ISOLATION AND IDENTIFICATION OF LUMINESCENT GLOWING BACTERIA IN MARINE SQUID, *LOLIGO SP.* FROM REMIS BEACH, KUALA SELANGOR, MALAYSIA

Rozila Alias, Jayanthi Elangovan and Hafidzatulnur Mohd Rathy
Institute of Bio-IT Selangor, Universiti Selangor,
7/A Zirkon Road, Section 7, 40000 Shah Alam, Selangor, Malaysia.
Email: rozila@unisel.edu.my, Tel: +60108143895

Mohd Taufik Mohd Zaid and Siti Hasmah Mohtar
Department of Science and Biotechnology, Faculty of Engineering and Life Science,
Universiti Selangor, Bestari Jaya, Selangor, Malaysia.
Email: hasmah@unisel.edu.my; Tel: +60133957392

Yusmi Mohd Yunus
Department of Biomedical and Health Science, Faculty of Engineering and Life Science,
Universiti Selangor, 7/A Zirkon Road, Section 7, 40000 Shah Alam, Selangor, Malaysia.
Email: yusmiyunus@unisel.edu.my; Tel: +60196927967

Rafidah Saadun
Industrial Biotechnology Research Centre, SIRIM Berhad,
No.1 Persiaran Dato' Menteri, P.O.Box 7035, Section 2, 40700 Shah Alam, Selangor, Malaysia
Email: srafidah@sirim.my; Tel: +60196414201

Mohd Nizam Lani
School of Food Science and Technology,
Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia
Email: nizamlani@umt.edu.my; Tel: +60124988040

ABSTRACT

Luminescent bacteria had been broadly studied for its ability to convert chemical energy into light energy source by bioluminescent reaction. There are three main classifications of luminescent glowing bacteria which are mostly distinguished as Photobacterium, Vibrio, and Photorhabdus. Luminescent bacteria such as Photobacterium spp. and Vibrio spp. exist as symbionts within marine organisms such as squids, lantern fish, jelly fish, the angler fish, clams and the eel. Whereas, Photorhabdus spp. were found in earthbound insects like caterpillar and nematode as the intermediate host for the bacterial growth. The objective of this study is to isolate and to amplify the Lux-AB gene for confirmation of producing luminescent light bacteria from marine squid, Loligo spp. In this study conducted, the marine squids were brought from fisherman at Remis Beach, Kuala Selangor and the ink samples were obtained from its internal part containing ink sac. The ink sample was collected and was diluted from 10^{-1} to 10^{-5} and then was spread plate onto Luminescent agar (LA) medium. The LA plates were incubated at room temperature (~27°C) for 24 hours and every 6 hours the appearance of luminescent colonies was observed for the luminescene light in the dark room. The distinct 10 single colonies that were produced the most glow in the dark were randomly picked and further re-streaked to get pure culture onto LA plate and incubated again at room temperature (~27°C) for 24 hours. Gram stain test were done for the morphological study of luminescent bacteria. As result, 10 isolates showed as gram negative bacteria, pinkish color and it's a short rod shape morphology when view under 100x magnificent with immersion oil. Then, the isolates were sub-culture into Luminescent broth (LB) for overnight and the genomic extraction were done (Bioteke, Beijing) to amplify the Lux-AB gene cassette by using primer 611R (reverse) and 66F (forward). The Lux-AB gene present a PCR product band at 561-567bp using different temperature at 39°C, 41°C, 43°C, and 45°C respectively for gene producing the luminescent light. All the isolates were successful produced expected band with different optimum temperature. These luminescent bacteria which contained Lux-AB gene proven to be transmitted the light when it's catalysed by the enzyme called luciferase. These luminescent bacteria obtained will be further studied for its potential bio-light application as biosensor, reporter gene and biolight source.

Keywords: Luminescent Bacteria, Lux-Ab Gene and Biolight

INTRODUCTION

Luminescent bacteria had been broadly studied for its ability to convert chemical energy into light energy by bioluminescent reaction (Tait and Dipper 1998). It can be found in sea water and living in the terrestrial or fresh water environment. Mostly, marine bioluminescence bacteria are visible as green to blue colour because of its shorter wavelength of light and enables it to travel through in both shallow and deep sea water. Bacterial luminescence glowing is due to the action of the enzyme called luciferase where its catalyses the removal of an electron from two compounds. Excess energy is liberated in this process. The energy is dissipated as luminescent blue-green light. In this reaction, it involves the oxidation of a long-chain aliphatic aldehyde and

reduced flavin mononucleotide ($FMNH_2$) with the liberation of excess free energy in the form of a blue-green light at 490nm. A suite of genes dubbed "Lux" genes code for the enzyme and other components of the luminescent system. There are several researches that had proven the beneficial application of bioluminescent bacteria as a biomarker in medical field and also as biosensor which can be highly practical in bioremediation studies. With the arising health related issues and environmental problems, extensive research on bioluminescent bacteria could provide further solution to those problems. However, there is still limited knowledge of the physiological requirements of most marine bioluminescent bacteria and a greater understanding of its conditions for growth will offer new insights into the complex world of marine microbiology. Malaysia is currently undergoing environment related issues such environmental pollution which leads to soil pollution, water pollution from raw sewage, soil degradation and others are trending issues nowadays that need to be overcome by means of times. The motivation of this research is to emphasize the bioluminescent bacteria's capabilities as a solution assisted by the above points in consideration. There are a number of strains of luminescent bacteria have been reported and isolated from ocean and sea samples (Fukasawa et al., 1984; Nawaz et al.,2011; Shanware et al., 2013; Yaser et al., 2014; Maureen et al., 2014; Werdani et al., 2015; Kola et al., 2017). The objective of this study is to isolate luminescent glowing bacteria from marine squid, *Loligo spp*. which could be further analyzed for its benefits in scientific field.

MATERIALS & METHODS

SAMPLING

Three marine squids were collected fresh from the fisherman at jetty of Remis Beach, (straits of Malacca), Kuala Selangor, Selangor, Malaysia during the period of January 2017 early in the morning between 4:00 am to 6:00 am to obtain freshly caught squids samples. Then the squids were kept in the ice-box which half submerged of sea water for several hours at 18^oC to 22^oC and then immediately brought to the Nanobiotechnology Research Laboratory, Institute of Bio-IT Selangor at Universiti Selangor (UNISEL), Shah Alam, Selangor, Malaysia. The squids were first washed slightly with distilled water to remove any mud or sand particles. Then the squids were laid into sea water half submerged for several hours at 18^oC to 22^oC, or refrigerated at 4^oC to 8^oC for incubation. The squids were kept in cold to impair growth of any unwanted bacterium and to prevent decomposition.

Figure 1: Fresh Squids were collected from fisherman at Jetty of Remis Beach (Straits of Malacca), Kuala Selangor, Selangor, Malaysia.

PREPARATION OF LUMINESCENT MEDIA

The minor modified of luminescent agar (LA) and luminescent broth (LB) (Nealson 1978) was used in this study. The composition of luminescent agar/broth is as follows; 10g of peptone, 60% Glycerol, 1g of $MgSO_4$, 4g of K_2HPO_4, 30g of NaCl and 15g of bacteriological agar in one Litre. The media then was autoclaved at 121°C for 15 minutes.

ISOLATION AND SUB-CULTURING

The intestinal part of squids was dissected to obtain ink samples from the ink sac of the squids. 1 ml of the ink sample was mixed with 9 ml of LB in a falcon tube to obtain 10^{-1}. 1 ml from this dilution was taken and added to another 9 ml of LB in falcon tubes for 10^{-2} and repeated similarly until to get 10^{-5} dilution.The ink sample was diluted with LB from 10^{-1} to 10^{-5} and then 0.1 ml from each serial dilution was spread plate onto LA plates.The plates were then incubated overnight in room temperature, ~27°C for 24 hours and were observed and recorded at every 6 hours the appearances light of luminescent colonies in the dark room. The distinct 10 single colonies from the dilution between 10^{-4} and 10^{-5} plates producing the most glowing light in the dark were randomly picked up and further re-streaked onto LA plate and incubated again at room temperature, ~27°C for 24 hours.

SUBCULTURE OF SELECTED ISOLATES

The distinct isolated luminescent colonies of bacteria were marked while observing for luminescence and were further purified by sub-culturing in luminescent agar plates following standard bacterial isolation by repeated streaking on luminescence agar and store in luminescent broth for the growth of pure culture bacteria. By using pipette, 5uL of luminescent broth solution were streaked on luminescent agar. Gram staining and microscopic examination were done to observe the general morphology of the cell.

GROWTH OF LUMINESCENT BACTERIA IN TCBS MEDIUM

TCBS medium is a selective medium that allows the selective growth of bacteria belonging to the genera *Vibrio* spp. TCBS medium was prepared and poured in petri plates and 10 different strains were streaked and the result were observed after 24 hours. Appearance of yellow color colonies in this medium indicates the bacterial strain as *Vibrio* sp. The ten isolates obtained and identified by gram staining and biochemical tests. According to "Bergey's manual of determinative bacteriology, 9th ed.," Luminescent, there are three genera and five species of luminous bacteria, *Vibrio cholerae biotype, Pseudomonas, Vibrio fischeri, Lucibacterium harveyi, Photobacterium phosphoreum* and *Photobacterium mandapamensis*.

GRAM STAINING

For the confirmation of pure bacterial culture, gram staining was done. The morphology of bacterial cells was examined viewed under the compound microscope 100x magnificent plus immersion oil. Then, these 10 pure cultures were stored in LB added with 60% of glycerol to be kept as storage culture in -20°C refrigerator.

GENOMIC DNA EXTRACTION

The genomic DNA of 10 pure cultures of Luminescent bacteria were extracted according to manual manufacturing protocol using Bioteke, Beijing. The genomic was electrophoresed by 0.8% agarose gel (included 2ul of Gelview stain, Bioteke, Beijing) at 75 V and 30 mins. The gel was viewed under the blue light transluminator (Junyi Electrophoresis, Beijing) (Figure 4).

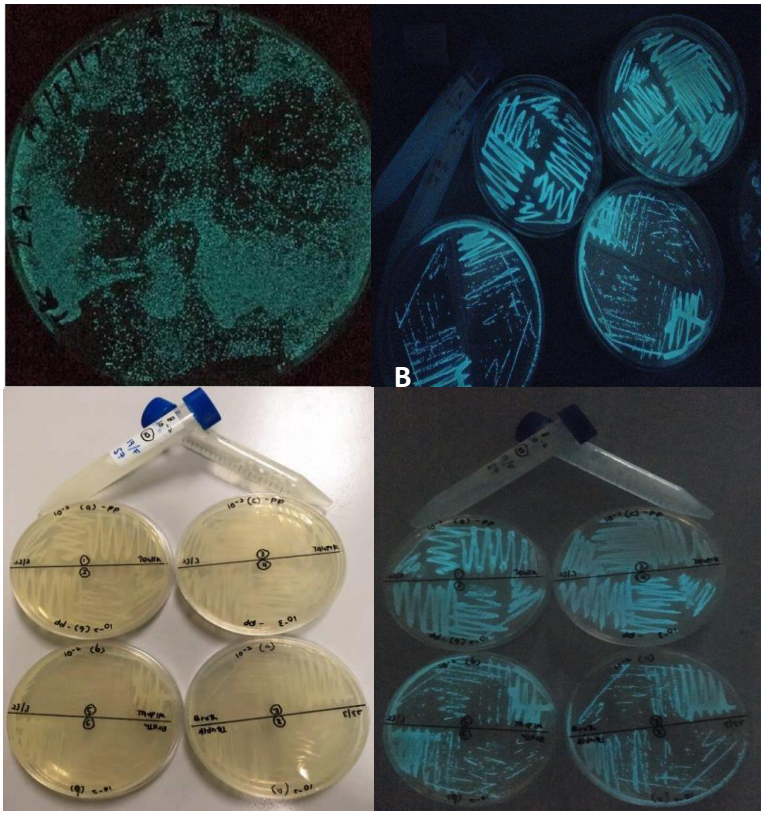
LUX-AB AMPLIFICATION USING POLYMERASE CHAIN REACTION

The DNA extraction was further proceeded to amplify the Lux-AB gene with 2x power taq PCR Master Mix (Bioteke, Beijing) and universal primers with PCR conditions using the following for 20 cycles: 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 60 seconds. The Lux-AB gene with 611R reverse primer (3'-AACRAAATCWYKCCATTGRCCTTTAT-5) and 66F forward primer (3'-CAAATGTGRAAAGGTCGTTTTAATTTTGG-5') (Gabriela et al., 2009) used for five different temperature at 37°C, 39°C, 41°C, 43°C, and 45°C to produce PCR band performed in 2% agarose gel electrophoresis at 75 V for 40 minutes. 100bp and 1kb of DNA ladder (Bioteke, Beijing) used as a gel view marker.

RESULTS AND DISCUSSIONS

Through spread plate technique, more than 300 colonies glowing were observed on the luminescent agar plate with serial dilution of 10^{-1} until 10^{-3} after 12 hours of incubation. Only 10 prominently glowing colonies of luminescent bacterial were randomly picked up and purified onto LA plate. (Figure 2). The luminescent bacteria were still glowing in the dark room within 12-24 hours except isolates L1 still glowing until 48 hours. Microscopic observation shows they are in pink colour, gram negative bacteria, actively motile and a rod shape (Figure 3). Their culture growth on TCBS agar shows it is expected to be *Vibrio spp.* According to Yaser et al., 2014, they also found the bioluminescent gram negative bacteria from seawater and squids which are *Vibrio sp*. and *Photobacterium leiognothi*.

Figure 2: Spread plate and sub-cultured luminescent bacteria on the luminescent agar.



Note: A: Serial dilution 10^{-2} of LA plate; B: Luminescene bacteria streaked onto LA plate;

C: Luminescene bacteria in lbright-field;D: Luminescene bacteria in dark conditions.

Figure 3: Morphology of luminescence bacteria through gram staining under microscope with magnification 100x plus immersion oil.

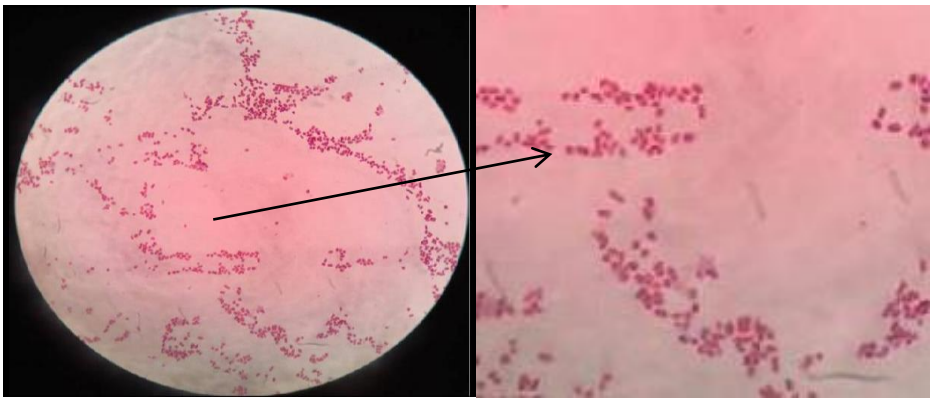


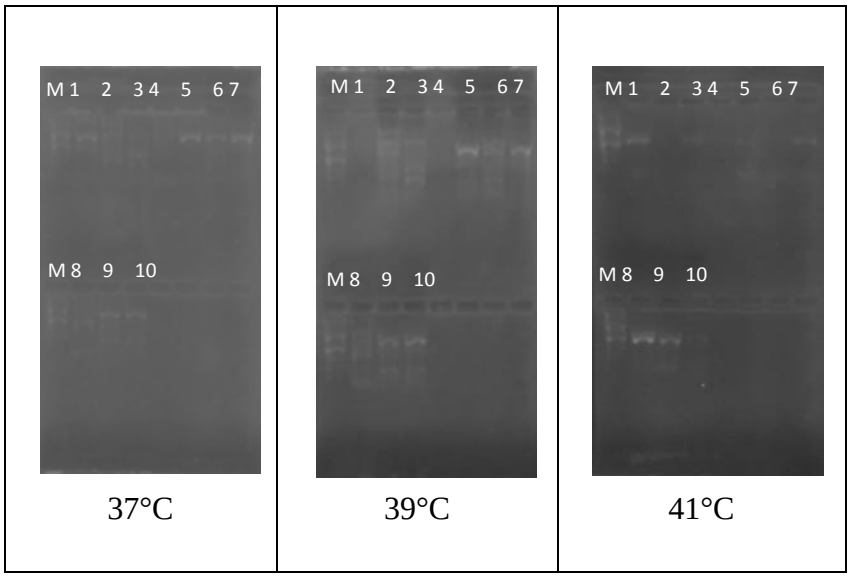
Table 1- Different temperature of PCR annealing temperature to obtain Lux-AB gene among the isolates.

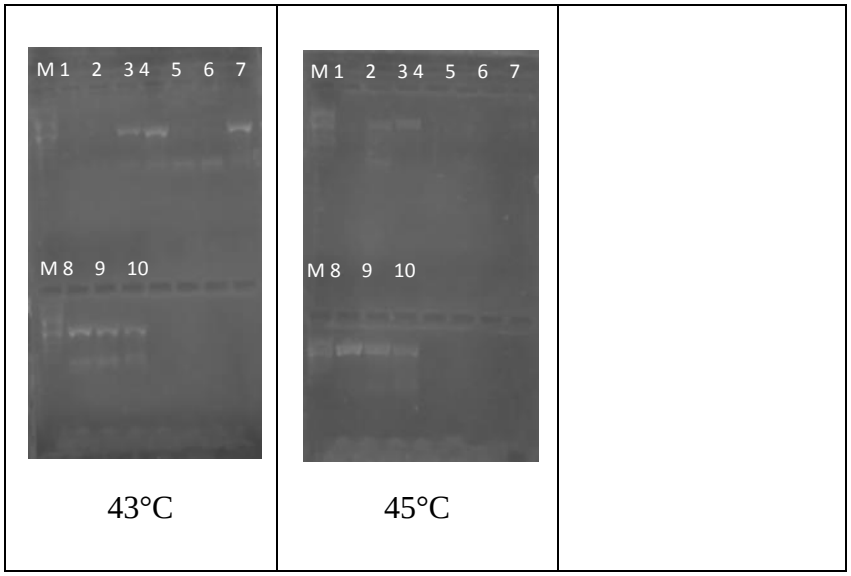
Isolates No.	Temperature (°C)				
	37°C	39°C	41°C	43°C	45°C
L1	√	-	√	-	-
L2	√	√	-	-	√
L3	√	√	√	√	√
L4	√	-	-	√	-
L5	√	√	√	-	-
L6	√	√	-	-	-
L7	√	√	√	√	-
L8	√	√	√	√	√
L9	√	√	√	√	√
L10	√	√	√	√	√

Notes: √ Amplified where the band is appear; - Not amplified where the band did not appear

The genomic DNA were successfully extracted (Figure 4) and proceeded with the Lux-AB gene amplification. The PCR product were obtained and appeared in different optimum temperature (Table 1). All the isolates were successful amplified Lux-AB and expected PCR band appeared mostly at 37^{o}C. Isolates L2, L3, L8, L9 and L10 are still able to amplify Lux-AB gene between 37^{o}C until 45^{o}C. This band indicated that they are having the Lux-AB gene between approximately 560bp which makes them glowing (Table 1).

Figure 4- PCR amplification of Lux-AB gene resolved on 2.0% agarose electrophoresis gel view.





Notes: Lane M: 100bp DNA Ladder; Lane 1-10: L1-L10

These luminescent bacteria contained Lux-AB gene are potential transmitter of the light with catalysed by luciferase enzyme. The enzyme catalyzes the three substrates which reduced flavinmononucleotide ($FMNH_2$), oxygen (O_2) and long-chain aldehyde (RCOH). That reaction frees flavin (FMN), long chain fatty acids (RCOOH), and water (H_2O) with the liberation of excess free energy in the form of a blue-green light at 490nm.

$FMNH_2 + RCHO + O_2$ ------> $FMN + RCOOH + H_2O$ + Light (490nm)

Luminescent bacteria are gram negative, motiles, rod shaped and there are three main classifications of luminescent glowing bacteria which are mostly distinguished as *Photobacterium, Vibrio,* and *Photorhabdus* (Dunlap and Kita-Tsukamoto 2006, Cheng Lin and Meighen 2009). Recent study stated that the largest prominent bioluminescent deep sea molluscs are cephalods such squids (Haddock, Moline & Case 2010). Marine squid such as *Loligo spp* have light organ which are biloped organs located on the ventral site of the ink sac near anus where the symbiotic affiliation occur (Guerrero-Ferreira and Nishiguchi 2009). These *Loligo spp*, commonly harbour luminescent bacteria from the family of Vibrionaceae more likely *V.harveyi* (Guerrero-Ferreira and Nishiguchi 2007, Schuster et al., 2010). According to McFall-Ngaj et al., 2011 that these squid-vibrio symbiosis has a unique system in playing its role of bioluminescence in a natural host environment. .To make the bacteria produce light, Lux genes are responsible comprising a regulon that encodes the proteins essential inside the bacterium such as *Vibrio fischeri.* The Lux regulon from luminescence bacteria consists of two divergently transcribed operons, L operon (left) and R operon (right), and at seven genes, LuxR in L operon and LuxICDABE in R operon and the intervening control region. The LuxA and LuxB genes are encode respectively the alpha and beta subunits of luciferase enzyme. Generally, the end product of luminescent bacteria is compared to respiration where it produces adenosine triphosphate (ATP) but in bacterial luminescent, it produce chemical compound emitting light termed luciferase (Danyluk et al., 2007). From the results, all the isolates were successfully detected the Lux-AB gene in different temperature which these Lux-AB genes are involved in producing luminescence light. In a natural way, the bioluminescence symbiosis between marine organisms and luminescent bacteria have many beneficial adaptations such as using counterilluminaton to protect themselves from predators, to lure their prey out and for attracting mates.

There are few limitations in this research such as methodology where the formulation of media needs to be optimize for the better luminescent capability of the bacteria for a longer period of time. Furthermore, the findings, discussions and informations about the physiology of luminescent bacteria is very limited whereby the correct amount of nutrition, culturable environment and other conditions that needed for culturing luminescent bacteria still have to be established and analyzed in order to further the researches on the bioluminescent bacteria. To obtain the optimum growth of luminescent bacteria, different carbon sources can be explored such as glycerol, gluconate, glucose, fructose, sucrose, starch, mannitol, lactose, galactose and maltose to ensure their growth are high and able to produce light in longer time. Hence, these findings of the paper on bioluminescent bacteria will be beneficial for environmental related industries in Malaysia. The construction of biosensor using bioluminescent bacteria is widely being applied in bioremediation where it is used to resolve the presence and concentration of specific pollutants along with distinguish between bioavailable forms of pollutants from those exist in the environment in inert, unavailable forms (Vania and Norma, 2003). Other than that, the microtox system which uses natural bioluminescent bacteria is a toxicity test where it evaluates the efficiency of toxicity reduction during waste water treatment, for initial screening of cyanobacteria blooms (Campbel et al., 1994). The system also monitors discharges from offshore installations at source within Offshore Oil & Gas Industry (Whale, 1994). However, bioluminescent bacterium is still being studied broadly for its beneficial application in various fields such as health and food industries. According to Kola S. and M.M, 2015 said that the Lux genes are widely used as molecular reporters since its non-hazardous, unlike radioactive isotopes The significant contribution of bioluminescent bacteria is

that it can be used as a bio-light in future which leads for substantially less carbon dioxide emissions, limit effect of light pollution and no need for electricity. Other than that, a bioluminescence bacterium is also could be used as a biomarker to detect tumors and disease in medical field. Clearly, a greater investment in the development of marine biotechnology will produce a novel compound such as bioluminescence bacteria that may contribute significantly towards betterment of human life in the coming years.

CONCLUSION

From the study we conclude that bioluminescent bacteria present during the isolation from the marine squid, *Loligo spp.* are expected to be from *Vibrio spp.* This study also shows the successful isolation and PCR amplification to detect the Lux-AB gene present in the glowing bacteria isolates by optimizing temperature where each of 10 isolates responded to different temperature thus proving the luminescent gene presence in all the isolates. A few studies could be focused in scientific field such as potential biosensor, biolight and as a reporter of gene expression application.

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