Effects of some physicochemical parameters and UV irradiation on Bioluminescence

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Abstract:

Culturable luminous bacterial strains serves as efficient biosensor in different monitoring systems specially radiation pollution. The aim of this study was to carry out an effective method for the isolation and characterization of novel luminescent bacterial species from sea water sample Tarkarli beach of Sindhudurg, India and efforts have been made to isolate luminescent bacterial species by using simplest possible medium and comparative assessment of the propensity to emit light under altering culture conditions such as temperature, medium composition, pH etc. The preliminary screening and molecular characterization was done following the standard techniques. Further, 16s rRNA amplified genes were sequenced and then analyzed by BLAST for similarity search followed by Phylogenetic analysis using MEGA software revealed that the strain is *Vibrio natriegens* which was successfully isolated from Tarkarli beach of Sindhudurg, India. By utilizing outcomes of luminescence intensity measurements of isolated bacteria acquired with luminometer, the strains and media providing stern and steady luminescence were endeavored in this study.

Keywords: Bioluminescent bacteria; *Vibrio natriegens*; 16S rRNA gene analysis; Biosensor.

Introduction:

Bioluminescence is natural phenomenon comprising in the generation of light by some living organisms as an outcome of their biochemical and enzymatic activity (Malave, Rubio, Rios 2010). It is proficient diverse group of organisms for various purposes which alters from one organism to other for instance defense against predators, for predation or for communication with their mates. Luminescence can be spotted in insects, certain plants and fungi but predominantly in bacteria. Bacteria are the most prolific bioluminescent organisms (Danyluk, Uchman, Konieczny, Bilska 2007; Agata, Jasiecki, Bogdan, Szpilewska, Grzegorz 2000). Bioluminescence emission of different luminescent organisms known to emit blue or green light catalyzed by

enzyme luciferase and regulated by *lux* gene operon. The blue and green light spectrum are the wavelengths that can diffuse through the brine most easily and hence the probable reasons that almost all aquatic light emission lies in the same range. Every organism's luminescence is unique in wavelength, duration, timing and consistency of flashes (Ramesh, Mohanraju 2015).

Bioluminescence is exhibited by all marine dimensions and has evolved several times beginning from bacteria to fish to intensely influence interactive and ecosystem buildups (Malave, Rubio, Rios 2010). Bacteria whereas are the most abundant among the bioluminescent species in marine ecosystem. While analyzing the chemistry and molecular biology of bioluminescence, it was detected that a light emitting molecule luciferin is oxidized in this reaction in which rate of reaction is controlled by luciferase enzyme which are of different types in fungi, insects and invertebrates. Essentially $FMNH_2$ which is a reduced riboflavin mononucleotide, activates Luciferase that further oxidizes a compound forming a highly stable complex with aldehyde that decades slowly by emitting luminous energy as a result of oxidation of the substrates. Consequently, emits a blue-green (490 nm) light that gives these natural light-producing molecules luminescent bacteria an important ecological role (Haddock, Moline, Case 2010). The investigations revealed that the presence of chemical pollutants and toxins disturbs cell metabolism of bioluminescent bacteria, reducing the intensity of the emitted light. Novel approaches understanding the chemistry and molecular biology such as transferring the bacterial bioluminescence genes (lux genes) have led to innumerable esteemed applications and a promising tool in the field of environmental biotechnology as these bioluminescent bacteria are nonpathogenic they are absolutely secure to work with as a microbe also they can be easily cultivated under laboratory environments and hence could serve as a bioindicator of pollution.

In the race of several countries to become most powerful have lead to such human activities which has augmented various types of pollution at an alarming rate. Despite of the threats of the major pollutions such as air, water, soil etc. exposure to radiation pollution has perilous effects on nature. The main radiation hazards in the environment come from ionizing and non-ionizing radiations. Their persistency and toxicity to most of the environmental biota has become a major issue of concern in recent decades. Radioactive material being unstable energy causes very fast and highly deleterious effects on not only human beings but also on plants and animals (Agata, Jasiecki, Bogdan, Szpilewska, Grzegorz 2000). Recently bioluminescent bacteria have been used as a bioassay because these assay systems are based on luminous bacteria are good candidates for scrutinizing the environmental toxicity. Here bacterial luminescent intensity that can be clearly measured instrumentally is the parameter used to examine the effect of hazardous UV radiations as these bioluminescent assays give rapid results also are very sensitive as well as availability of the devices for toxicity registration.

Materials and Methods:

Collection of Water Sample:

To accomplish the objective of this research, sampling was performed in 2016 in month of November so that it was not affected by rains from Tarkarli beach Sindhudurg, India. Sterile 500 ml bottle (stored in ice) bottles were used to contain enough water and after collection, the bottles were sealed and shipped immediately at 4^0C in cool boxes to the laboratory for further procedure.

Screening, isolation & purification of bioluminescent bacteria:

At the beginning, 300-500 µL of water samples were plated on nutrient agar medium supplemented with 2-3% glycerol and 75% sea water by using spread plate and then were kept at 20-22^0C in an incubator for 24-48 h. After incubation plates were examined in a dark room to observe bioluminescence. Positive colonies were picked up and transferred to other plates with same medium by streak plate method and subcultured further to obtain pure culture. The strains were further subjected to media optimization to obtain steady and stern luminescence.

Media optimization for Bioluminescent intensity assay:

In order to obtain definite growth and luminescence pattern the bacterial isolates were further tested on different types of media available for isolation bioluminescent bacteria. The media used were as LA medium, Luminescent Medium(LM), BOSS and Modified Nutrient agar (NA) media as depicted in Table 1. After using these different media, the strain showing highest luminescence was selected for further microbial and molecular characterization studies.

LA medium	NaCl 10g, Yeast extract 5g, Pepton (Bacto-peptone) 10g, Agar 15g, Made up with distilled water to 1000 ml.
BOSS medium	NaCl 30g, Glycerol 1g, Pepton (Bacxto-peptone) 10g, Meat extract 3g, Made up with distilled water to 1000ml.
LM medium	Yeast extract 3g, Glycerol 3g, $CaCO_3$ 1g, Trypton 3g, Made up with sea water to* 1000 ml.
Modified NA medium	Nutrient Agar 28g, Sea Salt 16.50g, Glycerol 3%.

Table 1: Varied media for bioluminescent bacterial isolation.

Scanning Electron Microscopy:

To characterize the parameters like morphological shape, size and external appearance of the bacteria were studied and confirmed using scanning electron microscopy (JEOL-JSM-2380- Analytical SEM). Overnight grown bacterial broth was centrifuged at 6000 rpm for 10 min. The pellet washed with phosphate buffer saline for 3 times. Few drops of 0.25% gluteraldehyde (in Na-phosphate, pH 7.2) added to bacterial pellet. Further pellet was incubated at room temperature for overnight. Overnight incubated bacterial pellet was further resuspended in Na- phosphate buffer and centrifuged at 6000 rpm for 5 min. This step was repeated for 3 times. Pellet was subjected to series dehydration with different ethanol concentrations, 10 min for each rinse (30%, 50%, 70%, 80% Ethanol). Finally, for complete dehydration bacterial pellet was incubated in 100% ethanol for 20 min. Further preparation of SEM stub was done by applying the adhesive tape and then adding the bacterial sample sputter coated with silver onto the tape. The samples were examined by Scanning Electron Microscopy at various resolution 5,000X, 10,000X and 12,000X.

Biochemical Characterization:

For characterization of biochemical parameters, a total of 14 biochemical assays such as: Oxidase, Nitrate reduction, Gelatinase, Simmons citrate, Indole, Methyl Red, Voges-proskauer, Catalase, Urease, Cytochrome oxidase, Amylase, Gelatin liquefaction and Hydrogen sulphide production test were performed, using standard protocols. For carbohydrate utilization test various sugars were used viz., glucose, lactose, mannitol, sucrose, maltose and pyruvate (George, 2005).

Identification of screened isolate:

The screened isolate was further subjected to molecular identification. DNA extraction was done using agarose gel electrophoresis. The extracted DNA was amplified using PCR. Details of primers and PCR programme are given below

8F: 5^0 AGA GTT TGA TCC TGG CTC AG 3^0 and

1492R: 5^0 ACG GCT ACC TTG TTA CGA CTT 3^0.

Amplification parameters were as follow: an initial denaturation of 94°C for 3 min., 30 cycles of 94°C 30 s, 52.7°C for 30 s, and 72°C for 1.30 min. Amplicon was confirmed by 1% Agarose Gel electrophoresis. The sequence was checked against the microbial nucleotide databases using BLASTN search algorithm and identified for genus and species. Most accurate sequences were further aligned to obtain the phylogram using the MEGA5 software.

Effect of temperature, pH and Salinity on the growth and luminescence of isolate Strain:

After optimizing media to enhance growth and luminescence, bacteria were subjected to alterations in various temperatures incubated at 4^0C, 22^0C, 37^0C, and 45^0C at different pH such as 6, 7 and 8 and salinity with (8.25, 16.50, 24.75, 33.00g sea salt/1000 ml) as 25%, 50%, 75% and 100% respectively to standardize the optimum temperature, pH and medium constitute. The flasks containing 100 ml of modified Nutrient Agar medium were inoculated loop full of 24 h old culture and kept on shaker incubator at 120 rpm with above mentioned variations in temperature, pH and salinity for 24 h. The culture flasks were placed in magnetic agitators for maintaining identical mixing conditions for bacterial suspensions as well as providing sufficient amounts of oxygen to cultures of the analyzed bacteria.

Effect of Ultra Violet ray irradiation on luminescence of isolated strain:

At very first to evaluate effects of UV irradiation on bioluminescence, bacterial strain was grown to a density of about 10^7 cells per ml. Cultures were diluted 10^4 times in a fresh medium (total volume of each culture was 10 ml),followed by incubation for 30 min. Bacteria were harvested by centrifugation (2000 × *g*, 5 min) and resuspended in an equal volume of 3% NaCl. Following UV irradiation (0-16 J/m^2) samples of 0.5 ml each were withdrawn in different time intervals up to 6 h after irradiation and luminescence was measured using a luminometer (Promega-Glomax MULTI JR Detection System) (Agata, Konrad, Wegerzyn 2002).

Results and Discussion:

Isolation & phenotypic characterization:

Discrete colonies were obtained by spread plate method of sea water samples. Amongst them desired vividly luminescent colonies were picked and inoculated further by streak plate method until purified luminescent bacterial strains were obtained in Fig.1. Here different types of mediums were used on obtained strains to enhance bacterial luminescence. Among them the strain HAM was showing steady enhanced luminescence for longer period and hence it was selected for further investigations.

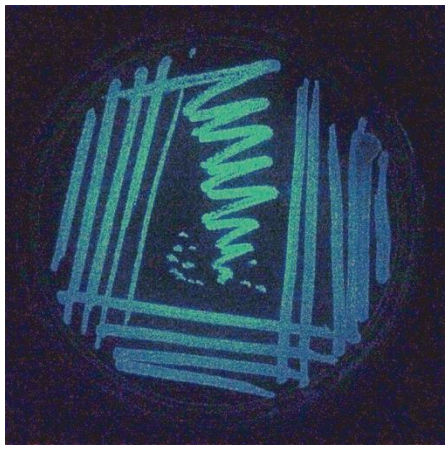

Figure 1: Isolated colonies of luminescent bacteria on Nutrient Agar Plate (white color is the luminescent bacteria)

Morphological and biochemical characterization of bacterial isolate:

The characterization of bacterial isolates under current exploration with regard to size, shape, arrangement of cells and motile features, colonial characteristics have been proved to be better for understanding of the morphology of bacterial isolate using different staining procedures. The isolate HAM was found to be highly motile. Also, the SEM analysis provided an additional perceptive to the external morphology and appearance of the bacterial cells. The isolated bacterium exhibited a basic morphology of elongated bacilli with 1µm size (Fig.2).

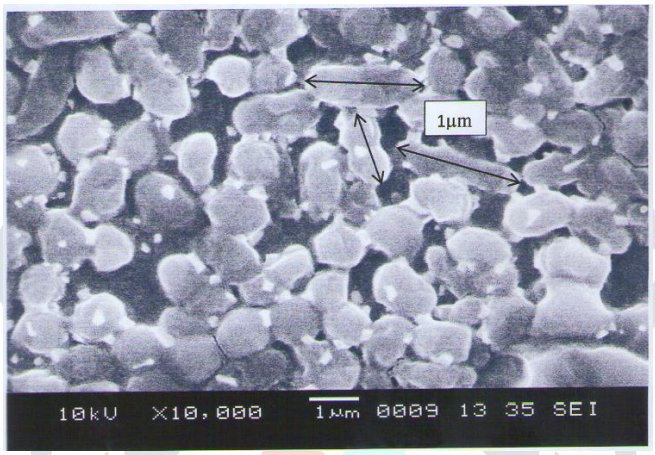

Figure 2: Scanning electron microscopy images of isolated bacteria

The biochemical profile of isolate as shown in table no.2 showed Positive Citrate, Indole, Oxidase, Amylase and Gelatinase. When considered the ability to metabolize carbohydrates, it was seen that Mannitol, Glucose, Sucrose, Maltose and Arabinose could be utilized by the isolate while Lactose and Pyruvate were not utilized by the isolate.

Sr.No.	Biochemical Test	Positive/Negative
1.	Citrate	Positive
2.	Indole	Positive
3.	Methyl Red	Negative
4.	Voges-Proskauer	Negative
5.	H_2S Production	Negative

6.	Catalase	Negative
7.	Oxidase	Positive
8.	Urease	Negative
9.	Amylase	Positive
10.	Gelatinase	Positive
11.	Motility	Positive
	Carbohydrates utilization Test	
12.	Mannitol	Positive
13.	Glucose	Positive
14.	Sucrose	Positive
15.	Pyruvate	Negative
16.	Maltose	Positive
17.	Arabinose	Positive
18.	lactose	Negative

Table 2: Biochemical Profiling of luminescent bacterial isolates

The isolated strain exhibiting high intensity, steady luminescence on modified Nutrient Agar Medium (with 3% glycerol and 25% sea water) when grown at 22^0C at pH 7 (Figures 3,4 and 5), while sluggish growth without luminescence was recorded at 37^0C and at 4^0C and 45^0C there was no appearance of growth (Figure 3). Effects of Sea Water Percentage at different percentages (25%, 50%, 75% and 100%) on the growth and luminescence of strain depicted that highest growth and luminescence at 25% while sluggish growth and less luminescence at 50%, 75% and 100% (Figure 5).

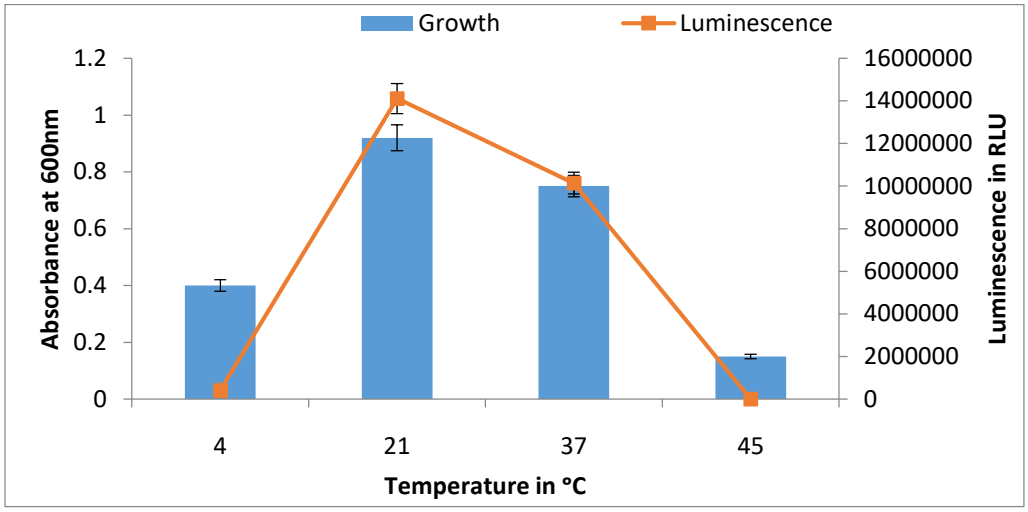

Figure 3: Effect of Temperature variation percentages on the growth and luminescence of Strain

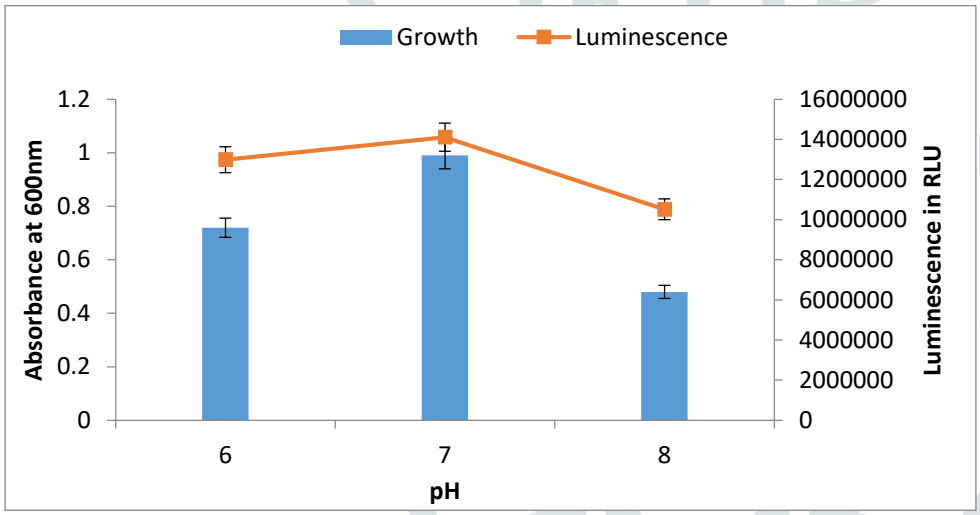

Figure 4: Effect of pH variation percentages on the growth and luminescence of Strain

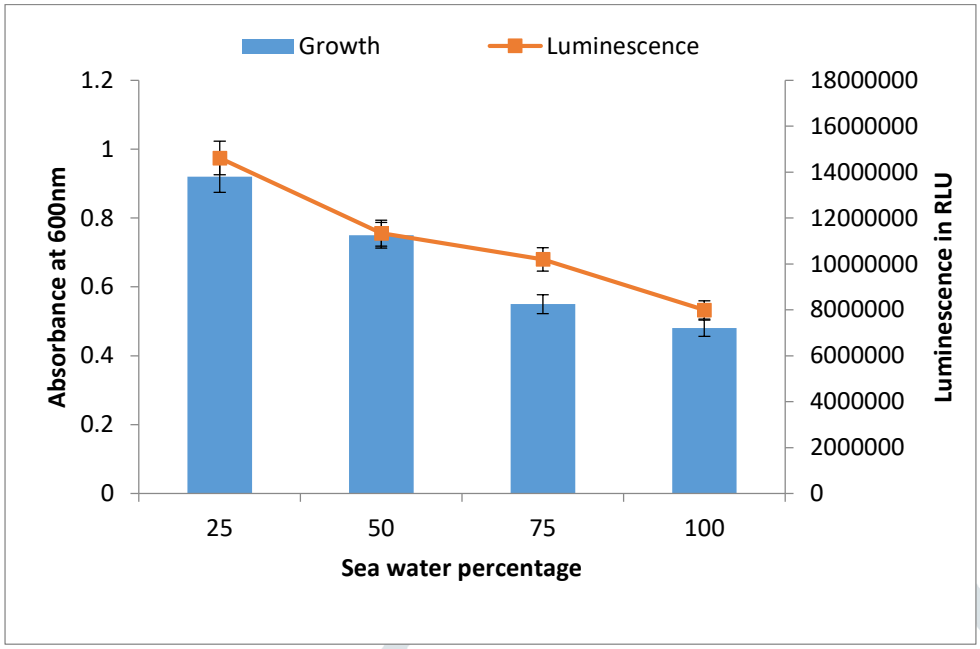

Figure 5: Effect of Sea Water Percentage variation percentages on the growth and luminescence of Strain.

PCR amplification of 16S rRNA gene:

PCR amplicon was electrophoresed on 1% Agarose Gel, as single band 1500 bp DNA has been observed when compared with 1 KB molecular marker (Figure 6). Consensus sequence of 1423 bp rDNA gene was generated from forward and reverse sequence data using aligner software.

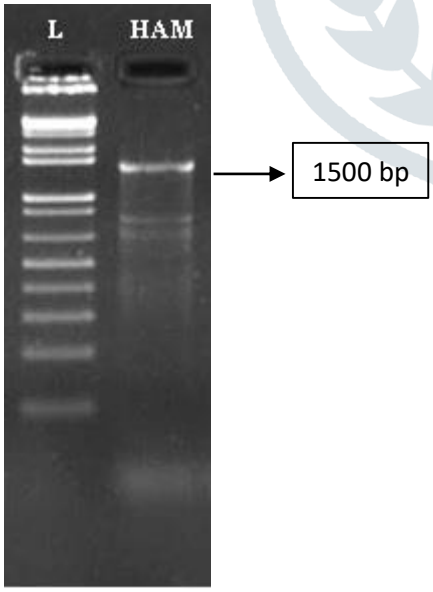

Figure 6: 16S rRNA gene amplification of isolated strain (HAM) resolved on the 1.2% agarose gel along with 1500 bp DNA marker (M).

Sequence alignments and homology:

The 16S rDNA gene sequence was used to carry out BLAST with the non redundant NCBI GenBank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program ClustalW. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA5. The isolate which was labeled as Strain HAM, based on nucleotide homology and phylogenetic analysis, was proved to be *Vibrio natriegens* as per close homology obtained with GenBank (Figure 7).

BLAST Homology

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Vibrio sp. CFZ12 16S ribosomal RNA gene, partial sequence	1459	1459	62%	0.0	96%	KT832698.1
Vibrio sp. YD8 16S ribosomal RNA gene, partial sequence	1458	1458	62%	0.0	96%	GU434166.1
Vibrio sp. ADZ10 16S ribosomal RNA gene, partial sequence	1454	1454	62%	0.0	96%	KX057342.1
Vibrio natriegens strain 534 16S ribosomal RNA gene, partial sequence	1454	1454	62%	0.0	96%	JN188424.1
Vibrio sp. JNU-H028 16S ribosomal RNA gene, partial sequence	1448	1448	63%	0.0	96%	KC121362.1
Vibrio diabolicus strain P4-1 16S ribosomal RNA gene, partial sequence	1447	1447	62%	0.0	96%	KC261281.1
Vibrio sp. CFZ9 16S ribosomal RNA gene, partial sequence	1443	1443	62%	0.0	96%	KT832695.1
Vibrio sp. CFZ5 16S ribosomal RNA gene, partial sequence	1443	1443	62%	0.0	96%	KT832692.1
Vibrio diabolicus strain SF16 16S ribosomal RNA gene, partial sequence	1443	1443	62%	0.0	96%	KF668280.1
Vibrio sp. JSA02 16S ribosomal RNA gene, partial sequence	1443	1443	62%	0.0	96%	KC012644.1

Phylogram

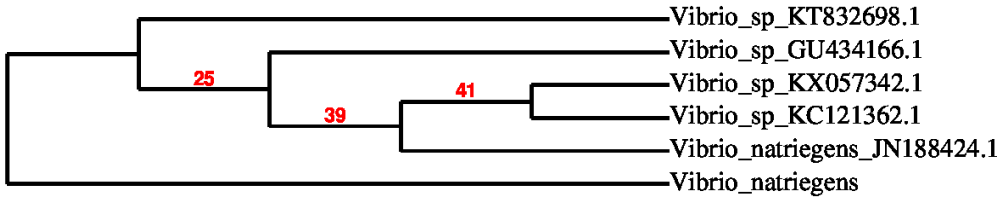

Figure 7:

BLAST homology and Phylogram of isolated Strain(HAM) proved to be *Vibrio natriegens* as per close homology obtained with Gene Bank.

Nucleotide sequence accession number:

16S rRNA gene sequence of *V. natriegens* has been deposited in the GenBank Database with accession number MG386478.

Effect of Ultra Violet ray irradiation on isolated strain:

Intensity of light emission by luminescent bacteria in response to UV irradiation was tested. A typical curve of luminescence intensity per cell after dilution of a dense *V. natriegens* culture is shown in Figure 8.

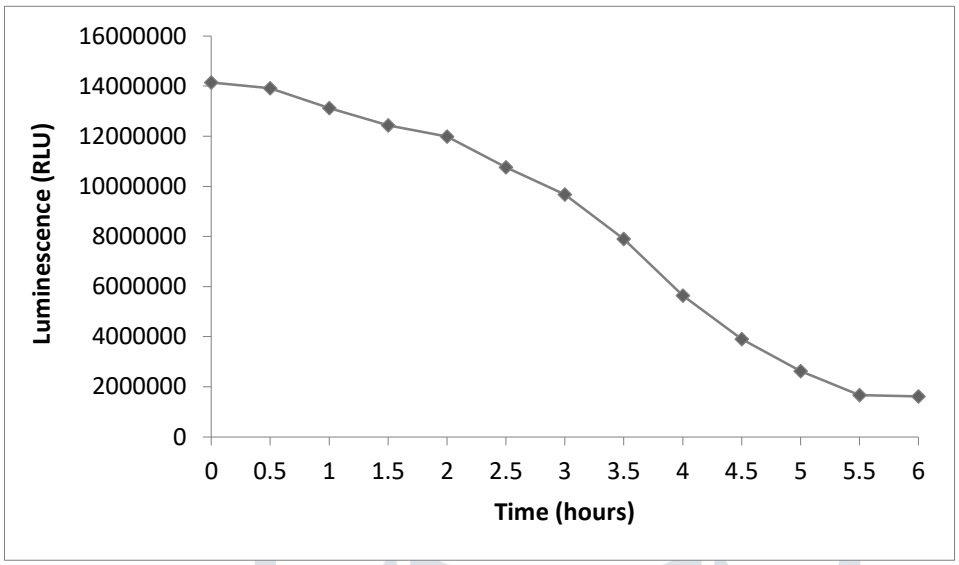

Figure 8: UV irradiation sensitivity profile of isolated *Vibrio natriegens* against time in hours.

This study demonstrates that luminescence of marine bacterial strain *V. natriegens* is significantly decreased by UV irradiation relatively shortly after dilution of cultures in respect to increase in time. Results presented in this study reveals that, bacterial luminescence systems, apart from being regulated by quorum sensing, are altered by UV irradiation. Such a regulation of expression of genes involved in bacterial luminescence has implications for the use of luminescent bacteria in several assay systems.

Conclusion:

Bioluminescence comprises generation of light by microorganisms which can be expended further to design on-spot censoring techniques and assays. The present investigation was an attempt to study the main features of bacterial bioluminescence and isolation of luminescent bacteria from marine various water samples among them most efficient strain was selected for studying effects of some physicochemical parameters which showed high intensity, steady luminescence on modified Nutrient Agar Medium with 3% glycerol and 25% sea water when grown at 22^0C at pH 7. It was found that *Vibrio natriegens* can be easily cultivated under in vitro and completely safe to work with as a nonpathogenic microbe. hence, it was chosen as an organism that could serve as a bio indicator to monitor radiation pollution. In the current study, preliminary characterization including microbiological and molecular approaches has been carried out to explore applications of bioluminescence in monitoring radiation pollution caused by Ultra Violet rays which revealed that bacterial strain *V. natriegens* had diminished luminescence after UV irradiation with increase in time. The altered luminescence by UV irradiation gives notion to use these luminescent bacteria as biosensor for further irradiation studies and its monitoring effects of other radiations also.

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Conflict of interest: Authors hereby declare that there is no conflict of interest. We have not received research grants from any company. This article does not contain any studies with human or animal subjects performed by the any of the authors.

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