Journal of Chemical Health Risks

www.jchr.org

JCHR (2024) 14(2), 3302-3307 | ISSN:2251-6727

Molecular Profiling of Isolated Bioluminescence Strain and its Optimization Studies

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(Received: 07 January 2024 Revised: 12 February 2024 Accepted: 06 March 2024)

KEYWORDS

bioluminescence, fluorescent bacteria, marine bacteria, DNA isolation, heavy metals, antibiotic resistance.

ABSTRACT:

Introduction: Bioluminescence, the emission of visible light by living organisms, has gained attention for its potential applications in environmental monitoring and biotechnology. Potential biotechnological uses for bioluminescent marine bacteria include the identification of hazardous and mutagenic substances in marine environments. For nearly fifty years, luciferase and oxidoreductase-producing fluorescent microorganisms have been used to create diverse preparations for drug biotesting.

Objectives: The study aimed to identify and characterize fluorescent bacteria from squid collected in Tamilnadu, India, for their potential utility in toxicity assessments and pollution detection

Methods: Samples of fresh squid were gathered from the Kavangarai fish market in Tamilnadu. Inoculate the sample in SWC agar plate with a swab, incubate it for 24 hours at 35°C, and look for bright colonies in the dark The morphological investigations were conducted using the gram staining method. the genus level determination via biochemical testing. The Biobee Spin EXpure Microbial DNA isolation kit, created by Bogar Bio Bee Shops Pvt Ltd, was used to isolate DNA from microbial samples. The optimization of pH, Glycerol and Nacl was noted. Various concentrations of heavy metals, including copper sulphate, ferrous sulphate, aluminium hydroxide, and zinc sulphate, were calculated using spectrophotometer. Using a disk diffusion assay, the isolates' susceptibility to antimicrobial drugs was investigated.

Results: DNA isolation revealed the presence of Gram-negative, rod-shaped bacteria closely aligned with *Vibrio harveyi*. The strain exhibited resistance to heavy metals, particularly aluminum sulfate, indicating their tolerance to environmental pollutants. The *V. harveyi* strain exhibited resistance to various antibiotics, while *V. harveyi* showed susceptibility to gentamycin. These findings suggest that these bacteria have developed adaptive mechanisms to withstand environmental stresses and antibiotic treatments

Conclusions: The research highlights the potential of bioluminescent bacteria as bioindicators for marine pollution and provides insights into their resilience to environmental contaminants.

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Introduction

The ocean, which makes up two thirds of the planet's surface, is the largest ecosystem. It is a multifactorial ecosystem that is home to 80% of all living forms on Earth but is still mostly undiscovered (Das *et al*., 2006). The ability of living things to release light at visible wavelengths due to a natural chemical interaction between their molecular biochemical makeup and enzyme activity is known as bioluminescence. The Latin term lumen, which means light, and the Greek word bios, which means "living light," are the sources of the name "bioluminescence."

Both marine and terrestrial bacteria, annelids or segmented worms (such as fire worms), beetles (such as ireflirs, click beetles, railroad worms), algae (such as dinoflagellates), crustaceans (such as sahrimp, ostracod), molluscs (such as squid, clams), coelenterates (such as jellyfish, seapansies, hydroids), bony fish (such as hatchet fish, flashlight fish, pony fish), and cartilaginous fish (such as sharks) are among the species that are fluorescent (Gauri and others, 2016).

Potential biotechnological uses for bioluminescent marine bacteria include the identification of hazardous and mutagenic substances in marine environments. For nearly fifty years, luciferase and oxidoreductase-producing fluorescent microorganisms have been used to create diverse preparations for drug biotesting. Compared to other bioassays based on ciliates, daphnia, algae, and fish, bioassays based on luminous bacteria quantify toxicity and are frequently faster, easier, more accurate, and sensitive. They are used to keep an eye on numerous toxicants. Photobacterium and Vibrio (P. phosphoreum, P. leiognathi, V. Fischeri and V. Harveyi) are two naturally occurring marine genera of bacteria that are frequently employed for this purpose (Roda *et al*., 2004).

The process that produces light emission involves the oxidation of long chain aliphatic aldehyde (RCHO), reduced flavin mononucleotide (FMNH2), and oxygen (O2) by the enzyme luciferase, resulting in the formation of long chain aliphatic acid (RCOOH), oxidized flavin mononucleotide (FMN), and water (H2O). There are three genera that contain all luminous bacteria. Xenorhabdus, Vibrio, and Photobacterium 4. But one more genera had lately emerged.The lux operon gene sequence is present in these bacteria. The lux operon in Vibrio fischeri is made up of the luxCDABE genes, of which luxCDE produces proteins necessary for aldehyde production and luxA and luxB encode bacterial luciferase components (Lior and others, 2018) Bioluminescent microorganisms are employed in environmental toxicity assessments and pollution detection.

Objectives

The goal of the current investigation was to maximize the potential culture and separate the bioluminescent bacteria from the squid sample

Methods

1.1. Study area and Sample Collection

Samples of fresh squid were gathered from the Kavangarai fish market in Tamilnadu. After the sample was obtained, it was quickly placed in a Ziplock bag and safely transported to the lab for additional processing in less than an hour.

1.2. Isolation of bioluminescent bacteria

To remove any debris, sterile distilled water was used to wash the collected sample. After that, inoculate a SWC agar plate with a swab, incubate it for 24 hours at 35°C, and look for bright colonies in the dark. The bright colonies were permitted to be maintained and cleansed. (Muhammad and associates, 2010)

1.3. Screening the intensity of isolates

Squid ink was smeared onto prepared boss material. Keep the plate in the room temperature incubator and record the amount of time the bacteria glow.

1.4. Genus level identification of Bioluminescent bacteria

The morphological investigations were conducted using the gram staining method. the genus level determination via biochemical testing. The media from TCBS, SWC, and the boss were used to identify the cultural traits.

1.5. Molecular Profiling

The Biobee Spin EXpure Microbial DNA isolation kit, created by Bogar Bio Bee Shops Pvt Ltd, was used to isolate DNA from microbial samples. Castresana (2000) Polymerase Chain Reaction (PCR) is a technique that makes use of primers and a very special enzyme to amplify particular genomic or cloned DNA sequences. Using a single-stranded DNA template, the enzyme

Journal of Chemical Health Risks

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JCHR (2024) 14(2), 3302-3307 | ISSN:2251-6727

DNA polymerase controls the synthesis of DNA from deoxynucleotide substrates in PCR (Talavera 2007). When an oligonucleotide is annealed to a longer template DNA, DNA polymerase adds nucleotides to its 3~ end. Therefore, DNA polymerase can employ a synthetic oligonucleotide as a primer and elongate its 3' end to create an extended area of double-stranded DNA if the oligonucleotide is annealed to a single-stranded template that contains a region complementary to the oligonucleotide (Nei M. and Tamura K, 1993)

Different Sequence Using the MEGA X software's Muscle program, the 16S rRNA gene alignment of the bacterial isolate was produced (Kumar S, 2018)

Using universal primers for rRNA below 16 s, single-pass sequencing was carried out on every template. Using an ethanol precipitation procedure, the fluorescently tagged fragments were separated from the unincorporated terminators. The samples underwent electrophoresis on an ABI 3730xl sequencer (Applied Biosystems) after being resuspended in distilled water.

1.6. Optimization of pH, Glycerol and Nacl

The pH was varied to 2, 3, 5, 6, 7, 8, and 9 by employing buffers such as strong hydrochloric acid and sodium hydroxide. After adding a culture to the tubes, they were incubated for a full day. The impact of glycerol was assessed at several concentrations, including 1%, 2%, 3%, 4%, and 5%. Glycerol was not added to one test tube, which served as a blank. The tubes were incubated at room temperature for a whole day. The impact of different concentrations of NaCl, including 1%, 2%, 3%, 4%, and 5%. After that, the tubes received a culture inoculation and were left to incubate for 24 hours at room temperature. Following incubation, each tube's optical density was measured using a colorimeter (Praytino S, B and Latch ford. J.W, 1995).

1.7. Heavy metal tolerance

The boss medium is ready. Various concentrations of heavy metals, including copper sulphate, ferrous sulphate, aluminium hydroxide, and zinc sulphate, were taken. Growth was calculated using optical density (OD), which was obtained at 620 nm using a spectrophotometer.

1.8. Antibiotic sensitivity test

The isolates were put through the antibiotic susceptibility test, which was conducted in compliance with CLSI recommendations. Using a disk diffusion assay, the isolates' susceptibility to antimicrobial drugs was investigated. Every disk was bought from Hi-Media in India. For this test, Mueller-Hinton Agar medium was utilized. We used a disc diffusion approach to measure the antibacterial activity. Mueller-Hinton agar plates were swabbed with 0.5 McFarland standard of the test culture (Bauer *et al*., 1966). utilizing the removed template disks distributed in the Mueller-Hinton Agar that has been solidified using test organisms. Cultures of bacteria were incubated at 37°C for 24 hours. The test was run three times. The Antibiotic Zone Scale (Hi-Media) was utilized to measure the zone of inhibition.

Results

1.9. Isolation of biolumiscence bacteria

The squid ink samples were used for isolation of biolumiscence producing bacteria. Totally 15 isolates were screened for bioluminescence assay. Among this one isolate (S-5) only showed the high light emission.

Table 1: Isolation of bioluminescence bacteria from squid ink

ISOLATES	BIOLUMINESCENCE
S-1	Positive
S-2	Positive
S-3	-
S-4	-
S-5	Positive

1.10. Luminescence Intensity

The luminescence activity screened for different time period. The luminescence intensity results are categorized into Dim luminescence, Dull luminescence, Good luminescence and Luxuriant luminescence. At the end of the results, time interval of 2nd 6 hours at 2 am showing the good luminescence activity.

Table 2: Determination of Luminescence intensity

S.No	TIME INTERVAL	TIME	INTENSITY
1.	1st 6 hours	8 pm	(++)
2.	**2 nd 6 hours**	**2 am**	**(+++)**
3.	After 18 hours	8 am	(+)
4.	At 24 hours	2 am	(-)

(-) Dim luminescence, (+) Dull luminescence, (++) Good luminescence, (+++) Luxuriant luminescence

Journal of Chemical Health Risks
www.jchr.org
JCHR (2024) 14(2), 3302-3307 | ISSN:2251-6727

1.11. Identification of S-5 strain by genus level

The selected S-5 strain showed the gram-negative coccobacilli in gram staining technique. It is motile, Capsulated. Biochemical test was confirmed S-5 strain identified as Photobacterium genus level.

Table 3: Genus level identification of S-5 Strain

S.No	Test	Results
1.	Gram staining	Gram negative, curved rod
2.	Motility	Motile
3.	TCBS	Yellow color colonies
Biochemical reaction		
5.	Indole	Positive
6.	Methyl Red	Positive
7.	Voges Proskauer	Negative
8.	Citrate Utilization	Positive
9.	H2S	Negative
10.	Urease	Negative
11.	Catalase	Positive
12.	Oxidase	Positive

1.12. 16s rRNA Sequencing

The NCBI Nucleotide BLAST analysis shown the isolated bacteria with a code of SCFISH shown 66% of query coverage and 93.01% of Sequence Identity to Vibrio harveyi (MT605241.1). Multiple Sequence Alignment of 16S rRNA Gene of bacterial isolate was generated using Muscle program in MEGA X software. The following parameters used to build the sequence alignment (gapopen:-400.00, gapextend:0.00, maxmb:2048, maxiters:16, cluster1:upgma, cluster2:upgma, maxiters:16, seqtype:DNA).

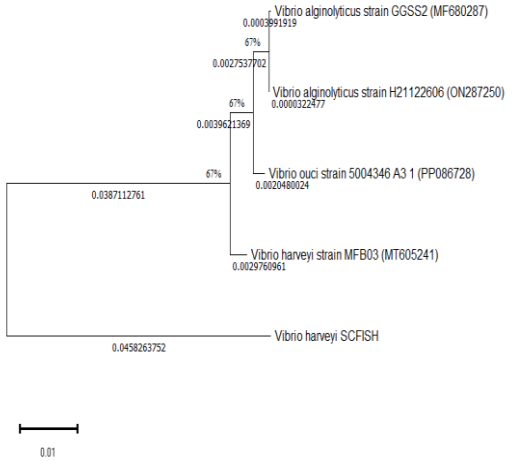

Figure No.1: Phylogenetic Tree was constructed to determine the relationship of SCFISH bacterial isolate (*Vibrio harveyi*).

1.13. Optimization

1.13.1. Optimization of pH

The potential strain S-5 was optimized by different pH, glycerol and NaCl concentrations. The (Figure-2) represents the high luminescent effect in the optimum pH range of 9.

1.13.2. Optimization of glycerol

The figure 3 represents the effect of growth in different glycerol concentration. The increased glycerol concentration upto 5 % was found to induce the growth and luminescence production.

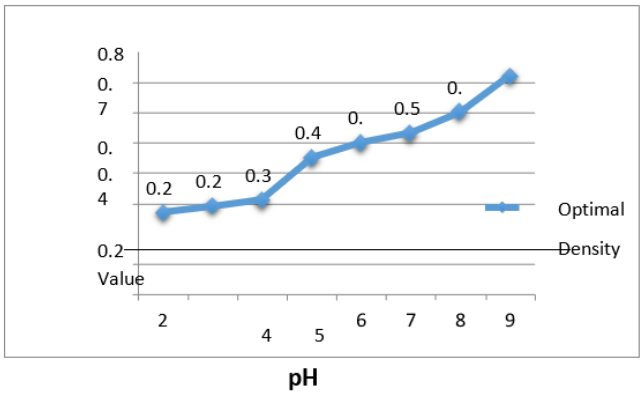

Figure 2: Optimization of pH

Journal of Chemical Health Risks
www.jchr.org
JCHR (2024) 14(2), 3302-3307 | ISSN:2251-6727

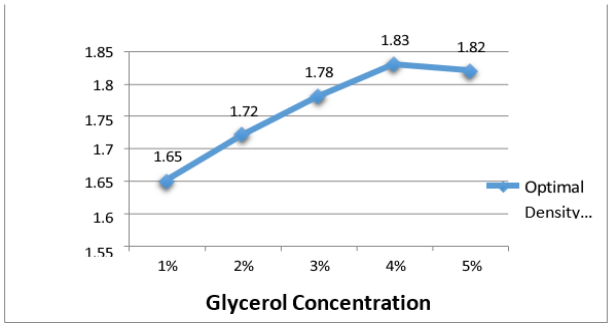

Figure 3: Optimization of glycerol

1.13.3. Optimization of Nacl

The Figure 4 showed the effect of growth in different salt concentration. The increased salt concentration upto 5 % was found to induce the growth and luminescence production. The optical density value determined as 1.79.

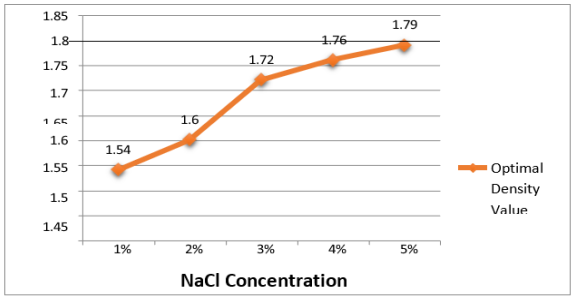

Figure 4: Optimization of Nacl

1.14. Heavy metal tolerance test

Heavy metal tolerance test was screened at different periods of time. The time interval taken for 0.5 minutes to 4 hours. Finally, the optical density value determined. All the heavy metals were showed the resistant at 4 hours. Among these four heavy metals, the ammonium sulphate showing the highest resistant value at 0.21. (Figure 4

Table.4. Screening the heavy metal tolerance

Time (hrs)	0.5	1	2	3
Zinc	0.09	0.1	0.12	0.15
Copper sulphate	0.043	0.053	0.072	0.09
Ferrous sulphate	0.04	0.07	0.09	0.13
Aluminium Sulphate	**0.09**	**0.13**	**0.15**	**0.17**

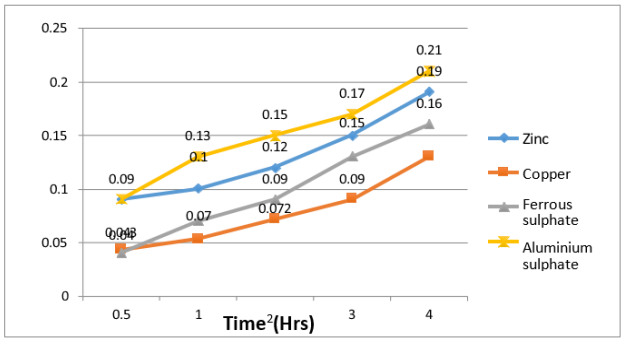

Figure 4: Heavy metal tolerance test

1.15. Screening of antibiotic resistance

The selected S-5 Strain were screened for antibiotic susceptibility test. Totally three antibiotics (Gentamycin, Penicillin and bacitracin). The S-5 strain shown the zone of inhibition (25 mm) against gentamycin and the penicillin and bacitracin antibiotics did not show the zone of inhibition. The AST test concluded the penicillin and gentamycin are resistant.

Table.5. Antibiotic Sensitivity Test

S.NO	ANTIBIOTICS	RESULT
1.	Gentamycin	S(25mm)
2.	Penicillin	R
3.	Bacitracin	R

S- Sensitive R- Resistant

DISCUSSION

Biolumiscence bacteria were isolated from squid samples obtained from the fish market in Kavangarai, Tamilnadu, for the current investigation. Only one S-5 strain had the highest relative luminescence intensity out of the five strains that were identified. Through the use of gram staining, biochemical testing, and cultural traits for genus-level identification, the isolated bioluminescent bacteria were identified. The results of the 16s rRNA sequencing revealed vibrio harveyi. In a Malaysian water sample, Yaser *et al*. (2014) isolated and identified Photobacterium leiognathid. The strong light intensity revealed the possible strain. Utilizing statistical techniques for surface design, the ideal concentrations of yeast extract (5.1 g/l) and NaCl (31.1 g/l) were determined. The chosen strain was optimized by varying the pH, glycerol, and Nacl. The optimization results indicated that the optimum pH of the strain is 9, the

Journal of Chemical Health Risks

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JCHR (2024) 14(2), 3302-3307 | ISSN:2251-6727

optimum glycerol and Nacl concentration is 5%. The optimization studies are crucial to the growth of potential strains and industrial purposes. The agar well diffusion method was used to assess the heavy metal toxicity with eight heavy metals, and both strains were sensitive to As(III), Cd(II), Ce(II), Cr(III), Cu(II), and Hg(II) while demonstrating resistance to Pb(II) and Sr(II). (Parmar, 2020). The present study concluded that the vibrio harveyi strain is highly tolerated the aluminium sulphate metal. Lastly, the antibiotic susceptibility test revealed the resistance.

Conclusion

The rod-shaped, gram-negative bacteria Vibrio harveyi (SCFISH) is a common member of the marine flora and an opportunistic pathogen in fishing hatchery habitats. Using 16S rRNA sequencing, it was located. On the other hand, an aerobe, mesophilic, motile bacteria called Vibrio harveyi (also known as Squid GK) was found. The strain demonstrated resistance to heavy metals and susceptibility to the antibiotic gentamycin.

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