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Research Article

Culture Parameters Affect the Light Emitting Property of Organisms Isolated from Two Marine Fishes

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Abstract

Organisms were isolated and cultured from two marine fishes (*Stolephorus indicus* and *Nemipterus japonicus*) that were exhibiting Bioluminescence. Identification and assessment of impact of five different culture media on the growth and light emission properties were studied. Isolates from both the fishes showed microbes to be gram negative Coccobacilli, Partial gene sequencing analysis suggested that the microbes belonged to *Vibrio* spp and *Photobacterium* spp. Biochemical analysis of both the bacterial isolates exhibited difference in sugar (Mannitol and Lactose), LDC, Indole and Urea content; suggesting that the two isolated colonies are different bioluminescent bacteria. Repeated subculture (to obtain pure colonies) of both the isolates resulted in gradual reduction in the intensity of luminescence of the bacteria and eventually loss of luminescence property occurred. However, luminescence was revived when they were grown in aerated condition.

Keywords: Bioluminescence; Anchovy Fish; Threadfin Bream Fish; Growth Curve; Lux Operon; Luciferase; Boss Medium

Introduction

Bioluminescence has attracted interest of researchers due to its immense applications in the near future. Bioluminescent organisms are being exploited for their utility as biosensors, biolighting, designing purpose, street lighting, detection of toxic compounds in environment and many more. Bioluminescence is the emission of light produced by microorganisms due to luciferase activity. These microorganisms are found as free living or as gut symbionts in marine environment. They are widely spread in terrestrial environment, fresh water and marine ecosystems.

Bioluminescence is a phenomenon that usually results from biochemical and enzymatic activity of living microorganisms. The reaction involves oxidation of long chain aliphatic aldehyde and reduced flavin mononucleotide (FMNH2) in the presence of enzyme luciferase. It basically involves a group of genes i.e. the lux operon. The lux operon constitutes the genes luxICDABEG. LuxA gene codes for α and LuxB gene codes for β subunits. The lux CDE genes are fatty acid reductase genes that codes for polypeptides. It is important and necessary for the conversion of fatty acids into the long chain aldehyde (fatty acid pathway) [1-3]. In the process of bioluminescence, the excess energy is liberated and emitted as a luminescent blue-green light at around 490 nm.

Many attempts have been made by researchers to apply bioluminescent reactions in practice including environmental protection, agriculture, food technology, biosensors and in genetic engineering. It was observed that bioluminescence is a property of certain bacteria found in abundance in marine ecosystems and that the presence of chemical pollutants and toxins disturbs cell metabolism of bioluminescent bacteria, reducing the intensity of the emitted light [Kießling and Rayner-Brandes 1998, BioTox™... 1996].

Luminescent bacteria are facultative aerobes. The capacity for bacteria to luminescence is closely related to the composition of the culture medium. In order for the bacteria to generate light the presence and access to oxygen are required. The end product of bacterial luminescence, which is frequently compared to respiration, is not adenosine triphosphate (ATP), but the excited chemical compound emitting light called luciferase. The bacterial luminescence is visible only after it reaches a certain cell density [2,3].

Many aquatic bioluminescent species such as fishes, crabs, jelly fishes etc. found in the marine environment [3,4]. The luminescence is due to the presence of symbiotic bacteria, which thrive on hosts.

From the scientific point of view advances in the isolation of bioluminescent bacteria as well as the determination of different media for optimal culture conditions and growth are of great importance. This research work mainly focuses on the study of isolated bioluminescent organisms from different sources and assessment of their light emission under different cultural conditions and parameters.

Materials and Methods

Source of procuring fish

Two marine species of fishes i.e. Threadfin bream (local name Raja Rani fish) and Anchovy fish (local name-Mandeli fish) were procured from the local market.

	Anchovy Fish	Threadfin bream Fish
Kingdom	Animalia	Animalia
Phylum	Chordata	Chordata
Class	Actinopterygii	Actinopterygii
Order	Clupeiformes	Perciformes
Family	Engraulidae	Nemipteridae
Genus	*Stolephorus*	*Nemipterus*
Species	*indicus*	*Japonicas*
Habitat	Widely distributed throughout the worlds ocean, mainly found in temperate waters, but rarely observed in cold or warm seas also.	Mostly found in tropical marine waters of Indian and western pacific oceans

Table 1: Classification of Anchovy and Threadfin bream Marine Fish.

Figure 1: (Left) Anchovy fish (Stolephorus indicus) local name is Mandeli and (Right) Threadfin bream fish (Nemipterus japonicus) locally known as Rani fish; collected from local market.

Isolation of bioluminescent bacteria

Fishes collected from local market were used for isolation of bioluminescent bacteria using five different media. The bacterial suspensions were prepared by scraping the surface of the fishes-Anchovy fish and threadfin bream fish using normal saline. This suspension was primarily used for isolation using the T- streak method and side streak method. The plates were incubated at room temperature 25°C ± 2°C for 24 hours. After 24 hours the plates were observed in dark room for isolated colonies that showed bioluminescence [5,6]. The bioluminescent colonies were then picked up and further subcultured several times on agar plate in order to obtain only bioluminescent colonies without any contamination.

Culture media used

Five different culture media were tried to isolate and culture Bioluminescent organisms from both the fishes (Table 2); they were - BOSS medium, LA medium, Photobacterium medium, LM medium and NCBE medium [3,10].

Observations

Morphological characterization, growth curve study, biochemical analysis of isolated bacteria and light emission by isolated microbes were recorded.

The microscopic characterization of the isolated bioluminescent bacteria was done to record its morphology i.e. shape (cocci, bacilli, rod etc.), size, consistency, colour and elevation. Gram staining was carried out to distinguish gram positive and gram negative bacteria.

Growth curve study of the isolates was carried out using 24 hours old culture. For growth curve study Boss medium was used. 25 ml of the medium was sterilized in a side arm flask and used for further studies. The optical density (OD) values were checked at regular intervals to observe the growth pattern of the isolates.

Moreover, standard biochemical content was also analyzed. The bioluminescent bacteria were successfully isolated from Anchovy and threadfin bream fish thriving in the marine waters of Mumbai. The isolates were labelled as isolate 1 and isolate 2 respectively. The biochemical analyses were done to differentiate between the two isolates.

Luminescence of microbial cultures growing on slants and petri plates were recorded.

Results

The growth curve of bacterial isolate from Anchovy fish and from Threadfin bream fish is presented in figure 2. The black line represents the normal growth curve of luminescence bacteria. The log phase of growth was recorded to be for 3 hours after subculture, whereas the green line represents the luminescence of the bacteria which takes place in the log phase. Both isolates exhibited similar growth curve pattern.

Boss medium	LA medium	Photobacterium medium	LM medium	NCBE medium
NaCl- 30g	NaCl- 10g	NaCl- 30.0g	$CaCO_3$- 1g	Yeast extract- 3g
Glycerol- 1g	Yeast extract- 5g	NH_4Cl- 0.30g	Glycerol- 3g	Bacto-peptone- 5g
Bacto-peptone- 10g	Bacto-peptone- 10g	$MgSO_4$- 0.30g	Yeast extract- 3g	Distilled water - 250 ml
Meat extract -3g	Agar-15g	$FeCl_3$- 0.01g	Tryptone- 3g	Sea water - 750 ml
Made up to 1000 ml with distilled water	Made up to 1000 ml with distilled water	$CaCO_3$- 1.00g	Made up to 1000 ml with sea water	
		KH_2PO_4- 3.00g		
		Na glycerophosphate- 23.50g		
		Casein hydrolysate - 5g		
		Yeast extract- 2.5g		
		Made up to 1000 ml with distilled water		

Table 2: Media composition.

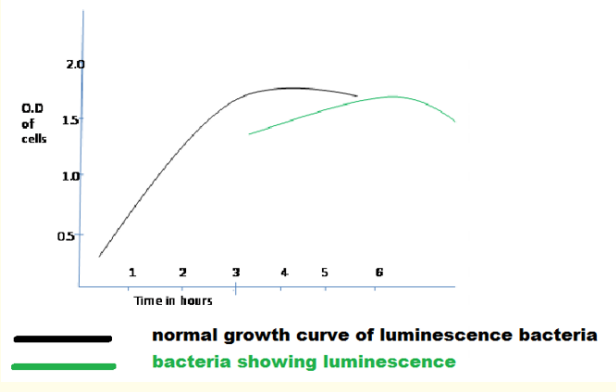

Figure 2: Growth curve pattern exhibited by both isolate 1 and isolate 2.

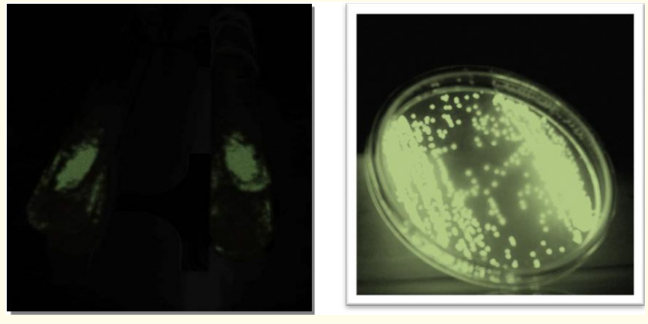

Figure 3: Light Emission by Isolated Microbes cultures on slant as well as petridish.

Biochemicals	Isolate 1	Isolate 2
Xylose	-ve	-ve
Mannitol*	-ve	+ve
Glucose	+ve	+ve
Lactose*	-ve	+ve
Maltose	+ve	+ve
Sucrose	+ve	+ve
LDC*	-ve	+ve
ODC	-ve	-ve
TSI	-ve	-ve
Indole*	-ve	+ve
MR	+ve	+ve
VP	+ve	+ve
Urea*	-ve	+ve

Table 3: Biochemical analysis of isolates 1and 2 taken from 2 fishes.

*: Biochemical analysis of organisms provides us the information regarding their differing metabolism thus differentiating their types. It mainly comprises of sugar utilization test, protein utilization and their detection based upon the end products.

Both the isolates were found to be gram negative Coccobacilli. The two isolates 1 and 2 were found to differ in the sugar utilization (Table 3) which proves that the two isolated colonies are different bioluminescent bacteria.

The bioluminescent organisms in study showed differences in their carbohydrate metabolism (sugars i.e. mannitol, lactose (Table 3) and the utilization of amino acid tryptophan which was detected by the production of indole using Kovac's reagent.

The isolates also differ in LDC i.e. lysine decarboxylase test - decarboxylases are a group of substrate specific enzymes that are capable of reacting with carboxyl portion of amino acids, forming alkaline reacting amines and by product carbon dioxide, increased pH of medium is detected by color change. Each carboxylase enzyme is specific for an amino acid. The isolated organisms vary in the conversion of lysine to cadaverine (Table 3).

Urea is the product of decarboxylation of amino acids. Hydrolysis of urea produces ammonia and CO2 thus increasing the alkalinity which is detected by color change. The isolates differ in urea test.

The luminescence intensity of bacteria was recorded using BIOTEK imaging reader. The best luminescence recorded was 12940576 which was around 10000 times more as compared to negative or non-luminescent culture. It was found that isolates obtained on BOSS media showed better luminescence as compared to other media (LM, PB, LA and NCBE media). Hence, BOSS media was used for further study. The isolated bacteria were named as isolate 1 and isolate 2 obtained from Anchovy fish and threadfin bream fish respectively. These isolates were repeatedly subcultured to obtain pure colonies. However, after several subcultures the luminescence of the bacteria was found to be reducing; as several subcultures result in the loss of luminescence property.

Several attempts were made to revive the cultures and its bioluminescence property using different medium and conditions. The attempt of reviving the bioluminescence of the culture came to fruition using Photobacterium media. The Photobacterium media consisted of various salts like Sodium chloride, ammonium chloride, magnesium sulphates, ferric chloride and calcium carbonate which enriches the medium and was thought to be aiding the bacteria to luminescent.

To revive the luminescence of the bacterial culture which had lost the luminescence, their growth was carried out in aerated condition. The intensity of luminescence was found to be related to the aeration of the culture. The greater the aeration the greater was the luminescence.

The biochemical studies of the isolates were done for the identification of the bacteria and isolates resembled the characteristics of *Vibrio* spp and *Photobacterium* spp. Further molecular studies done by Geneombio laboratories at Baner, Pune confirmed it. The results obtained for partial gene sequencing of both the isolates are described below.

FASTA FORMAT SEQ

Partial gene sequencing results for isolate obtained from threadfin bream fish (Rani fish)

ACGAGTTATCTGAACCTTCGGGGAACGATAACGGCGTCGAGCGGCGGACGGGTGAGTAATGC CTAGGAAATTGCCCTGATGTGGGGGATAACCATTGGAAACGATGGCTAATACCGCATAATGCCTACGGGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAG CTAGTTGGTGAGGTAAGGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCA GCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTCGTGAGGAAGGTAGTGTAGTTAATAGCTGCATTATTTGACGTTAGCGACAGA AGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCG GAATTACTGGGCGTAAAGCGCATGCAGGTGGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAATTGCATTTGAAACTGGCAGACTAGAGTACTGTAGAGGGGGG

Partial gene sequencing results for isolate obtained from Anchovy (Mandeli fish)

GGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGT GTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTCGTGAGGAAGGTAGTGTAGTTAATAGCTGCATTACTTGACGTTAGCGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGT AATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCATGCAGGTGGTTTGT TAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAATAGCATTTGAAACTGGCAGACTAGAGTACTGTAGAGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAGATACTGACACTCAGATGCGAAAGCGTGGGGAGC AAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTTGGAGGTTGTGGCC TTGAGCCGTGGCTTTCGGAGCTAACGCGTTAAGTAGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTTCCAGAGATGGATTGGTGC CTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTG

The study of bioluminescence from different biosystems provides us with a lot of information on the mechanism of bioluminescence. The property of bioluminescence is exploited in biotechnology, environmental microbiology and various fields to apply it as biosensors, microtox assays etc. on the basis of their light intensities [5,15]. Furthermore, the lux gene can be exploited for various genetic studies and as biosensors.

Discussion

The growth curve pattern shown by both the isolates indicates that as the culture reaches certain density it starts exhibiting luminescence especially in the logarithmic phase and continues to exhibit until the media starts depleting (quorum sensing). After few hours the culture media becomes more turbid due to excessive growth and starts reaching the death phase and impacts the luminescence of bacteria; however if the medium is replenished the luminescence can be maintained. The biochemical characterisation of the isolated shows difference in sugar utilization especially mannitol and lactose. Isolate 1 could not utilize sugars mannitol and

lactose whereas isolate 2 metabolizes mannitol and lactose. The protein metabolism also differs in both the isolates (Table 3). Isolate 1 was negative for LDC- which means cannot metabolize the amino acid Lysine, INDOLE- indicative of not metabolizing tryptophan into indole and UREA- indicative of not hydrolyzing urea whereas isolate 2 was positive for utilization of lysine decarboxylase, INDOLE and UREA. The difference shown in the biochemical characteristics is indicative that the two isolates are not same.

Furthermore, the molecular characterization results obtained by partial gene sequencing is also indicative of bacterial isolates being different. The study thus reveals the impact of various cultural parameters on the growth of bioluminescent bacteria. However further quantification and detailed analysis is in the process and will be represented in the next research paper [16-31].

Conclusion

The bioluminescence study, their cultural characteristics growth pattern provides information regarding the effect of repeated subcultures on bioluminescence, revival of luminescence depending on the aeration conditions or oxygen level and various salts that affect the luminescence intensity of bacteria.

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