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Isolation and Identification of Symbiotic Microorganisms from Bioluminescent Marine Life

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Authors' contributions

This work was carried out in collaboration among all authors. Author DP as the first author is a project coordinator, Author KD as second author contribute as fish expert and Author PA as squid expert. All authors read and approve the final manuscript.

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ABSTRACT

Bioluminescence means the ability of animals or plants to naturally produce light. The three known ways by which bioluminescence is produced are through specific cells called photocytes, bioluminescent glands in tissues and symbiotic bioluminescent microorganisms. Bioluminescence in *Loligo duvaucelii* is known to be caused by the presence of symbiotic microorganisms in bioluminescent sacs. There is a need to compile more information on bioluminescent symbiotic microorganisms on marine life in Indonesia and their potential. This study aims to determine the species of bioluminescent microorganisms on squid and fish, namely *Loligo sp.* and *Loligo edulis* from the waters of Jepara and the Bombay duck (*Harpadon nehereus*) from the Strait of Malacca, Indonesia and their potential. The samples were collected by isolating the microorganisms from the luminescent organs, after which the bioluminescent microorganisms were used in the research. This research consisted of antimicrobial tests against pathogenic microorganisms which were conducted qualitatively. The bioluminescent microorganisms were identified using biochemical assay and molecular assay (16S rRNA PCR). Tests results from *Loligo sp.* symbiotic microorganisms found

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two isolates which showed antimicrobial activities against pathogenic Multi Drug Resistant (MDR) microorganisms, namely uncultured bacterium clone 1P-1-G05 against *Escherichia coli* with 32.59 mm of inhibitory zone and Uncultured bacterium clone 3g10a against *Enterobacter* sp. with 28.44 mm of inhibitory zone. The bioluminescent symbiont microorganisms in *Loligo edulis*, which was identified to be *Photobacterium phosphoreum*, showed antimicrobial activities against *Vibrio harveyi*, *E. coli*, *Staphylococcus aureus*, and *Bacillus* sp. Bioluminescent symbiotic microorganisms on *H. nehereus* identified *Alteromonas macleodii*, which showed gamma hemolysis on the blood agar test.

Keywords: *Bioluminescent microorganisms; gamma hemolysis; Loligo sp.; loligo edulis; H. nehereus; MDR microorganisms.*

1. INTRODUCTION

Bioluminescence is the most important source of light in deep sea ecosystems. Resulted from the oxidation of substrate catalyzed by enzymes, it enables living organism to radiate light [1]. The emission of light that occurs benefits the organism because it is useful for foraging, avoiding predators, identification or communication with others, and for camouflage [2]. Bioluminescence in *Loligo duvaucelii* species is due to the presence of symbiont microorganisms in its bioluminescence organ [3]. Luminescent microorganisms have genes which allow them to produce luciferase enzymes and long aldehyde chains needed in bioluminescence chemical reactions [4]. This symbiosis is mutualistic because bioluminescent organisms get a variety of benefits from the light emitted, while microorganisms obtain a protected environment rich in nutrients [5].

In general, the reaction that causes the phenomenon of bioluminescence is influenced by two chemicals, namely luciferin and luciferase or photoprotein. Luciferin is a compound that can produce light [6]. Some organisms can produce luciferin compounds themselves, while some others get them from other organisms. External luciferin is obtained from food or through a symbiotic relationship. In addition to luciferin, the chemical that contributes to the phenomenon of bioluminescence is luciferase. Luciferase is an enzyme that interacts with the substrate to influence the rate of chemical reactions. The bioluminescence reaction requires the luciferase enzyme to catalyze three substrates, namely reduced flavin mononucleotide (FMNH₂), oxygen (O₂), and long chain aldehyde (R-COH). This reaction will free flavin (FMN), long chain fatty acids (R-COOH), water molecules (H₂O), and accompanied by visible light (hv) emission [7]. The bioluminescence phenomenon occurs because of certain different

chemical reactions in every animal. In this chemical reaction, luciferin acts as a substrate that helps bind oxygen to organic molecules. The interaction of luciferin oxidized by luciferase forms a by-product in the form of high-energy molecules. This molecule is referred to as oxyluciferin. The reaction then releases light energy which gives a luminescent appearance on the animal.

Most of the biota have living bioluminescence in the sea. Of the 700 genera of biota with bioluminescence in nature, 80% of them are in the ocean [8]. In the vast ocean with various types of microorganisms, bioluminescence organs that are rich in nutrients certainly become ideal ecosystems for microorganisms, resulting in competition among the luminescent microbial communities. In addition to the ability to produce light to win in this competition, bioluminescence microorganism have the ability to protect hosts from pathogenic microorganisms. These properties allow the use of luminescent microbial isolates genes in the field of ecology as heavy metal biosensor agents in the aquatic environment [9]. Therefore, the potential of bioluminescence studies is very promising in the field of biotechnology and ecology. The source of the study can be obtained from bioluminescence organisms found in Indonesian waters as a source of potential luminescent microbial isolates. Indonesian waters are vast, with various types of marine organisms, including bioluminescence organisms, such as *Anomalops katoptron* from Banda Island [10] and *Loligo duvaucelii* from Jepara [3].

Therefore, this study aims to determine the species of bioluminescent organism on squid and fish, namely *Loligo* sp. and *Loligo edulis* from the waters of Jepara and the Bombay duck (*H. nehereus*) from the Strait of Malacca, Indonesia and their potential.

2. METHODOLOGY

2.1 Sampling and Isolation of Luminescent Microorganisms on Squids

Five squids were collected by catching using floating net mesh from 1-2 m depth from the waters of Jepara. The samples were kept in a cool box for further preparation in the laboratory. Isolation of luminescent microorganisms was performed by pulverizing the light organ of the squid, after which a 10^{-5} dilution was made. The microbial suspension was then spread onto Zobell 2216E media composed of 2.5 g of peptone, 0.5 g of yeast extract, and 15 g of agar per liter. The microbial culture was then incubated for 2 days in room temperature. The resulting microbial colonies were isolated. The colonies producing the most luminescence were purified to obtain purified culture.

Determination of luminescent symbiotic microbial isolates in squid light organs was based on the morphology of the luminescence bacterial colonies grown on Nutrient Agar (NA) media in petri dishes as well as observing the emission of light by bacteria in a dark room. The resulting isolates were purified to obtain purified isolates of luminescent microorganisms. The observation for the morphology of the luminescence bacterial colony includes color, shape, surface and the edge of the colony and biochemical identification.

2.2 Sampling and Isolation of Luminescent Microorganisms on The Bombay Duck (*H. nehereus*)

Twenty Bombay duck fish samples, each approximately 23 cm in length, were collected from Tanjung Balai Karimun Island, Riau Islands, Indonesia. The collected samples were stored in a cool box prior to laboratory preparation. The samples were rinsed with sterilized saline water and were put in the open on a room temperature. After 8 hours of dark room observation, luminescent microorganisms occurred on the surface of the body. One gram of skin tissue from the samples was sampled for serial dilution. The resulting microbial suspension was then spread onto Zobell 2216E media composed of 2.5 g of peptone, 0.5 g of yeast extract, and 15 g of agar per liter. The microbial culture was then incubated for 2 x 24 hours in room temperature. Single purified colony with the most luminescence was inoculated into a new medium for molecular identification and hemolysis test.

2.3 Identification of Organism

Characterization of microorganisms were performed through TCBS (Thiosulfate-citrate-bile salts-sucrose) isolation, Oxidase test, Indole test, Oxidative fermentative (Sucrose and Lactose) test, Motility test, H₂S test, and Voges-Proskauer test. Gram staining was also performed according to standard method. Molecular analysis was performed in accordance to the following stages.

2.4 Amplification of 16S Partial Ribosomes from Isolated Luminescent Microorganisms

Genomic DNA from luminous bacterial microbial isolate was used as a template for PCR amplification. Amplification of 16S ribosomal RNA (rRNA) was performed using 27f (5'-AGAGTTT- GATCMTGGCTCAG-3') and 1492r (5'TACGGYTACCTTGTTACGACTT- 3') primers. Amplification took place in a thermal cyclor with 30 rounds followed by denaturation of DNA at 94°C for 1 minute, at 55°C for primary annealing for 1 minute, and extension at 72°C for 1 minute. The final extension was carried out at 72°C for 10 minutes. PCR products were analyzed using gel electrophoresis (1%) and the resulting images were analyzed using UVI Doc HD5 (UVITEC Cambridge).

The sequencing process was carried out at Genetika Science Inc., Indonesia. The result of the sequencing was the nucleotide of the 16S rRNA gene region data, which was then processed and edited using BioEdit software, and was submitted to the NCBI database that stores information on nucleotide sequences. The phylogenetic tree was made using Blast tool software and designed by NCBI database search tool.

2.5 Antimicrobial Activities of Luminous Microorganisms from *Loligo sp*

This process is carried out by an overlay method [11]. Purified culture of bioluminescence organism isolated from *Loligo sp.* was used against Multi Drug Resistant (MDR) pathogenic organism of the species *Klebsiella sp.*, *Enterobacter sp. 5*, *Enterobacter sp. 10*, *Coagulase Negative Staphylococcus* (CNS), *Pseudomonas sp.*, and *Escherichia coli*. Pathogenic organism samples were obtained from Dr. Kariadi Central General Hospital,

Semarang. MDR microorganisms were introduced in liquid Zobell 2216E plain media and then incubated for 24 hours at room temperature while being spun at 90 rpm. 1 mL of MDR microbial suspension was taken and put into 100 mL soft Zobell 2216E plain agar (1% of total volume), after which it was introduced to Zobell 2216E saline solid media which had been cultured with luminescent microbial isolates from the squid sample, then incubated at room temperature for 1- 2 days. The bacterial isolates which formed inhibition zones were sorted and were put into another iteration. Isolates which still showed viable result after iteration were taken and stored for further analysis. Quantitative tests using paper disks were then performed. Antimicrobial tests were carried out by streaking microorganisms isolate onto the paper disk with 20 µL concentration. The resulting inhibition zone was then measured with a caliper.

2.6 Antimicrobial Activities of Luminescent Microorganisms from *Loligo sp*

This process was performed using agar diffusion method according to Kirby-Bauer [12]. This test was performed both qualitatively and quantitatively using NA media. Purified culture of bioluminescence microorganisms isolated from *Loligo edulis* was used against vibriosis-causing pathogenic bacteria in animals, *V. harveyi* and *V. alginolyticus*, and pathogens which cause various types of infections in humans, *E. coli*, *S. aureus* and *Bacillus sp*. The qualitative test was performed first to determine whether the luminescent microorganisms isolate has the ability to inhibit the growth of the pathogenic microorganisms. Antimicrobial tests were carried out by streaking microorganisms isolate onto the paper disk with 20 µL concentration. The standard of microbial turbidity is equivalent to 0.5 McFarland. Pathogenic microorganisms sample was inoculated into NA media, after which paper disk was placed in the middle of the medium and was incubated for 4 x 24 hours at room temperature. The qualitative test result is a positive if transparent zone was formed around the paper disk. Otherwise, the test result is considered negative. Isolates showing positive result were put into quantitative testing. Quantitative test is performed to determine the diameter of inhibition zone. Quantitative test method is the same as the method used in qualitative tests. The formation of the barrier zone diameter was measured for each incubation period of 24 hours, 48 hours, 72 hours, and 96 hours using a caliper.

2.7 Hemolytic Activity of Luminescent Microorganisms Isolated from *H. nehereus*

Blood agar is a differential media enriched with 5% human blood. This medium is used to determine the pathogenic properties of microorganisms. The test was performed by inoculating the luminescent microorganisms isolated from *H. nehereus* into blood agar, and incubated in room temperature for 48 hours. After the observation period expired, observation was made for changes in coloration which indicate the ability of the microorganisms to destroy blood cells. Hemolysis alpha occurs when the agar surrounding the colony changes color into green or brown hue. Beta hemolysis indicated by the appearance of clear colors surrounding the colonies, whereas gamma hemolysis is indicated by no change in agar [13].

3. RESULTS AND DISCUSSION

3.1 Luminescent Microorganisms from *Loligo sp*.

Isolation of symbiont microorganisms of light organs from *Loligo sp*. resulted in 40 microbial isolates. After light emission observation, 2 microbial isolates were found to produce the most luminescence. Isolation results of microorganisms in *Loligo sp* light organ are presented in Fig. 1.

3.2 Luminescent Microorganisms from *Loligo edulis*

Isolation of symbiont microorganisms of light organs from *Loligo edulis* resulted in 1 microbial isolate. Determination of luminescent symbiotic microbial isolates in the light organ of *Loligo edulis* was based on the morphology of the luminescence bacterial colonies grown on Nutrient Agar (NA) media in petri dishes as well as observing the emission of light by bacteria in a dark room. The light produced was bluish in hue (Fig. 2). The resulting isolates were purified to obtain purified isolates of luminescent microorganisms. The microbial colony was milk white in color, with circular shape, convex surface, and even margin.

3.3 Luminescent Microorganisms from the Bombay Duck (*H. nehereus*)

Light emission from microorganisms obtained from the first serial dilution from the Bombay duck (*H. nehereus*) (Fig. 3). The dilution result

was introduced into agar media and the suspension was incubated for 48 hours. Based on the levels of light emitted, four colonies were selected to be purified. The purification resulted in one isolate which retained its capability in producing light (Fig. 4).

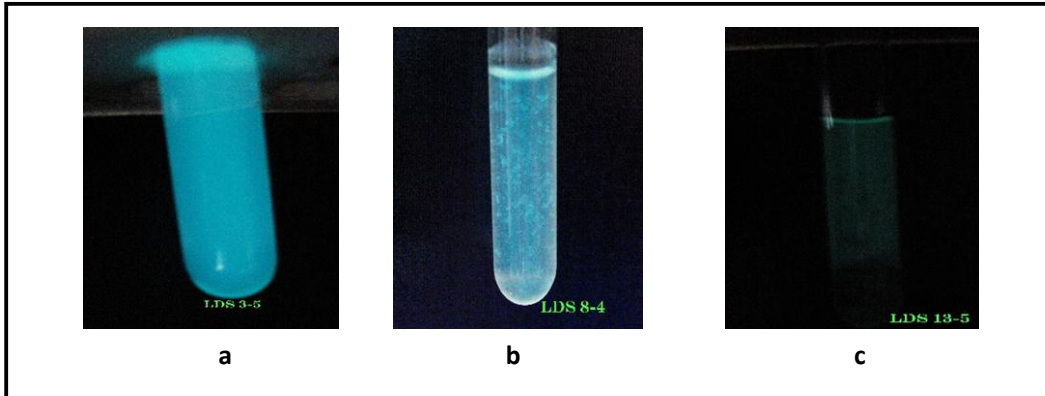


Fig. 1. Levels of light emitted by microbial isolates; (a) high, (b) medium, (c) low



Fig. 2. Light emitted by the microbial isolate on NA media in a dark room



a

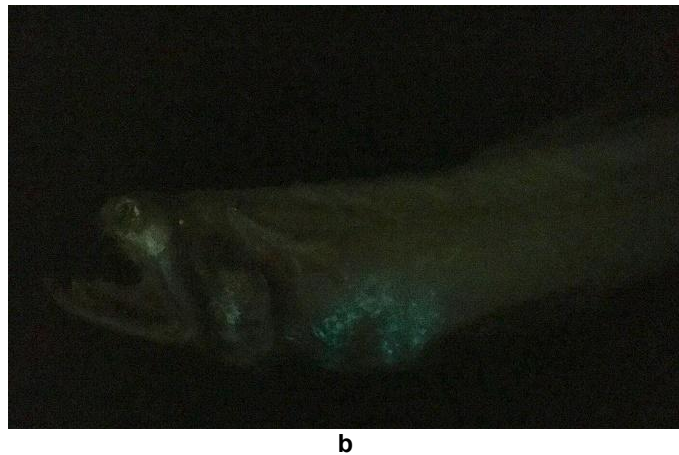


Fig. 3. Host of luminescent microorganisms (a) fresh Bombay duck (b) light-emitting Bombay duck

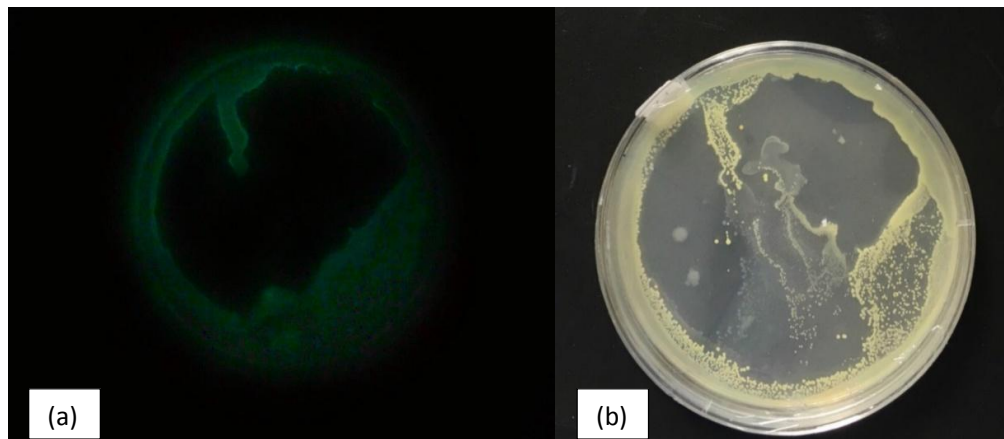


Fig. 4. Microbial isolate obtained from the Bombay duck (a) in a dark room (b) in a room with lighting

The luminescent microorganisms isolated from the Bombay duck was capable of emitting light in petri dish in the same way that it had done in its host (Fig. 4). Bioluminescence from the microbial isolate was obtained after 48 hours of incubation in room temperature. The light emission lasted for a period of 8 hours. The microorganisms was unstable in nature, which means that its bioluminescent property only occurred in the primary isolation. The microorganisms lost its ability to produce light after culture iteration from the isolate which had lost its bioluminescent property.

3.4 Molecular Identification of Luminous Microorganisms from *Loligo sp.*

The results of PCR 16S rDNA DNA amplification from 2 isolates obtained from *Loligo sp.* are

presented in Fig. 5.

The results of sequencing of LDS 12-4 and LDS 18-5 are as follows:

LDS 12-4 (765R)

```
CGACGGTTGGGTTTAAAGACCAAAGAGTCGC
CTTCGCCACTGGTGTTCCCTCCACATCTCTAC
GCATTTACCGCTACACGTGGAATTCCACTC
TCCTCTTCTGTAAGTCAAGTCCAGTTTCCA
ATGGCCCTCCACGGTTAAGCCGTGGGCTTTC
ACATCAGACTTAAAG
```

LDS 18-5 (765R)

```
CCCGTCGTTTCGACAGAGAGTCGCCTTCGCC
ACTGGTGTTCCCTCCACATCTCTACGCATTTCA
CCGCTACACGTGGAATTCCACTCTCTCTTC
```

TGTA CTCAAGTCCTCCAGTTTCCAATAGGCC
 CTCCACGGTTAAGCCGTGGGCTTTACATCA
 GACTTAAAGGACCGCCTGCGCGCGCTTT
 ACGCCGAATAATTCCGGACAACGCTTGCCCC
 CTACGTATTACCGGGGTGCTGGCAGGTAGT
 TAGCCGGGGCTTTCTGGTCAGGTACCGTCAA
 GGTGCCCGCGTATTTCGAACGGCACTTGTTCT
 TCCCTGACAACAAAGTTTTACAATCCAAAAAC
 CTTTCATCACTCAGCGGGCTTGCTCCGTCAA
 ACTTTCGTCCATTGCGAAAATTCCTACTGCT
 GCCTCCGGAAGGAGTCGGGGCCGGGTCTCA
 TTCCCAGGGGGGCCAATCACCGTCTCAGGT
 CGGCTACCCATCGTCCCCTTGTTAA

Homology analysis using BLAST is presented in Fig. 6. And Fig. 7.

Search results of each microbial isolate sequence using BLAST and phylogenetic tree. The phylogenetic tree is used to determine closest relative of the sample microbial isolate with homology. The search results are presented in Table 1.

The results of identification with the BLAST database indicate that LDS 12-4 has the closest homology of 98% with uncultured bacterium clone 1P-1-G05 and LDS 18-5 having the closest homology of 95% with uncultured bacterium clone 3g10a. The two isolates had the highest similarity levels of 98% and 95% respectively with microbial clones. The two clones were the result of a metagenomic study, in which both were derived from a mixture of DNA extracts isolated from nature and had not been isolated as pure extracts.

3.5 Identification of Luminous Microbial Isolate from *Loligo edulis* using Biochemical Assay

Luminescence bacterial isolates of the *L. edulis* (L2 isolate) were then identified based on the methods in [14,15], which involved observations of morphology and biochemical properties of bacteria. The biochemical assay results are presented in Table 2.

The biochemical assay determined that L2 isolate is gram negative, is light emitting in dark spaces, do not grow in TCBS test medium, produces oxidase, does not produce indole, is

oxidative towards carbohydrate (sucrose and lactose), is motile, does not produce H₂S during growth in SIM medium and can ferment 2,3-butanediol. The results of morphological observation on the luminescent microorganisms in *L. edulis* match that of the characteristics of *P. phosphoreum*. Based on Bergey's Manual of Systematic Bacteriology, the following is the taxonomical classification data of *P. phosphoreum*:

Division: Protophyta
 Class: Schizomycetes
 Order: Eubacteriales
 Family: Vibrionaceae
 Genus: Photobacterium
 Species: *P. phosphoreum*

3.6 Molecular Identification of Luminous Microbial Isolate from *H. nehereus*

One microbial isolate was obtained from the Bombay duck, based on its capability to produce light. The genetic information of the luminous microorganisms was successfully amplified using PCR (Fig. 8). The isolate identification was conducted using 16S rDNA gene. The result of identification showed that the luminous microorganisms showed 99% match with *Alteromonas macleodii* strain NBRC 102226. The construction of the phylogenetic tree was then analyzed using BLAST homology (Fig. 9).

Identification of luminescent symbiont microorganisms of marine animals capable of emitting light in Indonesian waters are summarized in Table 3.

3.7 Molecular Identification of Luminous Microbial Isolate from *H. nehereus*

Antimicrobial activity test were conducted on the two isolates against MDR microorganisms *Escherichia coli* and *Enterobacter sp.* 5, *Enterobacter sp.* 10, Coagulase Negative *Staphylococcus* (CNS), *Klebsiella sp.*, and *Pseudomonas sp.* Antimicrobial activity test results are presented in Table 4 and Table 5.

Table 1. Molecular identification of luminescent microorganisms isolates from *Loligo sp.*

No.	Isolate	Identification Result	Homology (%)	Access Ni.
1.	LDS 12-4	Uncultured bacterium clone 1P-1-G05	98	EU704793.1
2.	LDS 18-5	Uncultured bacterium clone 3g10a	95	EU873764.1

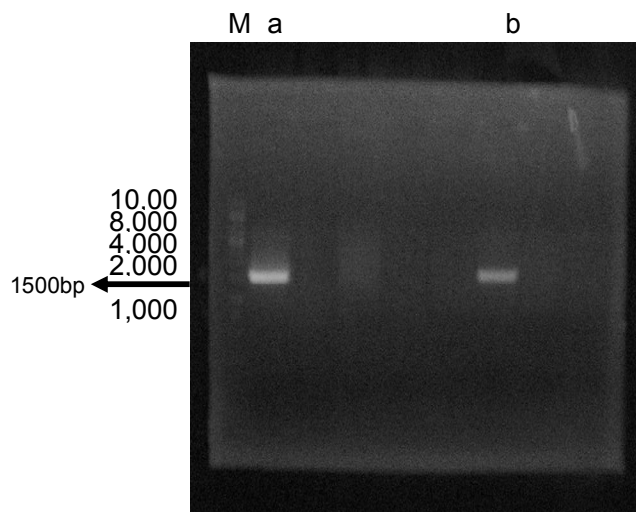


Fig. 5. The result of PCR 16S rDNA DNA amplification of (a) isolat LDS 18-5, (b) LDS 12-4, and M : Marker 1500 bp

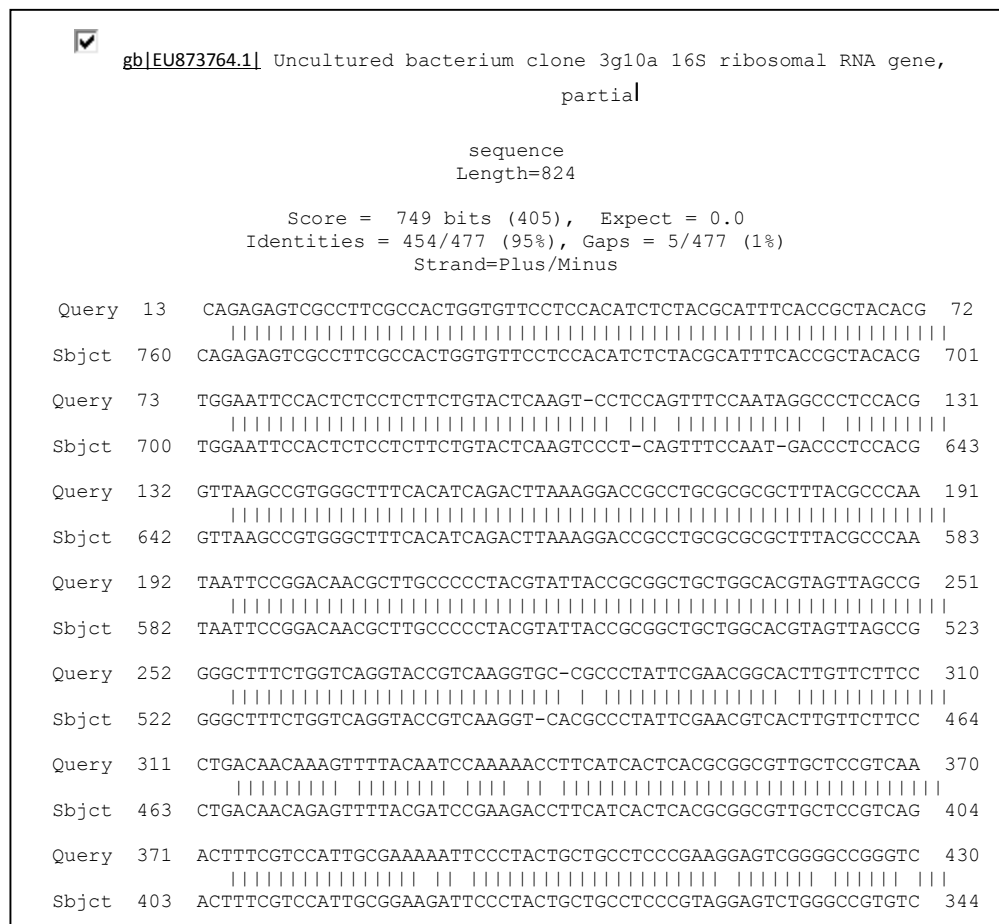


Fig. 6. Homology of isolate LDS 18-5 sequence using BLAST database. Symbol | indicates identical nucleotide

Table 2. Results of biochemical assay of luminescent microorganisms obtained from the light organ of *L. edulis*

Biochemical Tests	Isolate L2
Gram Staining	-
Luminescent	+
TCBS	-
Oxidase	+
Oxidative Fermentative Test	
Sucrose	-
Lactose	-
Motility	+
H ₂ S	-
Voges-Proskauer Test	+

Note: Positive (+): Reaction occurred, Negative (-): No reaction occurred

```

> gb|EU704793.1| Uncultured bacterium clone 1P-1-G05 16S ribosomal RNA gene,
partial
sequence
Length=1257

Score = 278 bits (150), Expect = 7e-72
Identities = 156/159 (98%), Gaps = 0/159 (0%)
Strand=Plus/Minus

Query 13  TTAAGACCAAAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTCACC 72
      ||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
Sbjct 663  TTACAGACCAGAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTCACC 604

Query 73  GCTACACGTGGAATTCCACTCTCCTCTTCTGTACTCAAGTCCTCCAGTTTCCAATGGCCC 132
      ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
Sbjct 603  GCTACACGTGGAATTCCACTCTCCTCTTCTGTACTCAAGTCCTCCAGTTTCCAATGACCC 544

Query 133 TCCACGGTTAAGCCGTGGGCTTTCACATCAGACTTAAAG 171
      ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
  
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Fig. 7. Homology of isolate LDS 12-4 sequence using BLAST database. Symbol |: indicates identical nucleotide

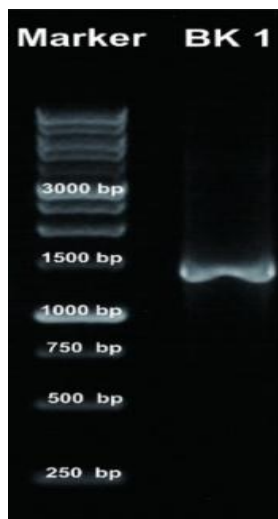


Fig. 8. Visualization of PCR product using electrophoresis with 0.8% agarose

3.8 Antimicrobial Activities of Luminous Microorganisms from *Loligo* sp

The sensitivity test of luminescent microbial isolate on the growth of pathogenic microorganisms showed that L2 bacterial isolate was bioactive or capable in inhibiting the growth of test microorganisms. This research uses *V. harveyi*, *V. alginolyticus*, *E. coli*, *S. aureus* and *Bacillus* sp. as test pathogens.

The qualitative test result of isolate L2 against the test pathogens (*V. harveyi*, *V. alginolyticus*, *E. coli*, *S. aureus* and *Bacillus* sp.) are presented in Table 6.

The qualitative test of L2 isolates resulted in positive (+) against *V. harveyi*, *E. coli*, *S. aureus* and *Bacillus* sp., whereas against *V. alginolyticus* the result was negative (-) (Table 6). This means

that L2 isolates are able to inhibit the growth of *V. harveyi*, *E. coli*, *S. aureus* and *Bacillus* sp. but it is not that of *V. alginolyticus*.

The quantitative sensitivity test result of isolate L2 against the test pathogens (*V. harveyi*, *V. alginolyticus*, *E. coli*, *S. aureus* and *Bacillus* sp.) are presented in Table 7.

3.9 Hemolysis Activity

Hemolysis activity is determined based on testing results in blood agar. Lumious microorganisms isolated from the Bombay duck (*H. nehereus*) showed hemolysis gamma with no change of color surrounding the growing colony on the blood agar. This shows that the microorganisms do not produce toxic substances capable of breaking down blood cells (Fig. 11).

Table 3. Isolated Bioluminescent Microorganisms

Strain code	Source	Sampling location	Species	Method	Luminescence properties
LDS 12-4	Squid (<i>Loligo</i> sp.)	Waters of Semarang	<i>Uncultured bacterium</i> clone 1P-1-G05	Molecular	Positive
LDS 18-5	Squid (<i>Loligo</i> sp.)	Waters of Semarang	<i>Uncultured bacterium</i> clone 3g10a	Molecular	Positive
L2	Squid (<i>Loligo edulis</i>)	Awur Bay, Jepara	<i>Photobacterium phosphoreum</i>	Biochemical (<i>Bergey's Manual of Systematic Bacteriology</i>)	Positive
BK	The Bombay duck (<i>H. nehereus</i>)	Waters of Tanjung Balai Karimun, Riau Islands	<i>Alteromonas macleodii</i>	Molecular	Positive

Table 4. Inhibition Zone of luminous microorganisms from *Loligo* sp. against MDR pathogens

No.	<i>Loligo</i> sp.	Antimicrobial activity against MDR microorganisms (mm)					
		<i>E. coli</i>	<i>Enterobacter</i> sp. 5	<i>Enterobacter</i> sp. 10	CNS	<i>Klebsiella</i> sp.	<i>Pseudomonas</i> sp.
1	LDS 12-4	+	-	-	-	-	-
2	LDS 18-5	-	+	-	-	-	-

Note: +: Inhibition zone formed; -: Inhibition zone not formed

Table 5. Antimicrobial activity test results

MDR Pathogenic Microorganisms	LDS 12-4	LDS 18-5
	Inhibition zone diameter (mm)	
<i>Escherichia coli</i>	32.59	-
<i>Enterobacter</i> sp.5	-	28.44

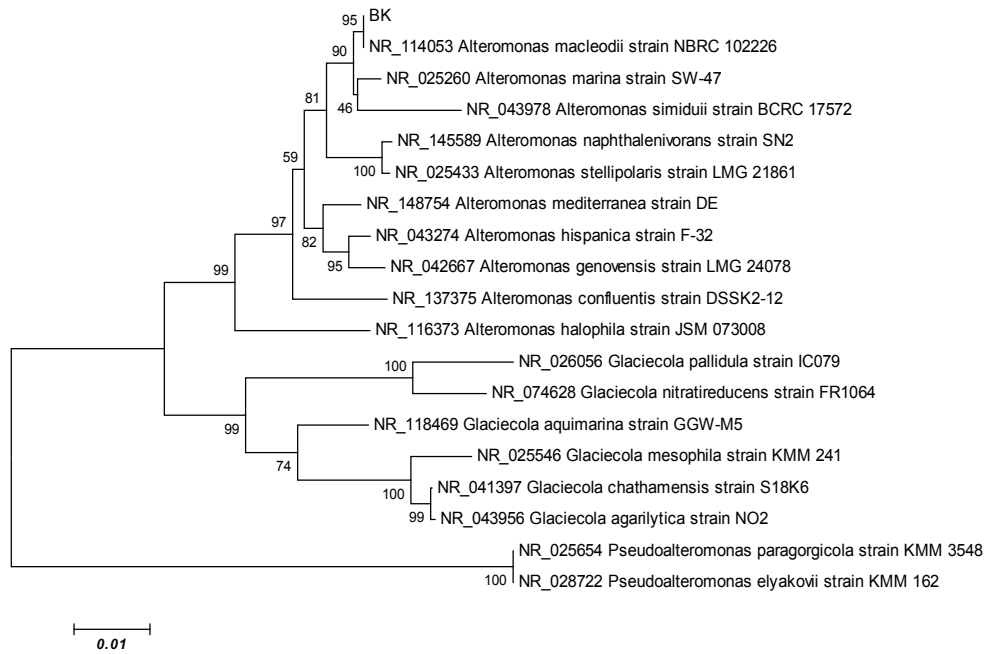


Fig. 9. The phylogenetic tree of the isolated microorganisms

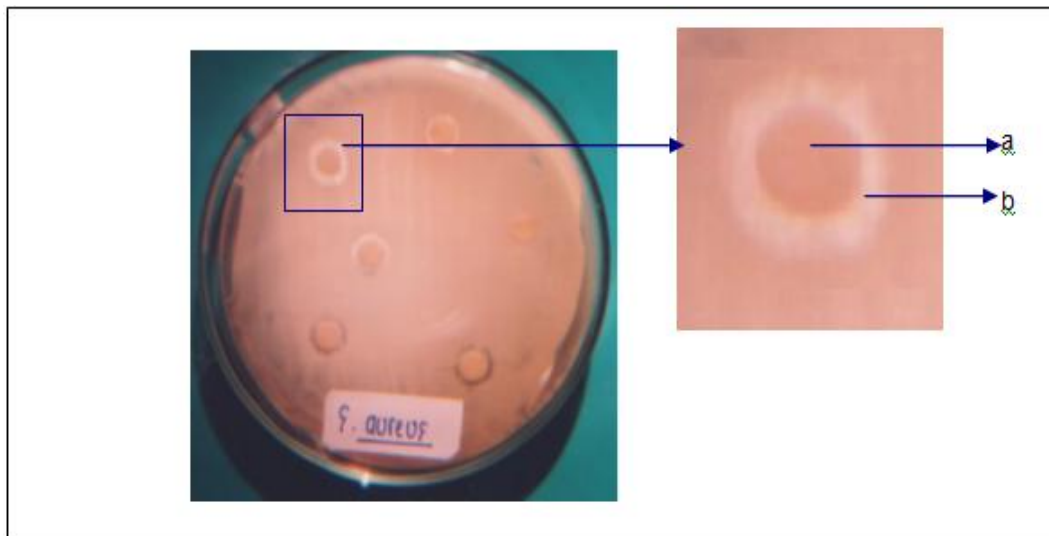


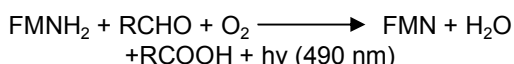
Fig. 10. Formation of inhibition zone diameter

Note: a: paper disk; b: inhibition zone

3.10 Discussion

Bioluminescence in marine animals can be produced through a photophore which inherent special cells called photocytes, or through glands [2], or can be produced by light organs of fish that are symbiotic with luminescent bacteria, Luminescent microorganisms naturally have

genes which allow them to produce luciferase enzymes and long aldehyde chains needed in bioluminescence chemical reactions [4]. Luciferase in an enzyme responsible in catalyzing luciferin substrate, which results in visible light thorough bioluminescence chemical reaction. The chemical reaction of bioluminesce is as follows:



Luminescent bacteria isolated from *Loligo* sp. produce blue colored luminescence in petri dishes, and luminescent bacteria isolated from *Loligo edulis* have strong blue luminescence. On the other hand, luminescent bacteria from *H. nehereus* have weak green luminescence. The blue-green color has a wavelength range of approximately 490-509 nm.

Luciferase isolated from various types of bacteria shows the maximum spectral range of bioluminescence around 490 nm to 509 nm which ranges in the blue-green range [16]. 470 nm, which translates into the color blue, is the range of spectra for species for the genus *Photobacterium* [17]. Blue-green is a color that is often produced from bioluminescence chemical reactions because most marine organisms have the most sensitive visual pigments in the blue-green region [18]. In addition to that, blue is the color with the farthest-reaching wavelength in sea water ($I_{\text{max}} \sim 475 \text{ nm}$) [19]. Green is the next most common color and is more commonly found in benthic and shallow coastal species, perhaps due to an increase in turbidity from particles in the water that spread blue light and support the transmission of longer wavelengths [18].

Two luminescent microbial isolates from *Loligo* sp. were identified as uncultured bacterium clone

1P-1-G05 and uncultured bacterium clone 3g10a, while luminescent microbial isolate from *Loligo edulis* was identified as *Photobacterium phosphoreum*. Luminescent bacteria from *H. nehereus* was identified as *Alteromonas macleodii* strain NBRC 102226. Luminescent bacteria isolated from *Loligo* sp. or *Loligo edulis* showed strong and stable luminescence, meaning they can be recultured. On the other hand luminescent microbial isolates from *H. nehereus* displayed weak and unstable luminescence, meaning that they will lose their luminosity if they are cultured from darkened colonies. Luminescent microorganisms naturally possess a gene called the Lux operon, consisting of luxCDABEG. This Lux operon is responsible for the production of light in bacteria. The complex Lux operon system is a microbial mechanism for quorum sensing [20]. Quorum sensing is a form of communication between microbial cells to form density and in this case produce light. From this very complex bioluminescence system, it is thought that several factors are the reason that several strains of luminescent bacteria carry lux genes when isolated from their host or natural habitat but these microorganisms do not express lux genes, case in point is *Vibrio cholerae* [21], or when isolated from its host the luminescent microorganisms loses its natural phenotype making luminescence only occurs during the first isolation but becomes dark after being re-cultured [22].

Table 6. Qualitative test results of isolate L2 against test pathogens

Isolate Code	Test Microorganisms				
	<i>V. harveyi</i>	<i>V. alginolyticus</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>Bacillus</i> sp.
L2	+	-	+	+	+

Note: +: Inhibition zone formed; -: Inhibition zone not formed

Table 7. Inhibition zone of luminescent microorganisms in *L. edulis* against pathogenic microorganisms

Pathogenic microorganisms	Incubation period (h)	Inhibition zone diameter (in mm)
<i>V. harveyi</i>	24	8.87
	48	8.79
<i>E. coli</i>	24	8.31
	48	8.21
<i>S. aureus</i>	24	9.02
	48	9.07
<i>Bacillus</i> sp	24	9.01
	48	8.83

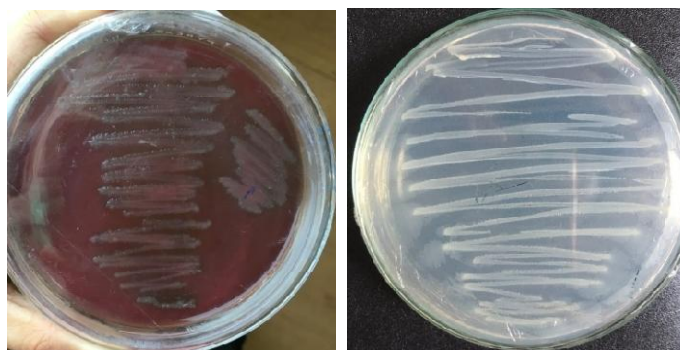


Fig. 11. (a) colony on blood agar (b) colony on Zobell agar

Luminescent microorganisms from the sea, both those living in symbiosis with light organs or living free, which have so far been mostly identified as *Vibrio*, *Photobacterium* and *Alteromonas* (shewanella) [23]. Exploration of luminescent microorganisms from various waters and bioluminescence organisms continues with so many reported new species that add to the list of luminescent microorganisms. Most of these species are identified as the genus *Vibrio* or *Photobacterium*. Four luminescent microorganisms isolated from the coastal region of Chah Bahar port, Sea of Oman, were identified as new strains and were reported to GeneBank. The four species mentioned are *Vibrio sp. Persian 1*, *Vibrio sp. Persian 2*, *Vibrio sp. Persian 3*, and *Vibrio sp. Persian 4* [24]. Isolation of luminescent bacteria from squid taken from Malaysian waters was identified as *Photobacterium leiognathi*, whereas free living luminescent microorganisms from these waters was identified as *Vibrio sp.* [25].

Symbion microorganisms have the potential to produce secondary metabolites to protect host organisms, as a defense against pathogenic microorganisms, and for the purposes of interspecies communication [26]. Some luminescent microorganisms are reported to have antimicrobial properties, although so far luminescent microorganisms have not been explored as new potential for sources of antimicrobial agents for sources of antimicrobial agents for emerging global threats of multi drug resistant microorganisms [27].

Isolate from *Loligo sp.* was identified as Uncultured bacterium clone 1P-1-G05 with antimicrobial potential against *E. coli* MDR pathogenic microorganisms, which was capable of forming 32.59 mm inhibition zone, whereas the luminescent microorganisms identified as

Uncultured bacterium clone 3g10a which displayed antimicrobial properties against *Enterobacter sp.5* MDR pathogenic microorganisms and formed 28.44 mm of inhibition zone. The luminescent microorganisms *Photobacterium phosphoreum* isolated from *L. edulis* showed antibacterial properties against *V. harveyi*, *E. coli*, *S. aureus*, and *Bacillus sp.* Several luminescent microorganisms have also been reported to show antimicrobial properties; [26] reported 7 species of luminous microorganisms (*V. harveyi*, *V. campbellii*, *V. owensii*, *V. rotiferianus*, *V. alginolyticus*, *P. damsela* and *P. leiognathi*) showing antimicrobial activity against one pathogenic bacterium with inhibition zones ranging from 7 to 25 mm. [28] reported that luminescent bacteria were isolated from the mangrove ecosystem of Roach Park, Tuticorin, India, generally from the species *Vibrio sp.* and had antibacterial activity against human pathogenic microorganisms *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis*. In addition, luminescent microorganisms isolated from *Lagocephalus spadiceus* (half-smooth golden puffer fish), *Leiognathus equulus* (ponyfish), *Scomber japonicus* (Pacific chub mackerel), and *Lutjanus argentimaculatus* (red snapper) were identified as *Vibrio sp.* (98%), *Vibrio anguillarum* strain X0906 (96%), *Vibrio sp.* (93%), and uncultured bacterium clone F2G (89%). The luminescent microorganisms also have the ability as antibacterial against MDR microorganisms *S. aureus* and *K. pneumonia* [27].

In general, the open surface area of marine organisms is rich in nutrients, giving rise to interest and competition among microbial communities [29]. This competition champions microorganisms that are able to produce metabolites that are beneficial for both the host and the microorganisms itself. It is known that

luminescent microorganisms are symbiotic, and that they mostly live inside light-producing organs, such as those found in the light organs of *Loligo sp.* and *L. edulis*. The cum-squid light organ is only intended for certain types of luminescent microorganisms and is not a residence for non-luminescent microorganisms [4]. So that luminescent microorganisms in symbiosis with light organs no longer need to compete with other microorganisms that do not have luminous properties. However, this study found that luminescent microorganisms from *Loligo sp.* was able to inhibit the growth of MDR microbial pathogens and luminescent microorganisms *L. written* was able to inhibit the growth of various human pathogenic microorganisms. [26] states that this ability is obtained because of the compounds produced by these microorganisms are beneficial for their survival through intra and interspecies chemical communication.

Microbial isolate from *H. nehereus* shows hemolysis gamma with no discoloration around the growing colony on the blood agar media. This shows that the microorganisms do not produce toxic substances capable of breaking down blood cells. Hemolysis is indicated by discoloration surrounding the colony on blood agar. This test is conducted to categorize bacteria in pathogenic and non-pathogenic groups by looking at the ability of bacteria to break down blood cells. Hemolysis alpha is the ability of bacteria to break down a portion of red blood cells, which occurs when the surrounding colonies turn green or brownish. Beta hemolysis is indicated by the appearance of clear colors surrounding the colonies, whereas gamma hemolysis is indicated by no change in agar [13]. In the future, the isolates of luminescent bacteria can be explored further for the potential they have given that these microorganisms do not produce blood cell breaking toxins.

Bioluminescence microorganisms have yet been explored intensively as new, potential antibiotic sources. So that later research can be further developed for such uses and to discover more properties of the antimicrobial compounds produced by luminescent microorganisms. Luminescent microorganisms have been known to have many benefits in the field of biotechnology, so further research to study bacterial self-defense mechanisms and bacterial evolution so that they can fight various pathogenic microorganisms is viable.

4. CONCLUSION

Microbial isolation from light organ of *Loligo sp.* found two isolates emitting light with bluish hue. Isolate LDS 12-4 was identified as Uncultured bacterium clone 1P-1-G05 with antimicrobial potential against *E. coli* MDR pathogenic microorganisms, which was capable of forming 32.59 mm inhibition zone. Isolate LDS 18-5 was identified as uncultured bacterium clone 3g10a which displayed antimicrobial properties against *Enterobacter sp.5* MDR pathogenic microorganisms and formed 28.44 mm of inhibition zone. The analysis of luminescent microorganisms from *L. edulis* indicated the microorganisms was *P. phosphoreum* of which strong light emitted was bluish in hue. This species also showed strong antimicrobial properties against *V. harveyi*, *E. coli*, *S. aureus*, and *Bacillus sp.* The analysis of luminescent microorganisms from *H. nehereus* the Bombay duck was *A. macleodii* and this species was found to be capable of producing light with bright greenish hue. The microorganisms also displayed gamma hemolysis.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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