawn growth	17
30	Lawn Growth	15
40	-	-
50	-	-
60	-	-

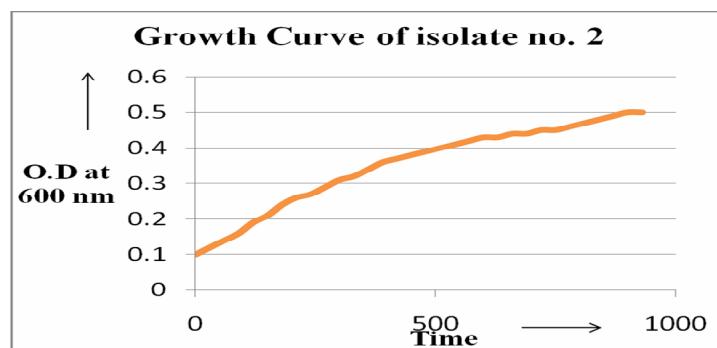
Photo.1 Glowing Bioluminescent bacterial isolated colonies of C1 and C2 on LA



Graph.1 Growth curve of Isolate C1



Graph.2 Growth curve of Isolate C2



Taken efforts were finally lead to successful identification of bioluminescent bacteria; two well isolated colonies of bioluminescent bacteria from gut flora of Indian Mackerel on luminous agar were identified as *Vibrio* (Berger's Manual, 9th edition). Growth on TCBS agar confirmed that the bioluminescent isolate belongs to the genus *Vibrio*. Growth on TCBS agar also state that one of the isolate C2 may be a late sucrose fermenter as we know there are some species that shows slow growth and also ferment sucrose slowly as they showed late green coloured colonies (Musa *et al.*, 2008; Ransanga *et al.*, 2012). The growth curve of the isolates gave the idea about the log phase of the organism. In this phase the bioluminescent intensity is prominent (Engebretsch *et al.*, 1983; Kumar, 2010, Gokhale *et al.*, 2012). As mentioned above isolate C2 showed steady growth in log phase i.e. steady intensity of light; so we prefer isolate C2 for further examination in

pollutant toxicity testing (Engebretsch *et al.*, 1983; Thomulka and Peck, 1995; Kuruvet *et al.*, 2005; Charrier *et al.*, 2010; Kumar, 2010, Gokhale *et al.*, 2012). This study helped to perform the TVC of the isolate C2

at specific concentration of 10^{-3} and 10^{-4} in

presence of pollutant chromium of concentration 1ppm (APHA, 2012; Tchobanoglou, 2004). Chromium is considered nonessential for plants, but an essential trace element in animals. Hexavalent compounds have been shown carcinogenic by inhalation and are corrosive to tissue. The chromium guidelines for natural water are linked to the hardness or alkalinity of the water (i.e., the softer the water, the lower the permitted level for chromium). The United Nations Food and Agriculture Organization recommended

maximum level for irrigation waters in 100 μ g/L (APHA, 2012; Tchobanoglous, 2004). This minute amount also affects the bacterial growth as the results showed declining number of colonies of organisms; we may say that the toxicity testing of chromium in wastewater by bioluminescent bacteria would be easy and risk free method (Thomulka and Peck, 1995; Lapota *et al.*, 1998; Boynton, 2009; Charrier *et al.*, 2010; Ranjith and Karthy, 2011). By this method we can also find out the concentration of the chromium in wastewater or other pollutants like metal salts zinc, lead acetate, ferrous sulphate etc. Modern technique including PCR can help to find out the identification of the isolates up to species level.

The victorious identification of both the bioluminescent isolates C1 and C2 to genus *Vibrio* states that the method for isolation and identification was found to be more efficient and cost effective, quite easy and simple. Though the method used for the toxicity testing was little bit tricky but helpful to get perfect results and proves the use of bioluminescent bacteria in toxicity testing is very much simple and harmless to the performer as well as the environment. The successful finding suggests that simplicity and beauty lies within the nature itself.

References

- APHA, 2012. Greenberg, A.E., Clesceri, L.S., Eaton, A.D. 2012. Oxidation of trivalent chromium: Standard methods for examination of water and wastewater, 22nd edn. American Public Health Association, American Waste Work Association, Water Environment Federation, Part-1000.
- Bergey's manual of determinative bacteriology, 9th edn, Pp. 190–192.
- Berleman, J. 2009. Analysis of inter-species interaction and metabolite excretion in bioluminescent marine bacteria isolates. MBC Microbial diversity. MA. University of Iowa, IA.
- Boynton, L. 2009. Using bioluminescent bacteria to detect water contaminants. *J. U.S. SJWP*, 4: 29–41.
- Cao, J.G., Meighen, E.A. 1989. Purification and structural identification of an Autoinducer for the luminescence system of *vibrio harveyi*. *J. Bio. Chem.*, 264(36): 21670–21676.
- Charrier, T., Durand, M.J., Affi, M., Jouanneau, S., Gezekel, H., Thouand, G. 2010. Bacterial bioluminescent biosensor characterization for on-line monitoring of heavy metals pollutions in waste water treatment plant effluents. *Intech J.*, Pp. 180–205.
- Eberhard, A. 1972. Inhibition and activation of bacterial luciferase synthesis. *J. Bacteriol.*, 109(3): 1101–1105.
- Engebrech, J., Nealson, K., Silvarman, M. 1983. Bacterial bioluminescence: isolation and genetic analysis from *Vibrio fischeri*. *Cell*, 32: 773–781.
- Gokhale, T., Wali, A., Parikh, S., Sood, N. 2012. Research of marine isolates in development of biosensor for environmental pollutants. *Eng. Rev.*, 32(1): 17–22.
- http://cibt.bio.cornell.edu/workshops_and_summer_programs/0610ccc/media.pdf
- <http://himedialabs.com/TD/M592.pdf>
- <http://microbiologyme.blogspot.in/2013/01/how-to-isolate-glowing-bacteria.html>
- http://www.biology.pl/bakterie_sw/bac_pod_en.html
- http://www.scharlabmagyarorszag.hu/katalogus/01-192_TDS_EN.pdf
- <https://www.sigmaaldrich.com/content/dam/sigmaaldrich/docs/Sigma/Datasheet/4/p1477dat.pdf>
- Kannahi, M., Sivshankari, 2014. Isolation and identification of bioluminescent bacteria from marine water at Nagapattanam sea shore. *Int. J. Pharm. Sci. Rev.*, 26(2): 346–351.

- Kumar, A. 2010. Isolation of bioluminescent bacteria from Bay of Bengal and their molecular characterization. PhD thesis. Dept of Biotech, AMET University, Chennai. India.
- Kurvet, I., Angela, I., Bondarenko, O., Sihtmae, M., Kahru, A. 2005. *LuxCDABE*—transformed constitutively bioluminescent *Escherichia coli* for toxicity screening: comparison with naturally luminous *Vibrio fischeri*. *Sensors J.*, 11: 7865–7878.
- Lapota, D., Liu, C.H., Rosenberger, D.E., Banu, J.I. 1998. Use of a rapid bioluminescent test (Qwiklite) using dinoflagellates to asses potential toxicity of sediment pore water, U.S. Patent number 5,565,360 (NC 76613).
- Lee, J. 2008. Bioluminescence: the First 3000 years, Dept of Biochem and MolBio., Pp. 194–204.
- Makemson, J.C. 1986. Luciferase-dependent oxygen consumption by bioluminescent Vibrios. *J. Bacteriol.*, 165: 461–466.
- Meighen, E.A. 1993. Bacterial Bioluminescence: organization, regulation and application of lux genes. *Faseb J.*, 7: 1016–1022.
- Musa, N., Wei, L.S., Wee, W. 2008. Phenotypic and genotypic characteristics of *vibrio harveyi* isolated from Black Tiger shrimp (*Penaeus monodon*). *World Appl. Sci. J.*, 3(6): 885–902.
- Nealson, K.H., Platt, T., Hastings, J.W. 1970. Cellular control of the synthesis and activity of the bacterial luminescent system. *J. Bacteriol.*, 104: 313–322.
- Nyholm, S.V., McFall-Ngai, M.J. 2004. The Winnowing: establishing the squid-*Vibrio* symbiosis. *Nature*, 2: 632–640.
- Orengo, M.J., Rubio-Marrero, N.R., Velazquez, R.C. 2010. Isolation and characterization of bioluminescent bacteria from marine environments of Puerto Rico. Current research, technology and educational topics in applied microbiology and microbial biotechnology.
- Ranjitha, P., Karthy, E.S. 2011. Detection of heavy metal resistance bioluminescence bacteria using microplate bioassay method. *J. Elixier Pollut.*, 40: 5108–5112.
- Ransanga, J., Lal, T.M., Al-Harbi, A. 2012. Characterization and experimental infection of *Vibrio harveyi* isolated from diseased Asian Seabass (*Lates calcarifer*). *Malaysian J. Microbiol.*, 8(2): 104–115.
- Rees, F., Wergeefose, B., Noiset, O., Dubuisson, M., Janssens, B., Thompson, E. 1998. The origins of marine bioluminescence: Turning oxygen defence mechanisms into deep-sea communication tools. *J. Exp. Biol.*, 201: 1211–1221.
- Roda, A., Pasini, P., Mirasoli, M., Michelini, E., Guardigli, M. 2004. Biotechnological applications of bioluminescence and chemiluminescence. *Trends Biotechnol.*, 22: 295–303.
- Tchobanoglous, G., Burton, F.L., Stensel, H.D., Metcalf, L., Eddy, H.P. 2004. Wastewater engineering- treatment and reuse, 4th edn. McGraw-Hill, Boston, Mass.
- Thomulka, K.W., Peck, L.H. 1995. Use of Bioluminescence in Detecting Biohazardous Substances in Water. *Assoc. Biol. Lab. Edu.*, 16: 219–227.
- Vedprakash, Rajpurohit, D., Dr. Suneetha, V. 2014. Bioluminescent bacteria as future torch light-screening, characterization. *Int. J. Sci. Eng. Res.*, 5: 949–951.
- Visick, K.L., Foster, J., Diano, J., McFall-Ngai, M., Ruby E.G. 2000. *Vibrio fischeri lux* genes play an important role in colonization and development of the host light organ. *J. Bacteriol.*, 182: 4578–4586.
- Walker, E.L., Bose, J.L., Stabb, E.V. 2006. Photolyase confers resistance to UV light but does not contribute to the symbiotic benefit of bioluminescence in *Vibrio fischeri* ES114. *Appl. Environ. Microbiol.*, 72: 6600–6606.