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## Screening and tentative identification of bioluminescence producing bacteria in fish

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### Abstract

The objective of this paper is to increase the availability of the sources for the detection methods because of its easy handling. The concept can prove useful for developing future torch lights and marker gene in the field of genetic engineering. For this the sample was collected from the Vellore fish market. The light producing organism was isolated from the fish fins, gut, head and tail. We were able to find the desired organism predominantly from gut and little from fins and area around the head. The organism was physically and biochemically characterized. Results showed the presence of the rod shaped gram positive bacteria with the feature of catalase positive and Indole negative. Spinach and marigold act as the most apposite substrate for the luciferin determination by using luciferase as the enzyme. The organism was sequenced giving the result of the presence of the *Kurthia* sp. which grows well at temperature 22-35 °C and pH 6 - 7.5.

**Keywords:** Bioluminescence, *Kurthia* sp, luciferase, Gram-positive.

### 1. Introduction

Bioluminescence is a phenomenon in which living organisms emit light. This light emission occurs as a result of a chemical reaction. This phenomenon of bioluminescence is shown by a wide range of organisms including bacteria, fungi, fish, insects, algae, and squid [1, 2, 3]. There are terms like chemiluminescence and phosphorescence which may create mystification among the three terms. Bioluminescence is a type of chemiluminescence where the energy is released in the form of light because of the atoms coming to their ground state. There are two components essential for the reaction, the substrate luciferin and the enzyme luciferase [3, 4]. Luciferin recycles as the specific activity remains the same for years. Not only luciferin, there are other compounds which keep on recycling to maintain the light emission phenomenon [5].

Luciferase is a heterodimer consisting of two polypeptide chain alpha and beta of different molecular weight encoded by two different genes luxA and luxB [6]. The bacteria are the most predominant bioluminescence producing organism and these can either be free living or in association with another organism [7]. In this paper, we are mainly focusing on bioluminescence shown by marine fish. The marine fishes usually show bioluminescence as a result of bacterial symbiosis. These bacteria usually live as symbiont in the alimentary canal of fishes and many times some other parts as well [8]. These luminescent bacteria mainly are of five types *Photobacterium (leiognathi, phosphoreum)*, *Vibrio (harveyi, fischeri)* and *Beneckea spp.* These bacteria typically are curved rods 1 to 3 microns long and motile by flagella. These bacteria are aerobic as well as facultative anaerobes [4].

The reaction occurring in the bacteria is below:



Where FMNH<sub>2</sub> is reduced mononucleotide, RCHO is long chain fatty aldehyde, FMN is oxidized mononucleotide and RCOOH is long chain of acid.

The above reaction is catalyzed by luciferase, which oxidizes Flavin mononucleotide (luciferin) and the fatty aldehyde and blue/green light is emitted in the visible range [9]. Light

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is only produced when the organisms are present at high cell densities. Quorum sensing helps the organism in bioluminescence by the release of the signal molecules which consequently maintains the light emission [10]. The equation above shows that the oxygen is a critical parameter for the reaction, but besides the oxygen there are few other components like salt concentration, carbon source etc which play a role in the process [11]. The Majority of the luminous organisms are present in the aphotic region (>800 m) of the sea [12]. Since there is less light available for the organism to serve their activities, bioluminescence proves useful in the deeper region of the sea. These Fishes and other marine organisms provide bacteria with the environment to support their growth and metabolism, and in turn use their light emitting property to camouflage, hunt their prey, or to attract their mates, etc. [13, 14, 15]. This study thus apart from providing a view to understand the interaction between microbes and other organism, it also provides us with strategies that can be utilized to treat various diseases, preventing antibiotic resistant bacterial infections, methods for biological monitoring the environment, toxicity testing etc. [2]. The bioluminescent system is highly sensitive to very minute amount of the pollutants which can cause serious diseases. These are best suited because of unfussiness, accurateness and sensitivity. These can also be used for developing some devices which can detect cancer and tumors [16, 17, 18]. In our study, we had collected fish from the Vellore fish market and the fish is called as Ornate Threadfin bream fish or called Rani Fish in the Hindi language exhibits bioluminescence.



**Fig 1:** Ornate threadfin bream fish

### Scientific classification

Kingdom	Phylum	Class	Order	Family
Animalia	Chordata	Actinopterygii	Perciformes	Nemipteridae

## 2. Materials and methods

### 2.1 Sample collection and processing:

The marine water fish sample was collected from the Vellore fish market, Tamil Nadu. These fishes are imported from the various places, including Andhra Pradesh, Kerala, Chennai and Nagapattinam. The fresh sample collected was dissected aseptically and various parts of the fish fin, gut, head and tail were kept in their respective containers containing the sea water, which was brought from Marina beach, Chennai. Now these were kept overnight at 4 °C.

Artificial sea water media (ASW) was used for the growth of the bioluminescent bacterial colonies.

### Composition for 200 ml medium

Sea water NaCl (5.7 g), KCl (0.154 g), CaCl<sub>2</sub>.2H<sub>2</sub>O (0.32 g), MgCl<sub>2</sub>.2H<sub>2</sub>O (0.96 g), NaHCO<sub>3</sub> (.022 g), MgSO<sub>4</sub>.7H<sub>2</sub>O (0.7 g) and Distilled water (200 ml). 150 ml of sea water is taken and to this following chemicals are added: Peptone (1 g), yeast extract (0.6 g), glycerol (0.6 ml), agar (3 g) and distilled water (50 ml) [19]. A Pour Plate technique is used for the culture of the colonies [20]. After almost 12- 14 hours colonies were visible. The colonies obtained were further streaked in order to get pure colonies for the physical and biochemical characterization.

### 2.2 Physical characterization

Gram staining was performed with the pure bioluminescent colonies. This staining technique is based on the differences in the bacterial cell walls distinguishes the gram positive and gram negative bacteria. Gram positive bacteria keep the stain whereas the gram negative species do not hold on the crystal violet dye and hence appear pink in color due to the counter stain (safranin).

### 2.3 Chemical characterization

#### 1.) Citrate utilization test

100 ml of Simon citrate agar medium was prepared and autoclaved, approximately 7ml of the medium was added in 4 ml test tube and the slant was prepared. The test tubes were inoculated and stabbed with respective colonies of fin, gut and head excluding control. These test tubes were kept at room temp (25-30 °C). After 48 hours the color was changed from dark green to blue color giving positive test.

#### 2) Methyl red and Voges Proskauer

100 ml of MR-VP medium was prepared and autoclaved. 7 ml of the medium was added in 8 different test tubes (4 for each). The respective colonies were inoculated in 6 test tubes excluding blanks. The test tubes were kept at room temperature .After 48 hours about 10 drops of methyl red and Barritt's reagent A+ Barritt's reagent B was added in MR and VP test tubes respectively and left for the color change for 10 minutes.

#### 3) Indole test

100 ml of trytone broth was prepared and autoclaved. 7 ml of the medium was added in 4 test tubes. The bacterial colonies were inoculated in the test tubes exclusive of blank. These test tubes were kept at room temperature for 48 hours. After 48 hours 10 drops of kovac's reagent was added and kept for the color change.

#### 4) Triple sugar Iron agar

100 ml of triple sugar Iron agar medium was prepared and autoclaved. 5 ml of medium was added in 4 test tubes and slants were prepared. Test tubes were inoculated with respective colonies, leaving blank and after that kept at room temperature for 48 hours for the color change to occur.

#### 5) Catalase test

The pure colonies were smeared on the clean and sterilized slides in separately. 2 drops of hydrogen peroxide were added on the smear. Presence or absence of oxygen gives

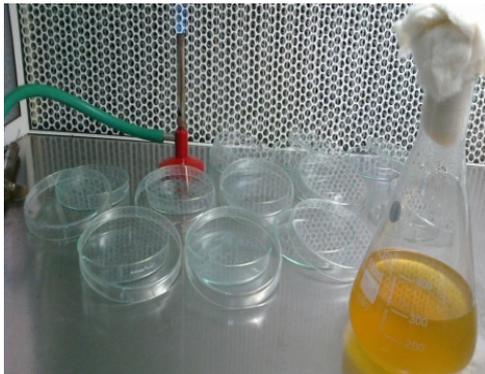
the result

### 6) Oxidase Test

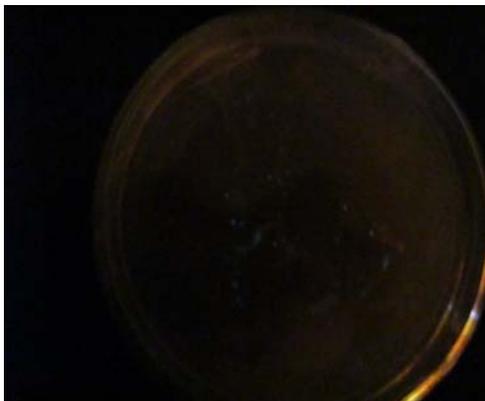
The oxidase discs are used for this test. The Single pure colony is smeared on each oxidase discs for fin, gut and head and kept for 10 minutes for the color change.

### 7) Mannitol salt agar test

100 ml of mannitol motility agar was prepared and sterilized. The medium was poured in 4 test tubes approximately 7 ml. It was allowed to solidify for 20-25 min. Once it gets solidify bacterial colony was inoculated in the test tubes except control and stabbed. The test tubes were kept for 48 hours to observe the color change.



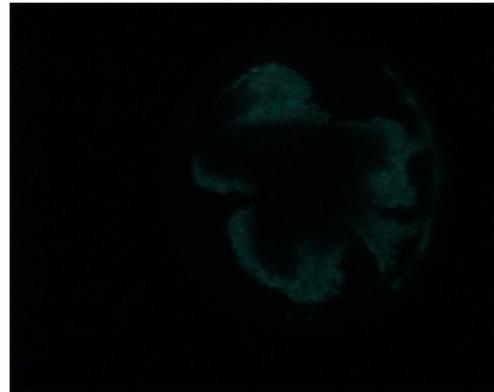
**Fig 2:** Artificial sea water media



**Fig 3:** Bioluminescent bacteria from fins



**Fig 4:** bioluminescent bacteria from gut



**Fig 5:** Bioluminescent bacteria from head

### 2.4 Temperature and pH optimization

Parameters like temperature and pH were optimized in order to find out the preferred conditions for the increased production. Optimum conditions are necessary for the activity of the luciferase enzyme which reacts with the substrate luciferin resulting in the product formation. Normally for the media the preferred pH is 7.5 and we tried to find out whether at some other pH the activity exists. Also, in the case of the temperature, at room temperature the bioluminescence can be seen but we are finding if at some other temperature the enzyme luciferase is activated. Firstly the bioluminescent media is made for the fin, gut and head of 100 ml each in 250 ml of round bottom flask. The media is inoculated with the colonies of the bacteria from the petri plates. This media is kept for the 12 hours incubation and then kept at 4 °C. After 12 hours again the 100 ml of the media was prepared and was inoculated with the previously incubated media. Remember to inoculate with the 1% of the media. Again incubate the flasks at different temperatures 4 °C, 35 °C and 50 °C. After 12 hours take OD at 610 nm. The flask with the highest OD value is the most preferred temperature of the luciferase enzyme. The same process is repeated for the pH except that the OD values for the pH are taken at 5,6,7,7.5 and 9.

### 2.5 Protein Estimation

#### 2.5.1 Preparation of substrate

Six substrates were taken marigold, snapdragon, rose, chrysanthemum, spinach and cabbage. The flowers were sun dried. After that 15 g of each flower was dipped in 100 ml of water for 1 hour before the estimation of the protein content. The contents were filtered. The filtrate was then used further for the Lowry method for protein estimation.

#### 2.5.2 Production media preparation

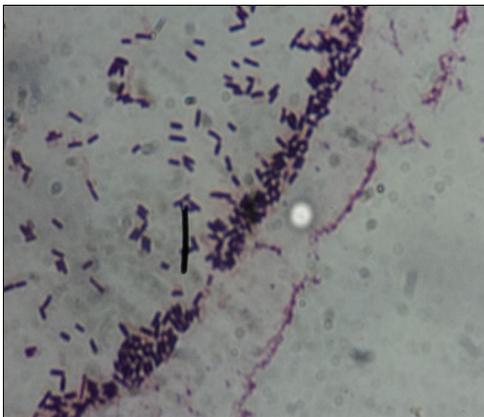
The bacterial colonies were used for the preparation of the production media for fin gut and head, which was kept in orbital shaker for the growth of the organism for 24 hours. After 24 hours for every substrate the production media are added from fins, gut and head in the test tubes containing the substrate and kept at three different temperatures 37 °C, 4 °C and -20 °C for 30 minutes. Trichloroacetic acid (TCA) is added in order to stop the reaction. For every substrate same procedure is repeated. 5 ml of Lowry Reagent A which is prepared freshly is added to the test tubes. Incubate for 10 minutes and then add 1 ml of folin ciocalteu reagent. Again incubate for 30 minutes. Now take OD at 660 nm.



**Fig 6:** Enzyme activity

### 3. Results and Discussion

Gram staining from the cultures of the fin, gut and head showed the presence of the purple rod shaped bacterial species confirming the existence of the gram positive bacillus species.



**Fig 7:** Organism from fish.

**Citrate utilization test:** The bacteria gave a positive test for the citrate utilization turning to blue color from dark green color. The color changes due to accumulation of the alkaline products. This indicates that the organism uses sodium citrate as the only carbon source and inorganic ammonium salts as the fixed nitrogen source.



**Fig 8:** Citrate utilization test

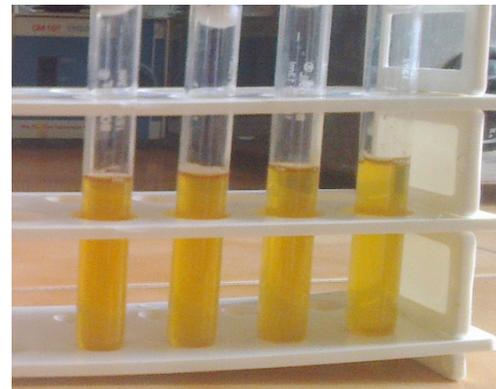
**MR-VP:** The organism gives Methyl red positive turning the culture into red color and Voges Proskauer negative

giving yellow color. These two tests give opposite results to each other. This suggests that the organism is able to ferment dextrose and releases acid in the medium which is indicated by red colour.



**Fig 9:** MR-VP Test

**Indole test:** The bioluminescent organism isolated from the fish is not able to convert tryptophan into Indole as no red ring forms on adding Kovac's reagent.



**Fig 10:** Indole test

**Catalase test:** The bacterium is catalase positive evolving out oxygen bubbles after adding hydrogen peroxide on the smear.



**Fig 11:** Catalase test

**Oxidase test:** It was found that the bacterium is oxidase positive turning the oxidase disc to purple color.

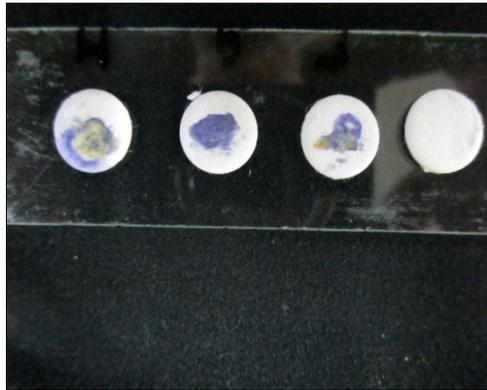


Fig 12: Oxidase test

**Triple sugar Iron test (TSI):** The result was positive with the release of hydrogen sulfide.



Fig 13: TSI test

Table 1: Results of the biochemical tests

Biochemical test	Result
Citrate test	+
Methyl red	+
Voges Proskauer	-
Indole test	-
Triple sugar iron	+
Mannitol salt agar	-
Catalase test	+
Oxidase test	+

**Optimization results**

Table 2: Temperature optimization

Temperatures(°C)	OD
4	negative
25	0.634
30	0.763
35	0.640
40	0.196
50	negative

Table 3: pH optimization

pH	OD
5	0.28
6	0.75
7	0.80
7.5	0.84
9	0.30

From the above two tables we can conclude that temperature of range 22 °C to 35 °C and pH of 6 to 7.5 are the preferred temperature and pH for the bacterial growth.

**Sequencing**

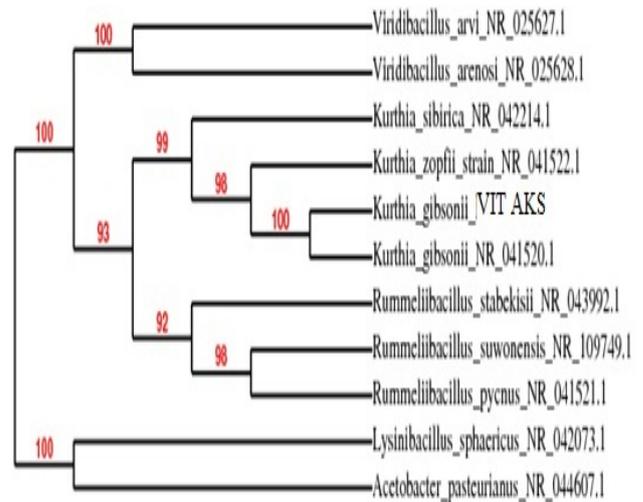


Fig 14: Result of sequencing

*Kurthia gibsonii* is the organism obtained from the Ornate threadfin bream fish responsible for the bioluminescence as shown by the results of the sequencing. This is an aerobic, gram positive and non pathogenic organism. The organism grows well at the room temperature [21].

**Enzyme activity**

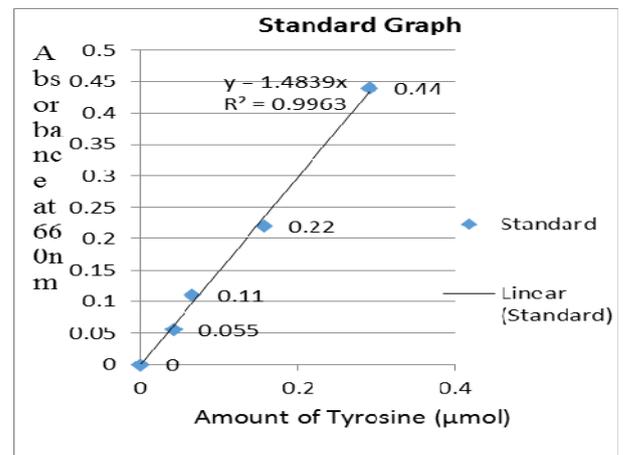


Fig 15: Standard tyrosine graph

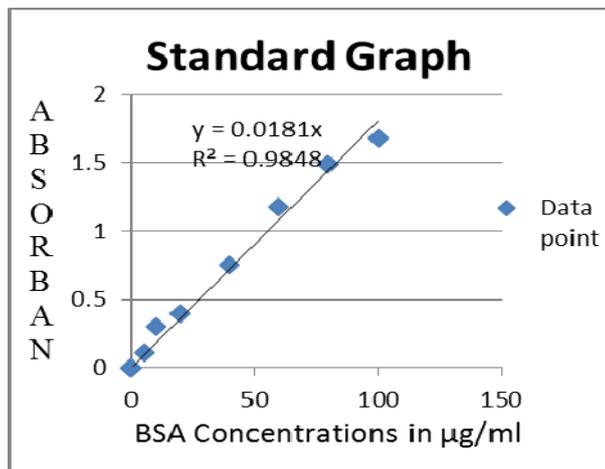


Fig 16: BSA standard graph

Here we have found that, marigold petals and spinach leaves may act as the suitable substrate for the enzyme luciferase. These substrate contains flavin mononucleotide (FMN) or some compound analogues to it. While doing the estimation, we are assuming that it is able to gain the hydride converting to FMNH<sub>2</sub> which reacts with oxygen giving out the desired reaction.

Substrate	Final OD	Enzyme activity(U/ml-min)
Marigold	0.123	0.0262
Spinach	0.104	0.022

The reason for the negative value could be possibly the absence of the substrate. As we all know that enzyme and substrate reactions are highly specific and the enzyme would not react with the unrelated substrate so may be the other flowers petals and cabbage besides marigold and spinach are not providing with the appropriate substrate or may be the enzyme gets denatured during the process resulting in the negative values. The OD is because of the tyrosine molecules present in the solution. When the reaction occurs in the case of the marigold and spinach the tyrosine is released from the substrate and the enzyme but in the other cases may be the tyrosine is released from the enzyme only increasing the blank OD and hence overall decreasing the values (actual OD).

**4. Conclusion**

The main objective of this project was to isolate an organism which has the property of the bioluminescence so that this very feature can be used for the detection instruments, as a marker in the genetic engineering or may be future torch light. The organism was isolated and characterized resulting in the presence of the gram positive bacteria from the *Kurthia sp.* These species are mostly found in the meat products with some specific feature of feathers like structure formation when plated on the gelatin or agar distinguishing the organism from the others. Parameters like temperature and pH were optimized for the organism. Results showed that the organism grows well at room temperature of about 35°C and pH of 6 to 7.5.

Regarding the estimation of the protein we have the presence of the FMN in the leafy vegetables and using spinach as substrate we were able to find the luciferin

content using luciferase enzyme of the bacteria from the production media. However since the purification process for the enzyme was absent, we still are not sure about the protein content but the Lowry method shows the activity of the enzyme and hence there is definitely some reaction occurring which is responsible for the enzyme activity. Marigold is also able to act as the substrate and determining the luciferin but the presence of the FMN in the marigold is still not known to us. There were other substrates as well which showed negative values and were not the appropriate substrate. We are assuming that the tyrosine content released from the substrate and enzyme increases the OD values in the case of the marigold and spinach whereas in the other cases the tyrosine is released from the enzyme only and hence the OD value is not large, ultimately giving negative value in the actual OD.

**5. Acknowledgement**

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