Isolation And Identification Of Bioluminescent Bacteria In Squid And Water Of Malaysia

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***Abstract*—** Bioluminescence is an emission of light that produce by chemical reaction of enzymatic activity and biochemical of the living organism. The most abundant and widely distributed light emitting organism is luminous bacteria either found as free-living in the ocean or gut symbiont in the host. These sets of species are diversely spread in terrestrial environment, freshwater and also in marine ecosystem. These species of bacteria emit blue-green light provoked by the enzyme luciferase and regulated by the *lux* gene operon and emitted at 490 nm. Some of the uses of the bioluminescence are as genetic reporters and biosensors. General microbiological techniques have been used to isolate bioluminescent bacteria from water samples and squid. The characterization of 6 isolates bioluminescent bacteria was conducted macroscopic and microscopically and was further analyses at molecular level using 16sDNA and sequenced for species identification.

***Keywords*—** bacteria, bioluminescence, lux operon, quorum sensing.

I. Introduction

BIOLUMINESCENT organisms with their attractive beauty and ease of detection have drawn interest among scientists to study about them. It is the ability of organisms to release visible light by using natural chemical reaction [11]. The emission of light is a result between the enzymatic activity and biochemical of the living organism. Bioluminescence has been found in diverse group of organisms ranging from microorganism like bacteria and protists to the macro organism such as fish and squid. Meighen has reviewed that the light emitting organisms can be found in terrestrial, freshwater and marine species [3].

The most abundant and widely distributed light emitting organism is luminous bacteria, and this will be found as free-living in the ocean, as gut symbionts in the digestive tracts of marine fish, as parasites in crustacean and insects, as light organ symbionts in teleost fish and also as saprophytes growing on dead fish or meat [3], [14]. These sets of species are diversely spread in terrestrial environment, freshwater and also in marine ecosystem.

These bioluminescent bacteria reported belong to the class *Gammaproteobacteria*. Among the bioluminescent bacteria reported, 17 bioluminescent species were currently known [7]. The luminous bacteria were also classified into three genera which are Vibrio, *Photobacterium* and *Xenorhabdus (Photorhabdus)* and the species within the genus *Vibrio* and *Photobacterium* are usually exist in marine environment and *Xenorhabdus* belong to terrestrial environment.

Some of the natural important of the bioluminescence in nature is in attracting prey, camouflage, deterring predators and in aiding in hunting [8].

Bioluminescence is the phenomenon that consists of light emission from the results of biochemical and enzymatic activity of living organism [3]. It is a group of genes that are responsible for this known as the *lux* operon found in the luciferase enzymes [1]. The reaction of bioluminescence involves the oxidation of a long-chain aliphatic aldehyde and reduced flavin mononucleotide ($FMNH_2$). This mechanism needs oxygen and is catalyzed by enzyme luciferase. In this process, the excess energy is liberated and emitted as a luminescent blue-green light at 490nm [9], [11].

The *lux* operon basically contains the genes *luxICDABEG*. *LuxA* gene and *LuxB* gene are the luciferase gene which codes for α and β subunits respectively. On the other hands, *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) [11], [13].

Therefore, the reaction of bioluminescence emission of light can be written as follows:

$$FMNH_2 + RCHO + O_2 \rightarrow FMN + H_2O + RCOOH + light\ (490\ nm)$$

Quorum sensing phenomenon is known as autoinduction phenomenon [15]. The emission of light controlled by the expression of genes which activated by the autoinduction and bacterial density.

Quorum sensing is the term that represents the cell-density dependent reaction. Luminescent bacteria use this system to activate the light emitting system only in the height concentration of bacteria. In the luminescence expression of gram negative bacteria, it is found coexist with cell-cell interactions and cell density. The cell density-dependent

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control the luminescent gene expression. The most study luminescent bacteria are *Photobacterium fisheri* (formerly known as *Vibrio fischeri*). The study of density-dependent regulation revealed that the bacteria do not emit light while in a small amount of bacteria or in the ocean. However, in large amount, these bacteria gathered in light emitting organ resulting in the emission of a blue-green light [2].

In the field of biotechnology, bioluminescence is applied in the construction of biosensors for detection of contaminants, measurement of pollutant toxicity, and monitoring of genetically engineered bacteria released into environment. Other application of biosensor is as the indicator of cellular metabolic activity and for detection of pathogen [15]. In the food industry, the application of bioluminescence was found to have started a few centuries earlier [5] where the bioluminescence were used to detect pathogens in food spoilage.

Due to its multiple advantages, we have conducted this study to compare the bioluminescence from Malaysian waters. In addition, the isolated bacteria that were identified using molecular approach will contribute to the diversity of bioluminescent bacteria community in Marine Malaysian water.

II. METHODOLOGY

A. Sample collection and materials

To accomplish the objective of this research, samples of marine water were collected from three locations which were Port Dickson Beach (2°30'7.77"N ,101°50'11.01"E), Sungai Sepang Besar Jetty (2°36'32.74"N ,101°42'11.97"E) and Pantai Leka,Parit Jawa (1°56'59.21"N ,102°37'59.08"E). Sterile 250ml Schott bottle (stored in ice) were used to collect the water. The other sample used was squid obtained from Pantai Leka, Parit Jawa Muar.

B. Isolation of bacteria

Luminescence Agar (LA; NaCl 30g, yeast extract 5g, peptone (bacto-peptone) 10g, agar 15g and distilled water 1000ml) [11] was used as media for each of water sample by using spread plate technique. After incubation period of 24 hours and at room temperature of 25^{o}C, the plates were examined in the dark room for presence of bioluminescence. Positive candidates were isolated into new plate by performing streak plate method to obtain pure colony for morphology examination.

For isolation of bioluminescent bacteria from squids, swabs from several parts of squids were used. Swabs were taken from the inner of the body, eyes, under the skin and also ink. Then, the cotton buds were streaked on the LA agar and incubated and observed as the same method as water samples.

C. Morphology Characterization

For macroscopic characterization, the bacteria were characterized based on the shape of bacteria, bacteria colony morphology, bacterial colony elevation, bacteria colony form, colour and size of colony. While for microscopic characterization, Gram staining was done to distinguish between Gram positive and Gram negative and to characterize the shape of individual organisms such as cocci, bacilli, curves, or spiral.

D. Primary Molecular Analysis

To confirm the species of bioluminescent bacteria, molecular analysis was conducted. The genomic DNA was performed using DNeasy Blood & Tissue Kit (Qiagen) and the viability and presence of genomic DNA was confirmed by performing 1% agarose gel electrophoresis [11].

The second step was the Polymerase Chain Reaction (PCR) which was used to amplify the 16S ribosomal unit with universal primers for bacteria (27F, 5 AGAGTTTGATCMTGGCTCAG 3 and R 1492, 5-TACGGYTACCTTGTTACGACT-3) [11]. The amplification has followed: an initial denaturation of 96ºC for 5min., 30 cycles of (96 ºC 30 sec., 55 ºC 30 sec., and 72 ºC 2 min. The positive control used was *Escherichia coli* (*e.coli*).

The reaction mixture was performed with 50 µl mixture which contains of Distilled water 19µl, 2.5µM of each primer, 1µl of DNA template and 25µl Paq5000 Hotstart PCR Master Mix (Agilent).

The PCR products were confirmed using the gel electrophoresis. The amplification of 16S rDNA was confirmed through 1% agarose gel electrophoresis which yielded a product of a 1.5 Kb [11].

E. Sequencing And Analysis

Sequencing of PCR product was done by BGI (Beijing Genome Institute). The sequence data obtained were analysed by using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) [14].

III. RESULT

Bioluminescent bacteria from water samples from marine Malaysian waters were successfully collected from four random locations. They were from Port Dickson, Sungai Sepang Besar and also Pantai Leka Parit Jawa Muar, Johor. In addition, bacteria from marine organism like squids were also successfully isolated. The squid sample came from Parit Jawa, Muar. Water samples collected from Port Dickson were taken by the beach side while for the other location water samples were taken from estuaries. TABLE 1 summarises the macroscopic and microscopic findings. The bioluminescent bacterial colonies can be seen in Figure 1 after 18 hours of incubation.

TABLE I
BIOLUMINESCENCE BACTERIA ISOLATED

Strain code	Source	Salinity (ppt)	Grams Nature	Luminescence properties
SQ1a	Squid	-	Gram negative rod	positive
SQ4a	Squid	-	Gram negative rod	positive
PD1	Port Dickson Beach 2°30'7.77"N 101°50'11.01"E	30.1	Gram negative rod	positive
SP1	Sungai Sepang Besar Jetty 2°36'32.74"N 101°42'11.97"E	29.8	Gram negative rod	positive
PJ1	Pantai Leka,Parit Jawa 1°56'59.21"N 102°37'59.08"	29.1	Gram negative rod	positive
PJ4	Pantai Leka,Parit Jawa 1°56'59.21"N 102°37'59.08"	29.1	Gram negative rod	positive

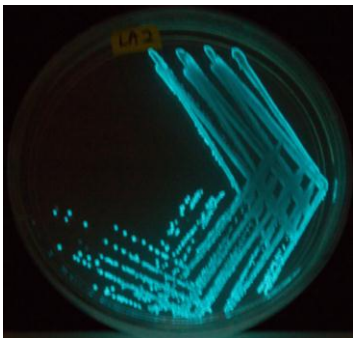

Fig.1 Streaked plate of bioluminescence as observed in a dark room in the absence of any light sources.

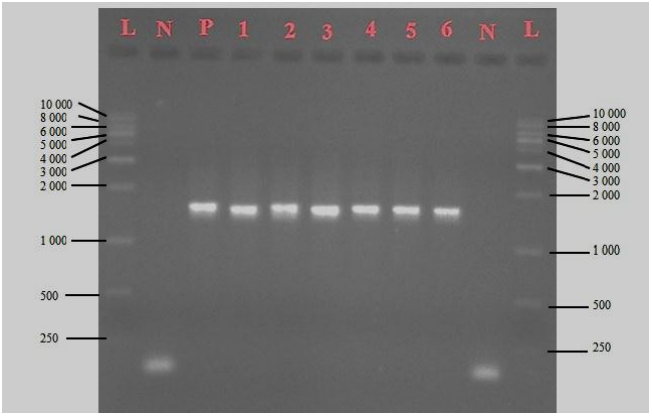

Fig. 2 Molecular characterization gel electrophoresis.
(L: 1 Kb Ladder, P: Posive control, N: Negative control, 1-6: samples)

From TABLE 1, all the bacteria isolates were found to be Gram negative rod bacteria. Fig.1 shows the example of bioluminescent bacteria streaked on the plate and observed in a dark room with the absence of light. The bioluminescent bacteria give off blue-green light in a dark.

Fig. 2 shows that the gel electrophoresis of PCR product of all the samples and positive control for 16S rNA gene identification. All the samples and positive control were amplified at 1.5kb through 1% agarose gel electrophoresis for 90 minutes at 90V. After sequencing and BLAST analysis, the samples from squid was found to be similar with *Photobacterium leiognathi* while the water samples was found to be most similar to *Vibrio sp.*

IV. DISCUSSION

In Malaysia, bioluminescence phenomenon where the beach glows at night does not occur as reported as such in Puerto Rico. However, the luminescent bacteria exist as the part of marine community. This was proven as water samples collected from marine waters contain bioluminescent as shown in Fig. 1.

In this study, all the bacteria from water samples were identified as *Vibrio sp*. and the bacteria isolated from squid were found to be similar to *Photobacterium leiognathi.* In all bioluminescent organisms, luminous bacteria are the most abundant organisms exist in nature. They are found ubiquitous in marine but less in freshwater and terrestrial environments. There are two common genera of bioluminescence in marine which are *Vibrio sp.* and *Photobacterium sp*.. The most studied luminescent bacterium is *Vibrio sp*. [6].

Most of the study revealed that in genera *vibrio*, *Vibrio fisheri* usually had been found either free living in the marine environment [14], symbiosis in light organ of Hawaiian bobtail squid *Euprymna scalopes* [16] and also *Vibrio phosphoreum* in squid [17]. For the other genera, *photobacterium* was reported to have been isolated from fish for example *Photobacterium phosphoreum* was isolated from migrating salmon in the Yukon River, Alaska. Other than that, *Photobacterium leiognathi* was reported been isolated from ponyfish, *Leiognathus nuchalis* [18].

Marine luminous bacteria are comprised of Gram negative rod bacteria. These bacteria have unique trait of the ability to emit light. In marine environment, this ability is used to attract prey, camouflage, to deter predators and also to aid in hunting [8]. These bacteria usually are symbiont with the host organisms where the host will provide nutrient rich environment for the bacteria and in return the bacteria provide benefits in the form of camouflage or protection from prey [4]. In certain organisms, luminous bacteria are found in the light organ such as squid where the light organ is located in the mantel of squid body [10].

Bacterial bioluminescence is common in the field of biology and biotechnology such as in the environmental microbiology as the biosensor [6], in the microtox system for toxicity study and others. Therefore, in order to apply bioluminescence for industrial used, isolation should be carried out from various sources to find the bioluminescence bacteria on the basis of strong light intensities. Beside that for biotechnology, *lux* gene from isolated bioluminescence bacteria can be explored further as it can be used as reporter gene in the construction of biosensor.

V. CONCLUSION

The isolation of the bioluminescent bacteria from Malaysia's marine water samples confirmed that the species is *Photobacterium leiognathi* found in squid while the luminous bacteria from marine water were Vibrio sp. This will help for further identification of bioluminescence from freshwater environment.

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