Journal of Marine Biosciences Vol. 1 (1), 33–49, 2015
Print ISSN 2454–3519 Online ISSN 2454–3527

JMBS

Factors Affecting Bioluminescence in Free Living *Photobacterium* spp. Isolated from Bay of Bengal, India

Alex Ranjit Kumar[1], M. Jayaprakashvel[2,3*], Elisabeth FeuK-Lagerstedt[1], A. Jaffar Hussain[2,3]

[1] School of Engineering, University College of Borås, Sweden.

[2]Department of Marine Biotechnology, AMET University, 135, East Coast Road, Kanathur, Chennai-603112, India.

[3]Centre for Marine Bioprospecting, AMET University, 135, East Coast Road, Kanathur, Chennai-603112, India.

*Corresponding Author: jayaprakashvel@gmail.com

Abstract

Luminescence is the emission of light by an object. Living organisms including certain bacteria are capable of luminescence. Bacteria are the most abundant luminescent organisms in nature. Bacterial luminescence has been studied most extensively in several marine bacteria. The luminescent bacteria exist in nature either as free living bacteria or in symbiotic association with certain marine organisms. Research on luminescent bacteria has always been a fascinating one. In the present study, twenty free living luminescent bacteria were isolated from Bay of Bengal, India using soft agar overlay method in sea water complex agar (SWCA). All the 20 strains were characterized for certain biochemical tests and tentatively identified that they are all *Photobacterium* spp. The effect of salinity, pH glycerol concentration and heavy metals on the growth and luminescence of these 20 strains was also studied using visual scoring to categorize the luminescence. In case of salinity, it has been found that up to 6% of NaCl the intensity of luminescence was good and thereafter it declined. Further, in some strains it was completely ceased beyond 9% of salinity. Luminescence was not greatly affected by pH in liquid medium however; the same was affected in solid medium. The intensity of luminescence has increased with increasing concentrations of glycerol ranging from 0.3 to 1.2%. All the 20 luminescent bacteria were characterized for their tolerance to heavy metals and antibiotics. Copper and zinc at 1 mg/ml concentration have inhibited the growth and luminescence of all the strains. Surprisingly, mercury at the same concentration has inhibited only two strains (AMET1913 and AMET1920). However, at 2 mg/ml concentration mercury has inhibited the growth and luminescence of all the 20 strains. Selected six luminescent bacterial strains were also characterized for their antibiotic susceptibility against six different antibiotics. It has been found that most of the strains were sensitive to all the six antibiotics tested. Since, the bioluminescence is regulated by quorum sensing, the effect of culture filtrate extracted with

dichloromethane was also tested for its effect on luminescence. These DCM extracts not influenced the luminescence much.

Keywords: Luminescence, *Photobacterium* spp, glycerol, heavy metals, quorum sensing

INTRODUCTION

Some bacteria possess a unique ability to produce light and are commonly described as phosphorescent, a term that implies that they absorb light energy, later releasing it when in the dark. They are, however, more properly described as luminescent, a term that indicates that they produce their own light. This ability may be lost just as virulence is sometimes lost on continued artificial cultivation of pathogenic bacteria; but this ability is normally regained rather readily if the organisms are cultivated on suitable media. This frequently means cultivation on neutral or slightly alkaline media prepared from sea water or containing equivalent amounts of the required salts. Bacteria able to emit light are common in the marine environment (Baumann and Baumann 1980). Luminous bacteria constitute a heterogeneous group of microorganisms, mainly representing the family Vibrionacea. Luminous bacteria occur in the sea as free-living organisms, as saprophytes, and as symbionts in light organs of certain fish and cephalopods. The biochemistry of the light reaction associated with luminous bacteria has been extensively studied, and many reviews on the topic have been published (Hastings 1968; Cormier and Totter 1968; Cormier *et al*., 1975).

Luminous bacteria can be isolated readily from the marine environment. They have been found as planktonic forms in seawater and associated with decaying animal material in the benthos (Nealson and Hastings, 1979). Luminescent bacteria occur in the intestinal tracts of marine animals (Ruby and Morin, 1978; O'Brien and Sizemore, 1979) and may be associated with luminous fecal pellets (Raymond and DeVries, 1976). Lesions on the chitinous exoskeleton of crustaceans can be caused by luminous bacteria (Baross et al., 1978). The functioning of light organs of certain fishes and cephalopods requires colonization by bacterial symbionts (Fitzgerald, 1977; Tebo et al., 1979; Hastings and Nealson, 1981). Despite the wide range of habitats occupied by luminous bacteria, little is known about the environmental factors affecting their populations (Yetinson and Shilo, 1979).Luminous marine isolates can be readily identified by application of a relatively few, simple, diagnostic traits (Baumann and Baumann 1983; Baumann et al, 1983). This fact has led to a number of ecological studies which have established the seasonal fluctuation of luminous species in sea water (Ruby and Nealson et al. 1978) their vertical distribution in the water column (Ruby et al. 1980), and the species specificity of the symbiotic association between luminous bacteria and marine animals (Ruby et al 1996).

Bacteria are the most abundant luminescent organisms in nature.Bacterial luminescence has been studied most extensively in several marine bacteria (e.g.,*Vibrioharveyi, Vibrio fischeri, Photobacterium*

phosphoreum, *Photobacteriumleiognathi*), and in *Xenorhabdus luminescens*, a bacteria that lives on land. In luminescent bacteria, the general scheme involves an enzyme that is dubbed "luciferase". A suite of genes dubbed "lux" genes code for the enzyme and other components of the luminescent system.The lux gene system responsible for bacterial luminescence has become an important research tool and commercial product. The incorporation of the luminescent genes into other bacteria allows the development of bacterial populations to be traced visually. Because luminescence can occur over and over again and because a bacterium's cycle of luminescence is very short (i.e., a cell is essentially blinking on and off), luminescence allows a near instantaneous (i.e., "real time") monitoring of bacterial behavior. The use of lux genes is being extended to eukaryotic cells. This development has created the potential for the use of luminescence to study eukaryotic cell density related conditions such as cancer. A simple set of diagnostic traits has been devised for the identification of these species (Bang et al., 1978) which has been recently applied in a number of ecological studies. Identification of luminous bacteria is based on specific methods (Williams and Wilkins *et al* 1984**).** In this scenario, the present work has been aimed to isolate marine luminescent bacteria and to characterize them using routine and some molecular tools. It has also attempted to determine the cellular interaction between them and the influence of environmental parameters on the growth and luminescence of the isolated luminescent bacteria.

METHODS AND MATERIALS

Sample Collection

100 ml of sea water was collected from the intertidal zones of Kovalam, Kanathur, Muttukadu in Chennai. The four different sea water samples which were collected from three different places were subjected to serial dilution. 10 ml of sea water sample was mixed with 90 ml of sterile distilled water in a 250 ml flask to obtain 10^{-1}. 1 ml from this dilution was taken and added to another 9 ml of sterile distilled water in test tubes from 10^{-2} and repeated once similarly to get 10^{-3} dilution. 0.1 ml from the10^{-3} dilution was used to spread plate in sea water complex agar(SWCA) medium (Peptone 5g, Yeast extract 3g, Glycerol 3ml, Agar 15g and50% Sea water per litre.) in petriplates. The plates were then incubated for 24 hrs and at every six hours the appearance of luminescent colonies were observed.

Agar overlay technique

One ml of the previously serially diluted sea water sample (from the10^{-3} dilution) was taken and mixed with 10 ml of soft SWCA (Peptone 0.25g, Yeast extract 0.15g, Glycerol 0.15 ml, and Agar 0.3g Per 50 ml) and mixed well. Then, this suspension was poured over pre-solidified regular SWCA plates and kept for incubation at room temperature incubated for 24 h and at every six hours the appearance of luminescent colonies were observed.

The distinct isolated luminescent colonies of bacteria were marked while observing for luminescence and were further purified by sub-culturing in SWCA plates. Each such isolatedpure colonies of bacterium

were given unique accession number starting with a prefix of AMET indicating the institute name. And these bacterial strains were stored in sterile sea water in eppendorf tubes at 4ºC.

Effect of salinity (Varying conecntrations of NaCl), pH and glycerol concentration

SWCA medium was prepared by adding different amounts of NaCl to obtain the final concentrations of salinity such as 0%, 3%, 6%, 9% and 12%. And same SWCA medium was prepared with four different pH values such as 5, 7, 9 and 11. The pH of the medium was adjusted with appropriate acid or base and once pH was adjusted the medium was added with respective amount of agar and then sterilized. Another SWCA medium was prepared by adding different amounts of glycerol to obtain the final concentrations of salinity such as 0.1%, 0.3%, 0.6% and 0.9%.The medium was poured in Petriplates and six bacteria were streaked per plate with clear divisions between them. Likewise all the isolated bacteria's were tested.The medium was poured in Petriplates and six bacteria were streaked per plate with clear divisions between them. Likewise all the Bacterial strains were tested. The plates were incubated for 24 h and the intensity of luminescence was assessed by visual scoring.

Simple staining for cell morphology

A clean grease free glass slide was taken then prepare thin smear of the test bacterial isolate to allow air dry and heat fix the smear, Cover with Saffranin for 1 minute.Drain the dye and rinse under running water. And observe under oil immersion objective 100 x and note the shape of the bacterial colonies.

Biochemical tests

The Gram Nature of the selected strains was determined by performing the KOH string test 3% KOH solution was prepared by dissolving 3 g of Potassium Hydroxide in 100 ml of distilled water. The 3% KOH String Test was done using a drop of 3% Potassium Hydroxide on a clean grease free glass slide. A visible loopful of cells from a single, well-isolated colony is mixed into the drop. If the mixtures becomes viscous within 45 seconds of mixing and produce a string when lifted using the loop (KOH-positive) then the colony is considered gram-negative (Ciufecu et al., 1986).A drop of medium containing cells to be observed is allowed to hang in the cavity of slide. The advantage of this preparation over the wet mount preparation is the increased capacity of aeration as the drop is surrounded by an air space. This is the best method available for the routine use to observe the motility of bacteria. Place a drop of 3% hydrogen peroxide onto a clean microscope slide. Touch an isolated colony with an inoculating loop, carrying some of the isolate, into the drop of hydrogen peroxide and observe the slide for the evolution of bubbles.

The reaction is positive if oxygen bubbles form rapidly.Organisms containing the catalase enzyme will form oxygen bubbles when exposed to hydrogen peroxide. The citrate test utilizes Simmon's citrate medium to determine if a bacterium can grow utilizing citrate as its sole carbon and energy source. Simmon's media contains

bromthymol blue, a pH indicator with a range of 6.0 to 7.6. Bromthymol blue is yellow at acidic pH's (around 6), and gradually changes to blue at more alkaline pH's (around 7.6).

Uninoculated Simmon's citrate agar has a pH of 6.9, so it is an intermediate green color. Prepare slants and allow solidifying. Streak the test cultures and incubate for 24 hours. Growth of bacteria in the media leads to development of a Prussian blue color (positive citrate). The test organism is inoculated into tryptone broth, a rich source of the amino acid tryptophan.When Kovac's reagent (p-dimethylaminobenzaldehyde) is added to a broth with indole in it, a dark pink color develops. The indole test must be read by 48 hours of incubation because the indole can be further degraded if prolonged incubation occurs. The methyl red (MR) and Voges-Proskauer (VP) tests are read from a single inoculated tube of MR-VP broth. After 24-48 hours of incubation the MR-VP broth is split into two tubes. One tube is used for the MR test; the other is used for the VP test. When the pH indicator methyl red is added to this acidic broth it will be cherry red (a positive MR test).

Growth of luminascent bacteria in TCBS medium

TCBS medium is a selective medium that allows the selective growth of bacteria belonging to the genera Vibrio. 100 ml of TCBS agar medium was prepared and poured in petri plates and isolated different strains were streaked and observe the result after 24 hours. Appearance of yellow color colonies in this medium indicates the bacterial strain as Vibrio spp.

Effect of heavymetals

Each bacterium was grown previously in sea water complex (SWC) broth meddium and 24 h old broth culture was used in this experiment. 100 microliters of each broth culture was swabbed over the smooth surface of SWCA plates and air dired aseptically in a laminar air flow chamber. Then, wells of size 8 mm dia were made in the seeded agar plates using sterile cork borer. The heavy metals such as copper zinc and mercury were prepared at two different concentrations viz., 1 mg/ml and 2 mg/ml. 100 microliters from the all the three heavymetals were taken and poured in the wells made in bacteria seeded SWCA plates. The plates were incubated for 24 h and the intensity of luminescence was assessed by visual scoring.

Antibiotic disc assay

SWCA medium was prepared as usual and was poured in Petriplates. Each bacterium was grown previously in SWC broth meddium and 24 h old broth culture was used in this experiment. 100 microliters of each broth culture was swabbed over the smooth surface of SWCA plates and air dired aseptically in a laminar air flow chamber. Hexadiscs of antibiotics obtained from himedia Mumbai, India were placed over the dried surface of bacterria seeded agar plates. The plates were incubated for 24 h after which the zone of inhibition, if any around the antibiotics discs which is the indicative of susceptibility or resistance of luminascent bacteria to the particular antibiotic was recorded.

Bioassay for autoinducers

Sea Water complex broth was prepared, transfer 10 ml of the medium in each test tube and inoculate the bacterial culture in each test tubes and leave it for overnight .The culture was taken and it was transformed into the centrifuge tube and centrifuge for 10 min at 4°C. Transfer the supernatant in another test tube and an equal volume of Dichloro methane (DCM) was added and it was shaken well. Once again discard the supernatant and take 5ml of organic phase. The organic phase supposed to contain autoinduces of acylatedomoserine lactone (AHL) type was dried to solidness using slow evaporation method in watch glasses. Finally, the solvent extracts were re-dissolved in 0.5 ml of DCM. SWA complex were prepared and poured in petri plates and 3 wells for 8 mm dia were made.

In the center well 100 microliter of solvent DCM was added, the top and bottom wells were loaded with DCM extract of respective bacteria. In a plate two bacteria were tested. Both these two bacterial autoinducer extracts were loaded in top and bottom wells. In the side of the wells, both the bacteria were streaked. Shortly, the experiment has carefully designed to check both the self-inductive and cross inductive effects of autoinducers. The intensity of luminescence was assessed by visual scoring

Isolation of genomic DNA from luminascent bacteria

1.5 µl cultures were centrifuged and supernatant was discarded. Add 1.5 µl of saturated NaCl was added to the pellet and mixed well until the pellet is dissolved completely and centrifuge for 1 min. Discard the supernatant and add 0.75 of distilled water, 0.75 of lysis buffer and 30 µl of protease K, mix well and keep it in the water bath for 30 min. Transfer the supernatant into another eppendorf and add saturated phenol .Transfer the aqueous layer to another test tube and store the DNA at 4° C.

RESULTS

0ut of the 45 morphologically different colonies selected, almost 45% were obtained from serial dilution and remaining from agar over lay technique20 colonies were obtained which constitute about 55% of the total count obtained. Isolated pure colonies of bacterium were given unique accession number starting with a prefix of AMET indicating the institute name. Strain numbers AMET1901- AMET1920 were given these bacterial strains.

All cultures were tested positive (string formation were observed) so its gram negative. On the basis of simple staining performed, the isolates were determined to be rods in singles.

A preliminary identification of the isolates as a variety of luminescent bacteria can be made. Different concentration of SWCA medium was prepared with various salt concentration, AMET 1901, AMET1905, AMET1908, AMET1915, AMET1918 andAMET1920, Same Medium with various pH concentration, AMET 1902,AMET1903,AMET1904,AMET1913,

AMET1914 and AMET1920 shows immediate luminescent after 5 hours incubation. Different concentration of SWCA medium was prepared with various glycerol concentration. Immediate luminescent were observed from AMET 1901 to AMET1920 in higher concentration of glycerol 0.9% and 1.2%.

Table 1. Effect of different concentrations of NaCl on the luminescence of luminescent bacteria

Isolate code	Luminescence in different concentration of NaCl (%)				
	0	3	6	9	12
AMET1901	++	++	++	-	-
AMET1902	++	++	++	-	-
AMET1903	++	++	+++	-	-
AMET1904	++	++	+++	-	-
AMET1905	++	++	++	-	-
AMET1906	++	+++	++	-	-
AMET1907	++	+	++	-	-
AMET1908	++	+	++	-	-
AMET1909	++	+++	++	-	-
AMET1910	++	+	+	-	-
AMET1911	++	+	++	-	-
AMET1912	++	+++	++	-	-
AMET1913	++	+++	++	-	-
AMET1914	++	+++	++	-	-
AMET1915	++	++	++	-	-
AMET1916	++	++	++	-	-
AMET1917	++	++	++	-	-
AMET1918	++	++	++	-	-
AMET1919	++	++	++	-	-
AMET1920	++	+++	+++	-	-

-: No luminascenmce; +: Dull luminescence

++: Good luminescence; +++ Luxuriant luminescence

Table 2. Effect of different pH on the luminescence of luminescent bacteria

Isolate code	Luminescence of bacteria in different pH			
	5	7	9	11
AMET1901	+	+	+	+
AMET1902	+	+ +	+	+
AMET1903	+	+	++	+
AMET1904	+	+	++	+
AMET1905	+	+	+	+
AMET1906	+	+	+	+
AMET1907	+	+	+	+
AMET1908	+	+	+	+
AMET1909	+	+	+	+
AMET1910	+	+	+	+
AMET1911	+	+	+	+
AMET1912	+	+	+	+
AMET1913	+	+	++	+
AMET1914	+	+	+	+
AMET1915	+	+	+	+
AMET1916	+	+	+	+
AMET1917	+	+	+	+
AMET1918	+	+	+	+
AMET1919	+	+	+	+
AMET1920	+	+	++	+

-: No luminascenmce; +: Dull luminescence

++: Good luminescence; +++ Luxuriant luminescence

Table 3. Effect of heavy metals (1 mg/ml) on the luminescence of luminescent bacteria

Isolate Number	Heavy metal (1 mg/ml) tolerance s			Zone of inhibition in 1mg/ml		
	Copper	Zinc	Mercury	Copper	Zinc	Mercury
AMET1901	S	S	R	1.8	1	0
AMET1902	S	S	R	2.2	1.6	0
AMET1903	S	S	R	2.2	1.5	0
AMET1904	S	R	R	2	0	0
AMET1905	S	S	R	2	1.9	0
AMET1906	S	S	R	2	1.9	0
AMET1907	S	S	R	1.8	1.5	0
AMET1908	S	S	R	1.9	1.2	0
AMET1909	S	S	R	2	1.1	0
AMET1910	S	S	R	2.2	1.3	0
AMET1911	S	S	R	1.9	0.6	0
AMET1912	S	S	R	2	0.6	0
AMET1913	S	S	S	2.2	1.2	1
AMET1914	S	S	R	1.9	1.2	0
AMET1915	S	S	R	0.6	0.7	0
AMET1916	S	S	R	1.9	2	0
AMET1917	S	S	R	2	1.7	0
AMET1918	S	R	R	2.2	0	0
AMET1919	S	S	R	2.1	1.1	0
AMET1920	S	S	S	0.8	2	0.5

R- resistant ; S – Susceptible.

Values in parentheses are zone of inhibition in cm

Table 4. Effect of heavy metals (2 mg/ml) on the luminescence of luminescent bacteria

Isolate Number	Zone of inhibition in cm (2mg/ml)		Mercury	Copper
	Mercury	Copper		
AMET1901	1.5	1	S	S
AMET1902	1.2	1.1	S	S
AMET1903	1.4	0	S	R
AMET1904	1.3	1.6	S	S
AMET1905	1.1	1.3	S	S
AMET1906	1.5	1.1	S	S
AMET1907	1.3	1.3	S	S
AMET1908	1.4	1.6	S	S
AMET1909	1.2	2	S	S
AMET1910	1.1	1.7	S	S
AMET1911	1.2	1.6	S	S
AMET1912	1.2	1	S	S
AMET1913	1.2	0	S	R
AMET1914	1.1	2	S	S
AMET1915	1.2	1.1	S	S
AMET1916	1.4	0	S	R
AMET1917	1.3	0	S	R
AMET1918	1.1	0	S	R
AMET1919	1.5	1.6	S	S
AMET1920	1.2	0	S	R

R- resistant ; S – Susceptible. Values in parentheses are zone of inhibition in cm

Most organisms posess catalyze enzyme capable of breaking down hydrogen peroxide. Organisms containing the catalase enzyme will form oxygen bubbles when exposed to hydrogen peroxide. Bacteria produces catalase enzyme which splits H_2O_2 to h_20+0_2 which was exident from emerging bubble. Change in color of medium from green to blue indicates citrate utilization, but AMET 1908,AMET 1911,AMET 1914,AMET 1915,andAMET 1916 shows negative result. So there was no citrate production in these luminescent bacteria. In TCBS medium shows, all

cultures were grown, but AMET 1902, AMET 1903, AMET 1908, AMET 1915, AMET 1919 and AMET 1920 produce luminescent within 4 hours after streaking.

AMET (1901 to 1920) displayed maximum enzyme activity in the presence of Copper and Zinc and AMET 1903, AMET1918, and AMET 1920 shows lowest activity in the presence of Mercury Almost similar levels of activity were observed in case of ferric chloride and Magnesium. Therefore, it can be said that Copper promotes enhanced enzyme activity in AMET1901 to AMET 1920 whereas Mercury and Zinc inhibit activity. AMET1902, AMET1905, AMET1910, AMET1913 and AMET1918 displayed maximum enzyme activity in the presence of copper and the lowest activity in the presence of Manganese and Copper. Almost similar levels of activity were observed in case of Ferric Chloride and Magnesuim. Therefore, it can be said that Iron promotes enhanced enzyme activity in AMET1902, AMET1905, AMET1910, AMET1913 andAMET1918 whereas Copper and zinc lead to inhibition of activity. All 20 strains were tested for heavy metals resistance. The strain AMET1902, AMET1903, AMET1910 andAMET1918 has exhibited relatively resistance to all the five heavy metals tested. Antibiotic resistance is one of desire qualities of bacteria for bioremediation process. All those 6 selected bacteria were tested for six antibiotic testAMET1901 did not produce any zone and it was sensitive to AK and zone of inhibition were found in other antibiotic, AMET1905 which was sensitive to AK, NA30 and CF5, other culture showed zone of inhibition and which was sensitive to antibiotic. However in the present study, all the tested three bacterial strains are susceptible to all the six antibiotics tested.

DISCUSSION

Luminescence is an easily recognized characteristic feature for ecological studies; colonies are readily recognized and counted. The issue addressed by Blaise (1991) who has discussed the role of small scale microbiotests in aquatic toxicology. Bioluminescence has been observed in various insects, fish, and bacteria (Campbell, 1989; Hastings, 1986).Squids use the light produced from the bacteria for a behavior known as counterillumination (Young and Roper, 1977; Young et al., 1980; Jones and Nishiguchi, 2004). Luminescence emitted from the light organ reduces the squid's silhouette to match the intensity and wavelength of down-welling light (Young and Roper, 1977). This provides squids with a mechanism that allows them to evade predators by camouflage. A similarity between the luminescent bacteria concerns the conditions that prompt the luminescence. A key factor is the number of bacteria that are present. This is also known as the cell density (i.e., the number of bacteria per given volume of solution or given weight of sample). A low cell density (e.g., less than 100 living bacteria per milliliter) does not induce luminescence, whereas luminescence is induced at a high cell density (e.g., 10^{10} to 10^{11} living bacteria per milliliter). Luminous bacteria were identified in accordance with recent recommendations for identification of bacteria of the family Vibrionacea (Williams and Wilkins, 1984). The following parameters were assessed: morphology, Gram stain (Meditsina, 1973), growth characteristics, and

bioluminescence of luminous bacteria at different temperatures, sugar consumption as measured by color changes of bromothymol blue, a pH indicator; and enzymatic properties (Meditsina, 1973).Bioluminescence is the product of two distinct enzymes, firefly luciferase and bacterial luciferase. The application of the firefly enzyme in the study of mycobacteria has been described by other groups (Jacobs et al., 1993). The bacterial luciferase enzyme is a dimer of approx 80 kDa, consisting of α- and β-subunit (Meighen 1991, Hastings and Presswood 1978).For many of the organisms, the biochemical mechanisms of light emission is reasonably well understood (Meighen, 1988).

Table 5. Effect of different concentrations of glycerol on the luminescence of luminescent bacteria

Isolate code	Luminescence in different concentrations of glycerol (%)			
	0.1%	0.3%	0.6%	0.9%
AMET1901	+	+	+++	+++
AMET1902	+	+	+++	+++
AMET1903	+	+	+++	+++
AMET1904	+	+	+++	+++
AMET1905	+	+	+++	+++
AMET1906	+	+	+++	+++
AMET1907	+	+	+++	+++
AMET1908	+	+	+++	+++
AMET1909	+	+	+++	+++
AMET1910	+	+	+++	+++
AMET1911	+	+	+++	+++
AMET1912	+	+	+++	+++
AMET1913	+	+	+++	+++
AMET1914	+	+	+++	+++
AMET1915	+	+	+++	+++
AMET1916	+	+	+++	+++
AMET1917	+	+	+++	+++
AMET1918	+	+	+++	+++
AMET1919	+	+	+++	+++
AMET1920	+	+	+++	+++

- No luminascenmce; + : Dull luminescence ++ Good luminescence; +++ Luxuriant luminescence

Table 6. Antibiotic susceptibility/resistance of Luminescent Bacterial strains

Isolate code	Antibiotic sensitivity spectrum					
	Amikacin 30 mcg	Nitrofurantoin 300 mcg	Natillin 30 mcg	Nalidixic acid 30 mcg	Ceftazidime 30 mcg	Ciprofloxacin 5 mcg
AMET 1901	R	S	S	S	S	S
AMET 1902	S	S	S	S	S	S
AMET 1903	S	S	S	S	S	S
AMET 1904	S	S	S	S	S	S
AMET 1905	R	S	S	R	S	R
AMET 1906	S	S	S	S	S	S

R- resistant ; S – Susceptible.

Values in parentheses are zone of inhibition in cm

Table 7. Antibiotic susceptibility/resistance of Luminescent Bacterial strains

Isolate code	Antibiotic sensitivity spectrum (Zone of inhibition in cm)					
	Amikacin 30 mcg	Nitrofurantoin 300 mcg	Natillin 30 mcg	Nalidixic acid 30 mcg	Ceftazidime 30 mcg	Cipro Floxacin 5 mcg
AMET 1901	0	1.6	1.5	1.7	1	2.5
AMET 1902	1.4	1.6	1.2	1.4	1.5	0.8
AMET 1903	1.2	1	1.4	1.2	1.7	2
AMET 1904	1.3	1.2	1.4	1	1.2	1.8
AMET 1905	0	1.2	1.2	0	0.8	0
AMET 1906	1.2	1.6	1.4	1	1	1.7

The several non-selective media are useful in routine cultivation of microorganisms. It can be used for the cultivation and enumeration of bacteria which are not particularly fastidious.In a complex medium, the bacteria remove an inhibitor from the medium, the presence of which inhibits the synthesis of luciferase (Kempner and Hanson, 1968). In this study, the SWCA medium containing Peptone provides the essential nutrients for growth: nitrogen, vitamins, minerals and amino acids. It forms the principle sources of organic nitrogen, particularly amino acids and long chained peptides. Yeast extract provide the necessary water soluble substances like nitrogen compounds, carbon, vitamins and also some trace ingredients necessary for the growth of bacteria. Glycerol is a carbon source which has great influence on the luminescence of microorganisms.

The Gram Nature of the selected strains was determined by performing the KOH string test. This test is an alternative to classic Gram staining procedure and exploits differences in the cell wall of bacteria to help determine their Gram character (Ciufecu et al, 1986). This test has the advantage of simplicity, and it can be performed on older cultures. The reaction depends on the lysis of the gram-negative cell in the dilute alkali solution releasing cellular DNA to turn the suspension viscous. This can serve as a valuable adjunct to the traditional gram stain method (von Graevenitz and Bucher 1983). Synthesis of luciferase, the enzyme that catalyzes this reaction, is subject to several controls. The synthesis of the luciferases of some strains is subject to catabolite repression (Nealson et al., 1972; Ulitzur and Yashphe, 1975; Ulitzur et al., 1976) and is affected by the phosphotransferase system (Lin et al., 1976) as well as by other systems (Waters and Hastings, 1977). The catabolite modulator factor of Ullman et al. (1976) is similar in many of its properties to this inhibitor. Finally, it appears that oxygen somehow exerts a controlling effect, since some strains produce maximal amounts of luciferase when they are growing in well aerated media, while other strains give maximal yields only when the oxygen tension is quite low (Nealson and Hastings, 1977).The citrate test utilizes Simmon's citrate medium to determine if a bacterium can grow utilizing citrate as its sole carbon and energy source.MR-VP media contains glucose and peptone. All enterics oxidize glucose for energy; however the end products vary depending on bacterial enzymes. Both the MR and VP tests are used to determine what end products result when the test organism degrades glucose.

CONCLUSIONS

In general increasing the heavy metal concentration has decreased the bacterial growth with some expections. Industrial wastes contain certain types of toxic chemicals. The major categories of these pollutants are heavy metals ions toxic aromatic hydrocarbons and pesticides. Increasing the environmental pollutant by heavy metal from increasing their

utilization process (Nriagu and Pacyna 1988). The microbes that are assigned for bioremediation are expected to be resistant to heavy metals. So to find whether the selected strains are capable of survival in a heavy metal in a metal containing medium, the heavy metal tolerance spectrum has been done.

Most species of luminous bacteria growing in a complex medium must secrete a sufficient concentration of an autoinducer before luciferase synthesis can begin (Nealson et al., 1970; Eberhard, 1972; Nealson, 1977).

Antibiotics and pharmaceuticals are used to improve the quality of life worldwide. However, incomplete metabolism in human has resulted in release of large amounts of pharmaceutical drug into municipal waste water. So, the bacteria intended to bioremediate the municipal wastewater are expected to be resistant to various antibiotics (schwatrz et al., 2006; Nagulapally et al., 2009).The isolation of genomic DNA from a microorganism generally comprises three stages: cultivation of the cells, disruption to release cell contents, and chemical purification of the DNA. Two widely used methods for the preparation of bacterial DNA are those described by (Marmur, 1961; Kirby 1964**),** but procedures are frequently modified to suit the particular organisms under study. The best DNA isolation techniques produce good yields of pure, high molecular weight, largely double-stranded DNA. It may be problematic to obtain sufficient DNA from some bacteria if they are difficult to grow or to break open, or if they have small genome sizes.

ACKNOWLEDGEMENT

Authors ARK and EFL thank University of Boros for permissions and support. All the authors thank AMET University for facilities and support.

REFERENCES

Baltimore: Williams and Wilkins, 1984. Bergey‟s Manual Systematic Bacteriology, vol. 1.

Bang, S. S., Baumann, P., Nealson, K. H. 1978. Phenotypic characterization of PhotobacteriumIogei {sp. nov.), a species related to P. fischeri. Current Microbiology 1:285-288.

Baross, J. A., P. A. Tester, andR. Y. Morita. 1978. Incidence, microscopy and etiology of exoskeleton lesions in the tanner crab,Chionoecetestanneri.J. Fish. Res. Board Can. 35:1141–1149.

Baumann, P., Baumann, L, 1980. The marine Gram-negative eubacteria, in: Start, M. P., Stolp, H., Triper, H G. Balows, A., Schlegel, H. G. (eds.), The prokaryotes: A handbook on habitats, isolation, and identification of bacteria. New York, Heidelberg, Berlin: SpringerVerlag. In press

Baumann, P., Baumann, L. 1983. Genus Photobacterium. In: Krieg, N. R. (ed.), Bergey's Manual of Systematic Bacteriology, 9th ed. Baltimore: Williams and Wilkins Co., in press.

Baumann, P., Furniss, A. L., Lee, J. V. 1983. Genus Vibrio.

Baumann, P., Gauthier, M., Baumann, L. 1983. Genus Alteromonas. in: Krieg, N. R. (ed.), Bergey's Manual of Systematic Bacteriology, 9th ed. Baltimore: Williams and Wilkins Co., in press.

Blaise, C. 1991.Microbiotest in Aquatic Ecotoxicology: Characteristics, Utility, and Prospects. Environ. Toxicol. Wat. Qual. 6, 145-155.

Campbell, A.K. 1989. Living Light: Biochemistry, Function and Biomedical Applications. Essays in Biochem. Vol 24, 41-81.

Ciufecu C, Cedru C, Năcescu N, Israil, A.1984. Rapid method of determining the presence or absence of gram constituents. Rev IgBacteriolVirusolParazitolEpidemiol PneumoftiziolBacteriolVirusolParazito lEpidemiol. 29(3):279-82. Romanian. PubMed PMID: 6390654.

Ciufecu C, Cedru C,Israil, A. 1986.AThe 3% potassium hydroxide test, an alternative to gram staining II.RevIgBacteriolVirusolParazitolEpid emiolPneumoftiziolBacteriolVirusolPa razitolEpidemiol. Unbound Medline. 31(4):379-80

Cormier, M. J., Lee, J., Wampler, J. 1975. Biolumiuescence: recent advances. Ann. Rev. Biochem. 44, 255-272.

Cormier, M. J., Totter, J. R. 1968. Bioluminescence: enzymic aspects. Photophysiology 4, 315- 353

Eberhard A. 1972. Inhibition and activation of bacterial luciferase synthesis. J Bacteriol.109(3):1101–1105.

Fitzgerald, J. M. 1977. Classification of luminous bacteria from the light organ of the Australian pinecone fish, *Cleidopus gloria-maris* .Arch. Microbiol. 12:153–156.

Hastings JW, Presswood RP (1978). "Bacterial luciferase: FMNH2-aldehyde oxidase". Methods Enzymol. Methods in Enzymology 53: 558–70.

Hastings, J. W. 1968.Bioluminescence. Ann. Rev. Biochem. 37, 597- 630.

Hastings, J. W. 1986. Bioluminescence in bacteria and dinoflagellates, p. 363-398. In A. J. Govindjee, J. Amesz, and D. C. Fork (ed.), light emission by plants and bacteria. Academic Press, New York, N.Y.

Hastings, J. W., andK. H. Nealson. 1981. The symbiotic luminous bacteria. p. 1332–1346.In M. P. Starr, H. Stolp, H. G. Truper, A. Balows, and H. G. Schiegel (eds.), Prokaryotes, Vol. II. Springer-Verlag, New York.

Jacobs, W.R..Jr_. Barletta, R.G., Udani, R., Chart, J., Kal-kut. G. Sosne, G., Kieser, T.M., Sarkis, G.J., Hatfult, G.F. & Bloom, B.R, (1993), Rapid assessment of drug susceptibilities of *Mycobacterium tuberculosis* by means of luciferase reporter phages. Science, 260, 819-822.

Jones. B.W, Nishiguchi. M.K. 2004. Counterillumination in the Hawaiian bobtail squid, *Euprymnas colpes* Berry (Mollusca: Cephalopoda). Marine Biology. 144:1151-1155

Kempner, E. S., and F. E. Hanson. 1968. Aspects of light production by *Photobacterium fischeri*. J. Bacteriol. 95:975-979. Losick, R., and D. Kaiser. 1997. Why and how bacteria communicate. Sci. Am. 276:68-73.

Kirby, K. S. 1964. Isolation and fractionation of nucleic acids. Progr. Nucl. AcidsRes. 3,1.

Lebedeva M.N. 1973. Rukovodstvoprakyicheskimanyatiyam pomeditsinskojmicrobiologii. II M., Meditsina, 312 s. [In Russian].

Lin, P., Saier, M. H., Nealson, K. H. Regulation of the synthesis of the bioluminescent system in Beneckeaharveyi. Fed. Proc. 35,

Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms J Mol. Biol. 3,208-218

Meighen, E. A. 1988. Enzymes and Genes from the lux Operons of Bioluminescent Bacteria. Annual Review of Microbiology. 42:151.

Meighen. A, E. 1991. Molecular Biology of Bacterial Bioluminescence. MICROBIOLOGICAL REVIEWS, Vol. 55, No.1,p. 123-142.

Nagulapally, S. R., Ahmad, A., Henry, A., Marchin, G. L., Zurek, L., &Bhandari, A. (2009). Occurence of Ciprofloxacin-, Trimethoprim-Sulfamethoxazole-, and Vancomycin-Resistant Bacteria in a Municipal

Wastewater Treatment Plant. Water Environment Research: A Research Publication of the Water Environment Federation, 81(1), 82-90.

Nealson KH, Hastings JW. 1977. Low oxygen is optimal for luciferase synthesis in some bacteria. Ecological implications. Arch Microbiol.112(1):9–16.

Nealson KH, Markovitz A.1970. Mutant analysis and enzyme subunit complementation in bacterial bioluminescence in Photobacteriumfischeri. J Bacteriol. 104(1):300–312.

Nealson, K. H., Eberhard, A., Hastings, J. W. 1972. Catabolite repression of bacterial bioluminescence: Functional implications. Proc. Nat. Acad. Sci. USA 69, 1073-1076.

Nealson, K. H., Hastings, J. W. 1979. Bacterial bioluminescence; its control and ecological significance. Microbiological Reviews 43:496-518.

Nriagu, J.O., Pacyna, J.M., 1988. Quantitative assessment of worldwidecontamination of air, water and soil by trace metals. Nature 333, 134-139.

O'Brien, C. H., andR. K. Sizemore. 1979. Distribution of the luminous bacterium *Beneckea harveyi* in a semitropical estuarine environment. Appl. Environ. Microbiol. 38:928–933

Raymond, J. A., andA. L. DeVries. 1976. Bioluminescence in McMurdo Sound, Antarctica.Limnol. Oceanogr. 21:599–602.

Ruby EG, Greenberg EP, Hastings JW. 1980. Planktonic marine luminous bacteria: species distribution in the water column. Appl Environ Microbiol. 39(2):302–306.

Ruby, E. G., andK. H. Nealson. 1978. Seasonal changes in species composition of luminous bacteria in nearshoreseawater.Limnol. Oceanogr. 23:530–533.

Ruby, E. G., Morin, J. G. 1978. Specificity of symbiosis between deep-sea fishes and psychrotrophic luminous bacteria. Deep-Sea Research 25:161-167.

Ruby, G., E. 1996. LESSONS FROM A COOPERATIVE, BACTERIAL-ANIMAL ASSOCIATION:The *Vibrio fischeri–Euprymnas colopes* Light Organ Symbiosis. Annu. Rev. Microbiol. 50:591–624.

Schwartz, T., Volkmann, H., Kirchen, S., Kohnen, W., Schon-Holz, K., Jansen, B.,Obst, U., 2006. Real-time PCR detection of *Pseudomonas aeruginosa* in clinicaland municipal wastewater and genotyping of the ciprofloxacin-resistant.isolates. FEMS Microbiol. Ecol. 57, 158e16.

Tebo, B. M., D. S. Linthicum, andK. H. Nealson. 1979. Luminous bacteria and light emitting fish: ultrastructure of the symbiosis.BioSystems 11:269–280.

Ulitzur S, Yashphe J and J. W. Hastings. Inhibition and stimulation of the development of the bioluminescentsystem in Beneckeaharveyi by cyclic GM.Proc. Natl. Acad. Sci. USA. Vol. 73, No. 12, pp. 4454-4456.

Ulitzur S, Yashphe J. 1975. An adenosine 3', 5'-monophosphate-requiring mutant of the luminous bacteria Beneckeaharveyi. BiochimBiophysActa. 404(2):321–328

Ullmann, A., Tillier, F., Monod, J.1976.Catabolite modulator factor: a possible mediator of catabolite repression in bacteria. Proc. nat. Acad. Sci. (Wash.) **73**, 3476–3479.

Von Gravenitz, A and C Bucher. 1983. Accuracy of the KOH and Vancomycin Tests in Determining the Gram Reaction of Non-Enterobacterial Rods. J ClinMicrobiol. 16(4):983-985.

Waters CA, Hastings JW. 1977. Mutants of luminous bacteria with an altered control of luciferase synthesis. J Bacteriol. 131(2):519–525.

Williams and Wilkins, 1984. Bergey‟s Manual Systematic Bacteriology, vol. 1.

Yetinson, Y., andM. Shilo. 1979. Seasonal and geographic distribution of luminous bacteria in the eastern Mediterranean Sea and the Gulf of Elat.Appl. Environ. Microbiol. 37:1230–1238.

Young, R.E., Kampa, E.M., Maynard, S.D., Mencher, F.M., Roper, C.F.E., 1980. Counterillumination and the upper depth limits of midwater animals. Deep Sea Res. 27A, 671–691.

Young, R.E., Roper, C.F.E., 1977. Intensity regulation of bioluminescence during countershading in living midwater animals. Fish. Bull. 75, 239–252.