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Metal tolerance of bioluminescent bacteria isolated from marine organisms

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Bacterial Luciferin-Luciferase system is encoded by a set of genes (Lux CDABE). This type of bacteria was gram negative, facultative anaerobes and most luminescent bacteria have been in different genera *Vibrio*, *Beneckea, Shewanella* & *Photobacterium* and the bioluminescent bacteria were isolated by smashing the squid sample in the luminescent broth and observed at intervals for 24 hours. Bioluminescent colonies were streaked on luminescent agar medium, TCBS medium and luminescent broth. Further, the preliminary identification like Gram staining, Motility Test and Various biochemical tests were carried out for identifying the isolated bacteria and the phylogeny of the isolated bacteria were determined by performing 16SrRNA gene Sequencing Analysis. Metal toxicity test was done by using metals like Zinc sulphate, Copper chloride and Lead nitrate with different concentration of metals. It was concluded that the isolated BL-1 strain from the marine organism shows tolerance against certain heavy metals.

[**Keywords**: Bioluminescence, Marine, Luciferin, Luciferase, squid, Lux gene.]

Introduction

The term "Bioluminescence" was coined for the first time by [1] and the Mechanism is studied [2]. Bioluminescene refers to the production of light as a consequence of conversion of chemical energy within the living vertebrates and invertebrates and it is a ubiquitous but randomly dispersed natural phenomenon[3], and this phenomena are exhibited by bacteria of different genera, such as *Vibrio, Photobacterium, Beneckea and Shewanella* [4 & 5] .Bioluminescent bacteria are Gram-negative and facultative anaerobes that are present in shrimps and squids [6]**.** Bioluminescent bacteria are ample in nature when compare to the bioluminescent animals [7]. Distribution of the luminous bacteria depends on the climatic change, salinity and temperature [8]. For the emission of light the two important chemicals are required namely luciferin and luciferase and this was first discovered by Raphael Dubois. Luciferin is an organic molecule that is oxidized when the enzyme, luciferase is present and produces inactive oxyluciferin and light. Bacterial luciferin is found within the particular tissues of squids, shrimps and other fishes. A luciferin called coelenterazine is notably present in shrimps, squids, jellyfishes and fishes [9]. The reaction of bioluminescence is catalysed by the enzyme called luciferase and it is coded by the genes lux AB [10]**.** Bacterial luciferase is a 77 kilo Dalton chimeric protein of two identical subunits, namely A and B coded by 2 adjacent genes called lux A and lux B that forms the regulated lux operon .Later it was discovered that the fatty aldehydes and $FMNH_2$ are essential for the emission of light from the luminous bacteria.

The luminescent reaction was carried out by 2 steps.

$$FMN+NADH+H^{+}\text{----------}>FMNH_2+NAD^{+}$$

(FMN-Reductase)

$$FMNH_2+RCHO+O_2\text{----------}>FMN+RCOOH+H_2O+Light$$

(Luciferase)

This reaction was discovered by [11 & 12].The dissolved oxygen, the reduce flavin mononucleotide and a long chain aldehyde are the main key substrates for the light emission [13]. TCBS medium was used as the best selective medium for the isolation of *vibrio* species, and for some strains of *Shewanella* that may produce slight growth on it

[14]. Bioluminescent bacteria are heterophic bacteria and gram negative rods [15] & [16].

Materials and Methods

Different types of samples were collected from the Mahabalipuram beach and from the Marina beach, using sterile container and brought to the laboratory for further processing. Sea water, soil and Fish samples were collected from Chennai and brought to the laboratory for further processing. And a different variety of shrimps and squid were purchased from the market on seacoasts. Using mortar and pestle the variety of fish samples were smashed. Shrimps and squid were transferred to the separate 500 ml conical flask and added to the Luminescent medium broth (Sea water- 1000 ml, Yeast extract – 5.0 g, Glycerol – 3.0 g, Calcium chloride – 1.0 g, Tryptone- 5.0 g) and incubated under the anaerobic condition in the dark room and observed under dark. Transferred the sample to the luminescent broth and kept for incubation in the dark room. luminescent agar (Sodium chloride – 10.0 g, Yeast extract- 5.0 g, peptone- 10.0, Agar- 15.0, Distilled water- 1000 ml) was chosen as a selective medium to perform a spread plate technique. A quadrant streaking was performed in both luminescent agar plate and Thiosulphate citrate bile salt agar (TCBS) plate. (Tab.1, 2 and 3).

The morphological characterization was performed to determine the morphology of the bioluminescent bacteria and the Gram staining method was done for identification and Motility test was done using a hanging drop method. Biochemical assays such as catalase, oxidase, amylase urease, glucose, sucrose, lactose, maltose arabinose, indole, methyl red, Voge -proskauer and citrate test was performed. Antibiotic sensitivity test were done by using Kirby- Bauer disc diffusion method and 2% sodium chloride was added to the Muller Hinton agar [17].

Molecular Analysis

The genomic DNA was isolated from the bacterial culture and subjected to the PCR amplification and the 16SrRNA gene amplification was carried out using the primer sets 27F (5'-AGAGTTTGATCFCCTGGCTCAG-3' [18] and 1492R (5'-GGTTACCTTGTTACGACTT-3' [19]

PCR conditions:

95°C	94°C	55°C	72°C	72°C
5 min	1 min	1 min	1 min	1 min
1 cycle	30 cycles	30 cycles	30 cycles	1 cycle

The PCR amplification end product was electrophoresed on a 1% agarose gel with molecular markers. The targeted gene was sequenced using Applied Biosystems 3730 x 1 DNA Analyzer and the sequence data analysis was performed by using chromopro and sequencing analysis software. The BLAST tool was used to identify the homogeneity of the sequence [20].

Heavy metal Toxicity testing

The toxicity testing for heavy metals was performed using agar dilution method [21]and 2% of sodium chloride was added to the Muller Hinton agar [22] with various concentrations of metals like Zinc sulphate, Copper chloride and Lead nitrate. 20 ml of media was poured on to the plates and the isolated strain was inoculated and incubated at 37^0 C. (Tab. 4)

The metal toxicity test consists of three independent samples and it performed at three different concentrations for three times to obtain the triplet value and was recorded. Results were shown as mean ± S.D values.

Results

The isolation of bioluminescent bacteria was carried out in three different samples like sea water sample, soil sample and fish samples and finally the bioluminescent bacteria were isolated from the smashed squid sample with Luminescent broth and after the incubation period, the light emission was observed. (Fig. 1).

Fig. 1-Bioluminescent bacteria in dark

1 ml of sample was taken from smashed squid sample and after serial dilution it was transferred to the luminescent agar plate for performing spread plate technique and kept at room temperature for incubation period and it produces large, Mucoid, Salmon color colonies. (Fig. 2 and Fig. 3).

Fig. 3- Bacterial colonies in luminescent agar plate

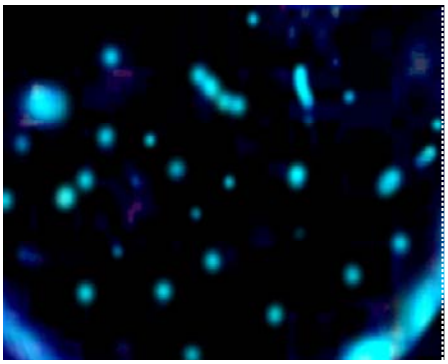


Fig.4- Bacterial colonies in darkness

The isolated colonies were picked from the luminescent agar plate and it was inoculated in the luminescent broth and kept at room temperature, and after incubation period, it emits light in the luminescent broth. (Fig. 4)

Fig. 5-Bioluminescent bacteria in luminescent broth

The colonies were taken from the luminescent agar plate and the quadrant streaking method was done in TCBS plate and after 24 hours of incubation, the yellow color colonies were observed.

Morphological Characterization

PRELIMINARY TEST	RESULT
Gram staining	Gram negative rods
Hanging drop method	Motile

Tab.1 – Preliminary Test

Biochemical characterization

BIOCHEMICAL TEST	RESULT
Catalase	Positive
Oxidase	Positive
Amylase	Negative
Urease	Negative
Glucose	Negative
Sucrose	Negative
Lactose	Negative
Maltose	Negative
Arabinose	Negative
Indole	Negative

Methyl Red	Negative
Voges Proskauer	Negative
Citrate	Negative

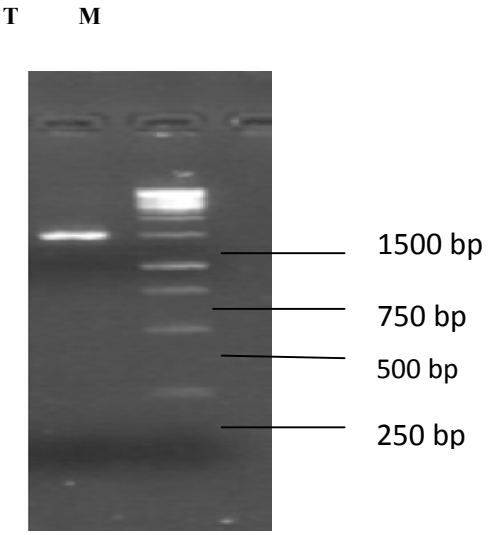
Tab. 2 – Biochemical Test

Disk Diffusion Method

ANTIBIOTICS	RESULT
Penicillin	Resistant
Ampicillin	Resistant
Tetracyclin	Susceptible
Amikacin	Susceptible

Tab. 3- Antibiotic Test

Molecular Characterization



T - Test sample

M- Molecular marker

Fig. 6 - PCR analysis of 16S rRNA gene DNA Band

The sequence was identified to be:

>OciSeq_Bl1_16SF_095.ab1
TTGGGAAAAGGATTGACCGAAGGCGGGCGCGACGGGTGAGTAATGGCCTGGGAATTTGCCCATTTGTGGGGGAT
AACAGT
TGGAAACGACTGCTAATACCGCATACGCCCTACGGGGGAAAGCAGGGGACCTTCGGGCCTTGCGCTGATGGATA
AGCCCA
GGTGGGATTAGCTAGTAGGTGAGGTAAAGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAG
CCACAC
TGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGA
TGCAGCC
ATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGGTGTAAGTTAATACC
TTGCAT
CTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCG
TTAATC
GGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTGTTAAGCGAGATGTGAAAGCCCCGGGCTCAACCTGGGAA
CCGCAT
TTCGAACTGGCAAACTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTG
GAGGAA
TACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGCACGAAAGCGTGGGGAGCAAACAGGATT
AGATACC
CTGGTAGTCCACGCCGTAAACGATGTCTACTCGGAGTTTGGTGTCTTGAACACTGGGCTCTCAAGCTAACGCATT
AAGTA
GACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCA
TGTGGTT
TAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCACAGAATCTTGGCAGAGATGCATCGGTGCCTT
CGGGA
ACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCA
ACCCCT
ATCCTACTTGCCAGCGGGTCATGCCGGGACTTAGGAGACTGCGTGATAAACCGAGAGGTGGGGACGACGTCAGT
CATCAT
GGCCTTACGAAGTAGGCTACACACACGTGCTAC

>OciSeq_Bl1_16SR_093.ab1
AAGAAATAGGAACCAGCCAAAAAAATAACGCCCTCACCAAGAACAATCCAACTACTTGCTGGTGCAGACCACTC
CCATGG
CGACGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTGGCATTCTGATCCACGATTACTAGCGATTC
CGACTT
CATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGACCAGCTTTATGGGATTAGCTCCACCTCGCGGCTTCGCA
ACCCT
CTGTACTGACCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCGTCCCCACCTTCC
TCCGG
TTTATCACCGGCAGTCTCCCTAAAGTTCCCGGCATGACCCGCTGGCAAGTAAGGATAGGGGTTGCGCTCGTTGCG
GGACT
TAACCCAACATTTCACAACACGAGCTGACGACAGCCATGCAGCACCTGTCTCACAGTTCCCGAAGGCACCGATG
CATCTC
TGGAAAATTCTGTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCT
TGTGC
GGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCTACTTAATGCGTTAGCTTGA
GAGC
CCAGTGTTCAAGACACCAAACTCCGAGTAGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGC
TCCCC
ACGCTTTCGTGCCTGAGCGTCAGTCTTTGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGC
ATTTC
ACCGCTACACCTGGAATTCTACCCCCCTCTACAAGACTCTAGTTTGCCAGTTCGAAATGCGGTTCCCAGGTTGAG
CCCGG
GGCTTTCACATCTCGCTTAACAAACCGCCTGCGCACGCTTTACGCCCAGTAATTCCGATTAACGCTCGCACCCTC
CGTAT
TACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGAGTAACGTCACAGATGCAAGGTATTAACTTAC
ACCCT
TTCCTCCTCGCTGAAAGTGCTTACACCCGAAAGGCCTTCTTCACACACGCGGCATGCTGCATCAGGTTTCCCCAT
TGTGC
ATATCCCCACTGCTGCCTCCCGTAGAGTCTGGGCGTGTCTCAGTCCATGGTGGCTGATCATCCTCCTCAGACCAG
CTAGG
GAATCGTCGCCCTAAGGGTGAGCC

In silico analysis of 16S rRNA sequencing of the isolates was performed using BLAST, which suggest BL-1 strain is 98% similar to *Shewanella algae.*

Name of the salts	Salt concentration (µg/ml)	Mean ± SD
Zinc Sulphate	100	46.33 ± 1.15
	500	47.33 ± 0.57
	1000	45.66 ± 0.57
Copper Chloride	100	47.33 ± 0.57
	500	44.00 ± 1.00
	1000	43.00 ± 1.73
Lead Nitrate	100	46.00 ± 1.00
	500	43.00 ± 1.73
	1000	37.66 ± 0.57

Tab. 4 - Heavy metal Toxicity Test

Discussion

Bioluminescence is the natural process of emission of light by the living organisms. Many of the prokaryotic organisms, especially bacteria were identified to be potential emmitants of bioluminescence. Bioluminescent bacteria were abundant in marine environment [23] and the marine luminous bacteria have been isolated from sea water [24] and extensively distributed in the coastal region. The bioluminescent bacteria phylogenetically belongs to the the Gamma Proteobacteria class with 14 species of marine organism in the family vibrionaceae ans shewanellaceae [25]. The bioluminescence exhibited by the bacteria in squid was studied. The Bioluminescent bacteria were isolated from squid sample and subjected to both microbiological and molecular characterization, which revealed that the bioluminescent bacteria (BL-1 strain) it to be 98% similar to *Shewanella algae*. It shows resistance to the heavy metals like Zinc sulphate, Copper chloride and Lead nitrate at various concentrations so it's concluded from this study that the isolated BL-1 strain shows resistance against the 3 heavy metals.

References

1. Harvey N E, Studies on bioluminescence: V. The chemistry of light production by the firefly, *American Journal of Physiology*. 42 (1916) 342-348.

2. William D. McElroy & Robert Ballentine, The Mechanisms of Bioluminescence. Proceedings of the National Academy of Sciencess. 40(1944): 377-382.

3. Lange C R and Lange S R. Biomonitoring of water. *Environmental Research*, 69 (1997) 900-915.

4. Baumann P, Baumann L, Woolkalis & Bang S, Evolutionary relationship in vibrio and photobacterium. A basis for a natural classification. *Annual Review of Microbiology*, 37 (1983) 369-398.

5. Campbell A K. Living light: biochemistry, function and biomedical applications. *Essays in Biochemistry,* 24 (1989) 41-76.

6. Nealson K H & Hasting J W, Bacterial bioluminescence; its control and ecological significance. *Microbiological Reviews*, 43(1979) 496-518.

7. Meighen E A, Genetics of bacterial bioluminescence. *Annual Review of Genetics*, 28 (1994) 117-139

8. Ruby E G & Nealson K H, Seasonal changes in the species composition of the luminous bacteria in near shore seawater. *Limnology and Oceanography*, 23(1978) 530-533.

9. Hori K & Cormier M J, Studies on the bioluminescence of Renilla reniformis. V. Absorption and fluorescence characteristics of chromatographically pure luciferin. *Biochemica et Biophysica Acta.*102 (1965) 386-396.

10. Blum L J, Bio, chemi-luminescent sensors. World Scientific publishing, Singapore (1997).

11. Cormier M J & Totter J R, Quantum efficiency determination on components of the bacterial luminescence system. *Biochemica et Biophysica Acta*, 25(1957) 229-237.

12. Hasting J W & Gibson QH, Intermediates in the bioluminescent oxidation of reduced flavin mononucleotide. *The Journal of Biological Chemistry*. 238 (1963) 229-237.

13. Meighen E A, Molecular biology of bacterial bioluminescence. *Microbiology and Molecular Biology Reviews,* 55 (1991) 123-142.

14. Nicholls K M, Lee J V & Donovan, An evaluation of commercial thiosulphate citrate bile salt sucrose agar. *Journal of Applied Microbiology*, 41(1976) 164-169.

15. Hendrie M S, Hodgkiss and Shewan J M, The identification, taxanomy and classification of luminous bacteria, *Journal of General Microbiology*, 64 (1970) 151-169.

16. Reichelt J L and Baumann L, Taxanomy of the marine luminous bacteria. *Archives of Microbiology*, 94 (1973) 283-330.

17. Julia P Galkiewicz, Zoe A Pratte, Michael A. Gray and Christina A. Kellogg, Characterization of culturable bacteria isolated from the cold water coral Lophelia pertusa. *FEMS Microbiology Ecology*, 17(2011) 333-346

18. Weisberg W G, Barns S M, Pelletier D A, Lane D J, 16 S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* 173 (1991) 697-703.

19. Reysenbach A L, Giver L J, Wickham G S, Pace N R, Differential amplification of r DNA genes by polymerase chain reaction. *Applied Environmental Microbiology,* 58 (1992) 3417-3418.

20. Altschul S F, Grish W, Miller W, Myers E W & Lipman D J, "Basic local alignment search tool. *Journal of Molecular Biology,* 215 (1990) 403-410.

21. Washington J A and Sutter V L, Dilution susceptibility test: agar and macro broth dilution procedures. In Lennette E H Balows A, hausler W J and Truant J P (ed.). Manual of clinical microbiology, rd ed. American Society for Microbiology, D C Washington (1980) 453-458.

22. Julia P Galkiewicz, Zoe A Pratte, Michael A. Gray and Christina A. Kellogg, Characterization of culturable bacteria isolated from the cold water coral Lophelia pertusa. *FEMS Microbiology Ecology*, 17(2011) 333-346

23. Nealson K H & Hasting J W. The luminous bacteria. In: Balows A, Truper H G, Dworkin M, Harder W, Schleifer K H (Eds.), . The prokaryotes. 2nd edition. Springer-Verlag, New York, (1992) 625-639.

24. Reichelt J L and Baumann L, Taxanomy of the marine luminous bacteria. *Archives of Microbiology*, 94 (1973) 283-330.

25. Kita-Tsukamoto K, Yao K, Kamiya A, Yoshizawa S, Uchiyama N, Kogure K, Wada M.. Rapid identification of marine bioluminescent bacteria by amplified 16S ribosomal RNA gene restriction analysis. *FEMS Microbiology Letters*. 258 (2006) 320.

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