Pakistan J. Zool., vol. 52(6), pp 2327-2331, 2020 DOI: https://dx.doi.org/10.17582/journal.pjz/20170510080532

Characterization of Bioluminescent Bacterial Strain Isolated from *Nemipterus japonicus* Fish of Arabian Sea

Aribah Naz[1]*, Uzma Badar[1], Afsheen Arif[2]*, Zaid Qureshi[1], Sondas Saeed[1], Erum Shoeb[1], Maqsood Ali Ansari[1] and Obaid Yusuf Khan[1]

[1]*Department of Genetics, University of Karachi, Karachi -75270, Pakistan*
[2]*Dr. A.Q khan Institute of Biotechnology and Genetic Engineering. University of Karachi, Karachi -75270, Pakistan.*

Article Information
Received 10 May 2017
Revised 23 May 2018
Accepted 11 October 2019
Available online 16 September 2020

Authors' Contribution
AN did lab work and wrote the manuscript. UB and AA supervised the project. SS and ZQ collected the samples. ES analysed the data. MAA and OYK supported financially.

Key words
Luminescence, Biochemical Analysis, 16S Identification, Gram negative, *Vibrio harveyi*

ABSTRACT

Luminescent bacteria are the most widely distributed organisms among glowing creatures, many of which are found in sea water while some are found in fresh water or terrestrial habitat. Marine micro flora possesses unique features that help them to survive under diverse environmental conditions including high salinity and low pH. Ease of detection is one of the prominent features of these glowing organisms to be the center of attention of many researchers and scientists. The focus of the study is to characterize and identify local Gram negative luminescent bacterial strain isolated from gut of *Nemipterus japonicus* commonly known as Japanese threadfin bream fish. Isolation, temperature optimization for growth and luminescence, growth curve, MTC for heavy metals and antibiotics were determined. Morphological, Biochemical and 16S ribosomal RNA gene sequencing have been done for the identification of the strain. 16S region was amplified by PCR and subjected to DNA sequencing. The sequence data analysis of the isolated strain showed 98% homology with *Vibrio harveyi* species. The sequence obtained has been submitted to NCBI under GenBank Accession# KY653092. Characterization of luminescent bacteria facilitates understanding of the environmental differences that favors the stability, survival and proliferation of species in their respective habitat.

INTRODUCTION

Marine microorganisms have unique characteristics which not only help them to survive but to proliferate in their respective habitat. Bacterial flora found in oceans are mostly Gram negative rods (Kumar, 2010).

Luminous bacteria are abundantly distributed among light-emitting marine organisms. They emit green blue light for defense, mating and communication purpose. These bacteria are usually facultative anaerobes (Nealson and Hastings, 1979). Light output from gram negative bacteria is usually higher because of controlled expression of lux genes is exhibited in Gram negatives (Griffiths, 2000). Luminescence involves the oxidation of $FMNH_2$ with a long chain aldehyde in the presence of enzyme luciferase.

$FMNH_2$ + R-CHO + O_2 FMN + R-COOH + H_2O + Light (~ 490 nm)

The studies have shown that all bacterial luciferases are heterodimers of ~ 80 KDa containing two subunits, alpha subunit of ~ 40 KDa and beta subunit of ~35 KDa. The bacteria use quorum sensing to coordinate their gene expression (Chaphalkar and Salunkhe, 2010).

The goal of this work was to identify and characterize the luminescent isolate.

MATERIALS AND METHOD

Isolation of luminescent strain

Nemipterus Japonicus caught from Arabian Sea was dissected carefully and different parts of fish (gut, mouth, fins, tails) was inoculated in separate autoclaved bottles containing 5ml LA (Bactopeptone 1%, NaCl 3%, and Yeast Extract 0.5%) broth and incubated at 22°C overnight. Loopful of broth from the overnight cultures were streaked on LA agar (Bactopeptone 1%, NaCl 3%, yeast extract 0.5%, and agar 1.5%) plates. After 24h, plates were examined in a dark room for the presence of luminescence. Luminescent bacterial culture (Fig. 1) was restreaked on LA agar plates to obtain isolated colonies. Purified luminescent colony inoculated in 10ml LA broth was used as a starter culture for further studies.

* Corresponding author: aribahnaz@hotmail.com
0030-9923/2020/0006-2327 $ 9.00/0

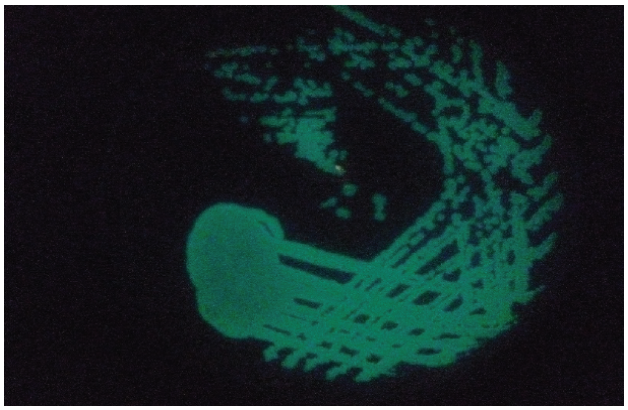

Fig. 1. Glowing culture of *Vibrio harveyi* bacterial strain (DGU300) in LA growth media.

Study of colonial and cellular morphology

In order to study colonial morphology, isolated colonies on LA agar plates were observed and data was recorded for the form, optical feature, elevation and margin of the colonies. The cellular morphology of selected bacterial strain was observed under light microscope using standard procedure of gram staining.

Optimization of temperature for growth and luminescence

To determine optimum temperature for growth and luminescence, 1ml of starter culture was inoculated in 10ml LA broth and loopful from the same was streaked on LA agar plates, followed by incubation under variable temperatures such as 20°C, 25°C, 30°C, 35°C and 40°C. The growth and luminescence were observed with naked eye on LA agar medium while Beckman Coulter UV/VIS spectrophotometer and Modulus luminometer of Turner Biosystem were used to record growth and luminescence of inoculated cultures in broth after 24 and 48h of incubation.

Growth curve

The growth was observed in LA (bactopeptone 1%, NaCl 3%, and yeast extract 0.5%) and boss (bactopeptone 1%, NaCl 3%, glycerol 0.3%, beef extract 3%) medium. The observation was recorded using Beckman Coulter spectrophotometer (UV-VIS) after every 30 minutes (for two days, 12 hours each day) to plot growth curve of local luminescent isolate.

Maximum tolerable concentration of heavy metals and antibiotics

In order to determine maximum tolerable concentration (MTC), 2ml of luminescent bacterial starter culture was inoculated in LA broth supplemented with various concentration of metals and antibiotics. Heavy metals salts such as cadmium ($CdCl_2$), zinc ($ZnSO_4$), nickel ($NiCl_2$), copper ($CuSO_4$) with the concentration of 0.1mM, 0.5mM, 1.0mM, 1.5mM, 2.0mM, 2.5mM and 3.0mM and antibiotics like Ampicillin (Amp), Streptomycin (Stm), Erythromycin (Ery), and Chloramphenicol (Cam) with the concentration of 25µg/ml, 50 µg/ml, 75 µg/ml, 100 µg/ml, 250 µg/ml, 500 µg/ml, 1000 µg/ml were used. After 24 hours, absorbance at 600nm was recorded to determine growth while luminescence was recorded using luminometer.

Biochemical test

Strain was sent to Department of Microbiology, University of Karachi for biochemical identification and was identified using API system of bacterial identification.

16SrRNA PCR identification

In order to identify the luminescent isolate, 16SrRNA analysis was done. Genomic DNA was extracted and its presence was confirmed by agarose gel electrophoresis. polymerase chain reaction (PCR) was performed with 16S rRNA universal primers 8F: 5'AGA GTT TGA TCC TGG CTC AG 3' and 1492R: 5' ACG GCT ACC TTG TTA CGA CTT 3'. The reaction mix was prepared with Biron Taq 2X Master Mix (Cat#101605), ~50ng of DNA and 10 picomole of each primer. Conditions for amplification were as follow: an initial denaturation of 94°C for 10 min., 35 cycles of (95°C for 30 sec., 60°C for 20 sec., and 72°C for 1 min) and final extension at 72°C for 5 minutes. The amplified product was sent to Macrogen, Seoul Korea for sequencing followed by alignment of the sequence with other bacterial species using BLAST tool to identify the bioluminescent isolate.

RESULTS

The bacterial colonies are small, opaque and round, after Gram staining bacteria appears red and comma shaped under electronic microscope (Fig. 2). The Optimum Temperature for the growth is 35-37°C and luminescence is almost independent of temperature (Table I). Absorbance (O.D) were plotted to draw the growth curves for both medium (LA and Boss), readings suggests both mediums are almost equally suitable for the growth (Fig. 3). Metal and antibiotic tolerance were determined qualitatively and quantitatively; strain was resistant against cadmium with persistent growth and luminescence even at the concentration of 2.0mM. Whereas for zinc and copper, culture medium supplemented with concentration greater than 1.0mM does not show growth and luminescence, the strain exhibits high sensitivity against nickel (Table II and Fig. 4).

Table I. Growth and Luminescence at various temperatures.

Temperature °C	24 h		48 h	
	Growth (O.D at 600 nm)	Luminescence (RLU)	Growth (O.D at 600 nm)	Luminescence (RLU)
20	0.586	6739	0.610	7780
25	0.609	7789	0.611	7800
30	0.599	6784	0.604	7763
35	0.611	7800	0.620	7918
40	0.591	6779	0.600	7796

Table II. Growth (O.D at 600nm) and Luminescence (RLU) with variable metal concentrations.

Metal salts	Concentration mM	0.1	0.5	1.0	1.5	2.0	2.5	3.0
Copper	Growth (O.D at 600 nm)	0.357	0.213	0.116	0.001	0.001	0.001	0.001
	Luminescence (RLU)	4198	1358	131	97	76	55	49
Nickel	Growth (O.D at 600 nm)	0.002	0.001	0.001	0.001	0.001	0.001	0.001
	Luminescence (RLU)	107	97	92	87	76	67	57
Zinc	Growth (O.D at 600 nm)	0.388	0.279	0.239	0.001	0.001	0.001	0.001
	Luminescence (RLU)	3871	2593	2189	119	103	97	73
Cadmium	Growth(O.D at 600 nm)	0.398	0.287	0.239	0.201	0.170	0.027	0.001
	Luminescence (RLU)	4871	3993	3589	3198	2373	166	101

Table III. Growth (O.D at 600nm) and Luminescence (RLU) with variable Antibiotic concentrations.

Antibiotics	Concentration (µg/ml)	25	50	75	100	250	500	1000
Ampicillin	Growth (O.D at 600 nm)	0.678	0.711	0.532	0.521	0.497	0.376	0.130
	Luminescence (RLU)	7818	8989	6899	6787	5678	4901	1061
Erythromycin	Growth (O.D at 600 nm)	0.001	0.001	0.001	0.001	0.001	0.001	0.001
	Luminescence (RLU)	111	85	87	87	76	67	57
Streptomycin	Growth (O.D at 600 nm)	0.347	0.234	0.126	0.043	0.026	0.019	0.002
	Luminescence (RLU)	4198	123	111	103	76	55	49
Chloramphenicol	Growth (O.D at 600 nm)	0.369	0.299	0.219	0.221	0.179	0.039	0.001
	Luminescence (RLU)	3871	2193	1089	998	173	66	53

The strain DGU300 is highly resistant to Ampicillin showing growth and luminescence even at the concentration of 1000µg/ml. Significant decrease in growth and absence of luminescence was recorded for a culture media supplemented with 1100µg/ml Ampicillin (Data not shown). Chloramphenicol and Streptomycin are not potential growth inhibitor, but luminescence was not observed at the concentration greater than 100 µg/ml and 25µg/ml respectively. Strain was highly sensitive to Erythromycin (Table III and Fig. 5).

Biochemical tests of strain were performed for preliminary identification, growth on TCBS agar plate and data for other parameters were recorded (Table IV), luminescent strain was found to be non-motile and non-fermenter of lactose, sucrose and salicin but can ferment glucose, fructose and maltose with acids only. 16SrRNA Sequences shows 99 percent similarity with *Vibrio harveyi* and was submitted to GenBank (GenBank Accession no. KY653092).

DISCUSSION

Characterization of glowing bacteria helps in understanding the differences that favors the stability, along with the environmental differences that the organism is subjected to in their respective habitat. Enriched media (LA and BOSS) were used to observe growth

and luminescence as minimal media is not appropriate for optimal growth and luminescence (Ansari, 2012). Composition of LA and BOSS is slightly different, not much difference was observed in term of growth and luminescence suggesting that these both can be used as culture medium for luminescent isolates.

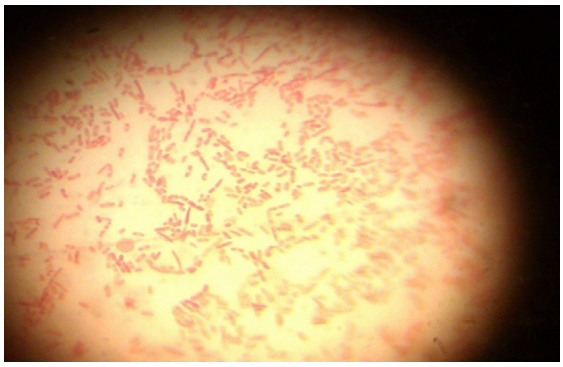

Fig. 2. 100X microscopic image of Gram negative luminescent isolate.

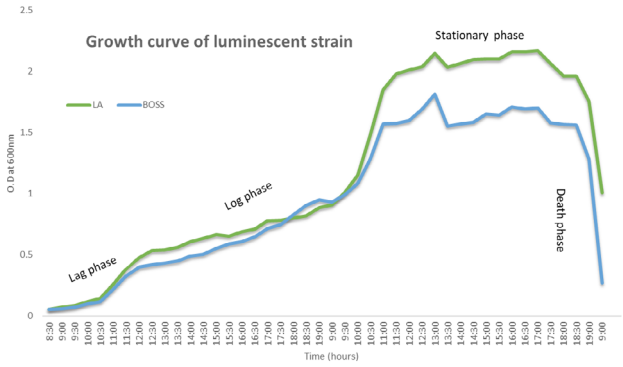

Fig. 3. Growth curve of luminescent isolate with LA and BOSS medium.

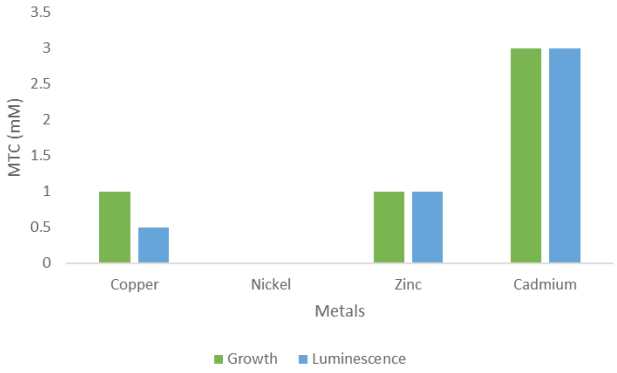

Fig. 4. Maximum tolerable concentration (MTC) for Strain DGU300 with respect to growth and luminescence against different metals.

Table IV. Biochemical tests for identification of luminescent strain isolated from Fish gut.

Tests	Result
Growth on TCBS	Yes
Growth on nutrient agar with 3% NaCl	Yes
Hanging drop (Motility test)	Non motile
Catalase	+
Oxidase	+
Indole	+
MR	+
VP	-
Citrate	+
Urease	+
Glucose	A
Sucrose	NF
Maltose	A
Fructose	A
Salicin	NF
Lactose	NF

A: Fermentation with Acid only; NF: Non fermenter.

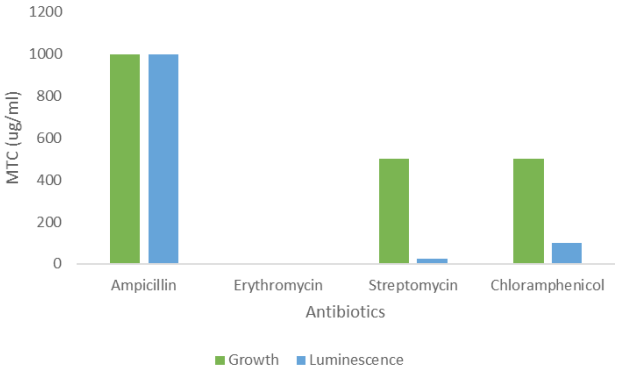

Fig. 5. Maximum tolerable concentration (MTC) for Strain DGU300 w.r.t growth and luminescence against different antibiotics.

As oceans and seas are greatly affected by the oil spills and factory wastes, it is not surprising that microorganism dwelling there must have develop resistance against those metal. Contaminated water with industrial pollutants increases the number of resistant aquatic life forms (An *et al.*, 2010; Zhao *et al.*, 2012). Luminescent bacterial isolate DGU300 isolated from the fish gut is resistant to cadmium (≤2.0mM), zinc (≤1.0mM) and copper (≤0.5mM) and complete sensitivity to nickel, indicating long time exposure to environment contaminated with metals like cadmium, zinc and copper.

Spread of antibiotic resistance is a serious threat to humans, one of the important sources could be microorganisms that are present in water or food products. It is important to study the antibiotic resistance of food borne microorganism. Most of the *vibrio* strains are resistant to Ampicillin and Streptomycin (Kang *et al*., 2014), our strain DGU300 also showed high resistance against Ampicillin (≤1000µg/ml), moderate resistance against Streptomycin (≤25µg/ml) and Chloramphenicol (≤100µg/ml) and complete sensitivity to Erythromycin.

CONCLUSION

The luminescent strain DGU300 isolated from fish gut was Gram negative. Considering growth and luminescence. MTC of the strain for cadmium, zinc and copper is 2.0 mM, 1.0mM, 0.5mM respectively whearas completely sensitive to nickel. MTC for Ampicillin, Chloramphenicol and Streptomycin is 1000µg/ml, 100µg/ml, 25µg/ml, respectively and completely sensitive to Erythromycin. Concentrations greater than MTC may affect growth or luminescence or both. LA and BOSS both are equally suitable medium for growth and luminescence. Biochemical and 16S identification of luminescent isolate confirms the organism is *Vibrio harveyi*.

REFERENCES

An, Q., Wu, Y., Wang, J. and Li, Z., 2010. Assessment of dissolved heavy metal in the Yangtze River estuary and its adjacent sea, China. *Environ. Monit. Assess.,* **164**: 173-187. https://doi.org/10.1007/s10661-009-0883-z

Ansari, M.A., 2012. Screening and characterization of luminescent bacterial strain. *J. Basic appl. Sci.*, **8**: 602-606.

Chaphalkar, A. and Salunkhe, N., 2010. Phylogenetic analysis of nitrogen-fixing and quorum sensing bacteria. *Int. J. Bioinf. Res*., **2**: 17-32. https://doi.org/10.9735/0975-3087.2.2.17-32

Griffiths, M.W., 2000. How novel methods can help discover more information about foodborne pathogens. *Can. J. Infect. Dis. Med. Microbiol*., **11**: 142-153. https://doi.org/10.1155/2000/364050

Kang, C.H., Kim, Y., Oh, S.J., Mok, J.S., Cho, M.H. and So, J.S.,2014. Antibiotic resistance of Vibrio harveyi isolated from seawater in Korea. *Mar. Pollut. Bull.*, **86**: 261-265. https://doi.org/10.1016/j.marpolbul.2014.07.008

Kumar, A., 2010. *Isolation of luminescent bacteria from Bay of Bengal and their molecular characterization*. PhD thesis, Department of Biotechnology, AMET University, Chennai India.

Nawaz, A. and Ahmed, N., 2011. Isolation and characterization of indigenous luminescent marine bacteria from Karachi coast. *Acad. Res. Int*., **1**: 74-83.

Nealson, K.H. and Hastings, J.W., 1979. Bacterial bioluminescence: its control and ecological significance. *Microbiol. Rev*., 43: 496-518. Engineering/Biotechnology, 1st edn. Springer, Berlin, Heidelberg. pp. 3-30.

Zhao, S., Feng, C., Quan, W., Chen, X., Niu, J. and Shen, Z., 2012. Role of living environments in the accumulation characteristics of heavy metals in fishes and crabs in the Yangtze River Estuary, China. *Mar. Pollut. Bull.*, **64**: 1163-1171. https://doi.org/10.1016/j.marpolbul.2012.03.023