International Journal of Frontline Research in
Science and Technology
Journal homepage: https://frontlinejournals.com/ijfrst/
ISSN: 2945-4859 (Online)

(RESEARCH ARTICLE)

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Evaluation of toxicity and resistant effects of heavy metals and antibiotics on the growth of marine bioluminescent bacteria

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International Journal of Frontline Research in Science and Technology, 2022, 01(02), 030–037

Publication history: Received on 07 November 2022; revised on 20 December 2022; accepted on 22 December 2022

Article DOI: https://doi.org/10.56355/ijfrst.2022.1.2.0041

Abstract

Luminescence is the emission of light by an object. Living organisms including certain bacteria are capable of luminescence. Bacteria are the most abundant luminescent organisms in nature. Bacterial luminescence has been studied most extensively in several marine bacteria. Bacterial luminescence is due to the action of the enzyme called luciferase. The luminescent bacteria exist in nature either as free-living bacteria or in symbiotic association with certain marine organisms. Research on luminescent bacteria has always been a fascinating one. In the present study, ten free living luminescent bacteria initially isolated from marine origin were characterized for their tolerance to heavy metals and antibiotics. Copper, zinc, cobalt and cadmium metals at 1 mg/mL concentration have inhibited the growth and luminescence of the all strains except strains 1, 2 and 7. Surprisingly, lead metal at the same concentration dd not inhibit any of the ten strains. However, at 2 mg/mL concentration, similar trend was observed on the growth and luminescence of all the 10 strains. Also, all the tested isolates were sensitive (1 cm >) to all Gram negative and positive antibiotics being tested except isolates 3, 6 and 8, respectively which were resistant (0 – 0.9 cm) to all the antibiotics tested. Thus, the strains isolated from the different sample types have good beneficial potentials such as heavy metal tolerance and antibiotic sensitivity.

Keywords: Antibiotics; Bioluminescence; Heavy metals; Marine; Toxicity evaluation

1 Introduction

Bioluminescence is the emission of light by living organisms and occurs in an array of organisms including fish, insects, jellyfish, and bacteria. The function of bioluminescence may vary from one organism to the other, such as defense against predators, predation or communication with their mates. Bioluminescent bacteria are the most abundant and widely distributed of all light-emitting organisms, occupying a wide variety of ecological niches (fish light organs, mammalian gut, nematode gut) and habitats (marine, freshwater, terrestrial, and symbiotic within a host). While most species of luminescent bacteria are capable of living free, the majority are found in nature associated in symbiosis with host organisms. Currently, only four genera of bacteria are known to naturally bioluminescence: *Vibrio*, *Photobacterium*, *Shewanella*, and *Photorhabdus* [1, 2].

In late 19th century Raphael Dubois experimentally extracted the two key components, enzyme luciferase and luciferine of bioluminescence reaction which are able to generate light [3]. Many bacteria regulate their set of *lux* genes by the mechanism of quorum sensing or autoinduction in which autoinducers (AI) evoke the characteristic response from the cell. In bioluminescence after achieving specific threshold value (conc. of AI 10 cell mL^{-1} in culture media) it triggers the synthesis of enzyme luciferase and other enzymes involved in luminescence. This sensing (AI level) helps to ensure that

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the luminescence production is enough high to cause an impact in environment and making the process cost effective. As the production of the luminous enzyme increases ultimately light generation also increases [4].

Due to the rapid industrial development, various wastes containing different metal ions are directly or indirectly discharged into the environment, bringing about serious environmental pollution and threatening marine life [5]. The toxic effect of heavy metals on aquatic biota is one of the main problems arising from the contamination of natural aquatic ecosystems [6]. Due to the issues raised by animal rights and human rights, the use of microbes like bioluminescent bacteria and algae gaining the attention for a toxicity evaluation as a model organism [4].

Bioluminescent bacterial tests have been successfully used previously in determining the toxicity of aquatic samples, sediments, and soils. Bioluminescent bacteria are easily available, efficient, cost effective, simple and widely used in toxicity assessment. There are few literatures and publications on bioluminescent studies from marine ecosystems in Nigeria with little or no information on the heavy metal toxicities on the growth and physiology of bioluminescent bacteria. This study was undertaken to evaluate the toxicity and resistant effects of heavy metals and antibiotics on the growth of marine bioluminescent bacteria.

2 Material and methods

2.1 Collection of samples

The water and sediment samples were collected from marine body located in Nembe City of Bayelsa State, Nigeria. This water source has history of oil contamination due anthropogenic activities such as transportation of petroleum products through speed boats and other petrochemical activities. The water and sediment samples were collected at three different points using sterile 4 L cylindrical and round bottom plastic containers that were rinsed with the samples thrice before collection [7]. The samples were placed into sterile polythene bags and transported to the laboratory for further processing and analysis.

2.2 Isolation of Marine Luminescent Bacteria

2.2.1 Serial dilution plating technique

The two different sea water and one sediment samples which were collected from three different points were subjected to serial dilution. In this study, 10 mL of sea water sample was mixed with 90 mL of sterile distilled water in a 250 mL flask to obtain 10^{-1}. Then, 1 mL from this dilution was taken and added to another 9 mL of sterile distilled water in test tubes from 10^{-2} and repeated once similarly to get 10^{-3} dilution. Thereafter, 0.1 mL from the10^{-3} dilution was used to spread plate in sea water complex agar (SWCA) medium composed of peptone 5 g, yeast extract 3 g, glycerol 3 mL, agar 15 g and 50 % sea water as well as photobacterium agar in Petri plates. The plates were then incubated for 24 hr and at every six hours the appearance of luminescent colonies was observed and counted. The colony forming unit (CFU) was calculated for each sample [8].

2.2.2 Subculture of selected strains

The distinct isolated luminescent colonies of bacteria were marked while observing for luminescence and were further purified by sub-culturing in SWCA plates. The bacterial strains were stored in sterile sea water in Bjou bottles at 4 °C. Whenever needed, subcultures were made from these stock cultures in SWCA. In all the experiments, 24 hr old bacterial cultures grown in SWCA were used, unless otherwise stated [7].

2.3 Characterization and Identification of Selected Microbial Isolates

The bacterial isolates were characterized microscopically through Gram staining and also biochemically through catalase, indole, motility, Voges – Proskauer, citrate, urease, gelatin liquefaction, nitrate reduction, hydrogen sulphide, sugar fermentation and oxidase tests by adopting the method of Cheesbrough [9]. The isolates were identified using Bergey's Manual for Determinative Bacteriology.

2.4 Preparation of Metal Solution

Two different concentrations namely 1 mg/mL and 2 mg/mL of the respective metal salts (zinc nitrate, cobalt nitrate, copper nitrate, cadmium oxide and nickel nitrate) were prepared in sterile distilled water and made ready for further analysis [8].

2.5 Toxicity Effect of Metal on Growth of Bioluminescent Bacteria

The method of Kumar [8] was adopted for this study. Five metal powders namely zinc, cobalt, copper, cadmium and nickel were used to test their effect on growth of the bioluminescent bacteria. To this, sea water complex agar (SWCA) medium (peptone 5 g, yeast extract 3 g, glycerol 3 mL, Agar 15 g and 50 % sea water) was prepared and poured in Petri plates. Each bacterium which had been grown previously in SWC broth medium and 24 hr old broth culture was used in this experiment. One hundred (100) microliters of each broth culture were swabbed over the smooth surface of SWCA plates and air dried aseptically in a laminar air flow chamber. Then, wells of size 8 mm diameter were made in the seeded agar plates using sterile corn borer. Also, 100 µL of the respective heavy metals as mentioned above were taken and poured in the wells made in bacteria seeded SWCA plates. The plates were incubated for 24 hr and the intensity of luminescence was assessed by visual scoring. Also, the zone of inhibition which is indicative of susceptibility or resistance of luminescent bacteria to the particular heavy metal was recorded.

2.6 Antibiotic Susceptibility Testing

The disc diffusion method of Clinical and Laboratory Standard Institute (CLSI) [10] was adopted for the antibiotic susceptibility test. The turbidity of the inocula of various isolates was made to be equivalent to 0.5 of McFarland standard and each of the isolates was inoculated onto the surface of Muller Hinton agar using sterile swab sticks. The antimicrobial agents tested were: ciproflaxin 10 µg, norfloxacin 10 µg, gentamycin 10 µg, tarivid 10 µg, reflacine 10 µg, ceporex 10 µg, amoxicillin 20 µg, rifampicin 20 µg, ampiclox 20 µg, levofloxacin 20 µg, erythromycin 20 µg, streptomycin 30 µg, chloramphenicol 30 µg, augmentin 30 µg, nalidixic acid, septrin 30 µg, ampicillin 30 µg) (Opton Disc, Nigeria). These discs were aseptically placed on the surface of the inoculated agar plates. After 30 mins of applying the discs, the agar plates were inverted and incubated for 24 hrs at room temperature [11]. The clear zones that developed around each disc were measured as the zones of inhibition on the basis of CLSI guidelines.

2.7 Statistical Analysis

The data obtained in this study were analyzed statistically using GraphPad Prism Software version 8.0. Analysis of variance using two factors was adopted to compare the means of the zones of inhibition of the antibiotics on the growth of the marine bioluminescent bacteria. The values less the threshold value of $P < 0.05$ were considered significant [7].

3 Results and discussion

Table 1 Sea H_2O agar growth distribution

Sample type	Colony forming count		Log CFU/mL
	10^4	10^6	
Marine H_2O A	TLTC	65	1.13
Marine H_2O B	TLTC	60	1.11
Marine sediment	TLTC	TLTC	

Key: TLTC = Too low to count

Table 2 Photobacterium agar growth distribution

Sample type	Colony forming count		Log CFU/mL
	10^4	10^6	
Marine H_2O A	TNTC	TNTC	
Marine H_2O B	TNTC	TNTC	
Marine sediment	TNTC	TNTC	

Key: TNT = Too numerous to count

Table 3 Biochemical tests for the tentative identification of luminescent bacteria

Isolate code /Parameters	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Indole	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Methyl red	-ve	+ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
Voges Proskauer	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve
Oxidase	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Citrate	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve
Nitrate	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Catalase	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Urease	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Gelatinase	+ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	-ve
Motility	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve
Maltose	+ve	+ve	+ve	+ve	+ve	+ ve	+ve	-ve	+ve	+ve
Fructose	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve
Mannitol	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve
Arabinose	-ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	+ve
Xylose	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve
TCBS	**No growth**	**Growth (Green)**	**No growth**	**No growth**	**Growth (Green)**	**Growth (Green)**	**Growth (Green)**	**Growth (Green)**	**Growth (Green)**	**Growth (Green)**
H_2S	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Sucrose	-ve	+ve	-ve	+ve	-ve	+ve	-ve	-ve	-ve	+ve
Glucose	-ve	-ve	+ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve
Galactose	+ ve	+ve	+ve	-ve	-ve	-ve	-ve	+ve	-ve	+ve
Gram reaction/shape	- Rod	- Rod	- Rod	- Rod	- Rod	- Rod	- Rod	- Rod	- Rod	- Rod
Identity	*Phot-obacter - ium*	*Vibrio* sp.	*Phot-obacter - ium*	*Phot-obacter - ium*	*Vibrio* sp.	*Vibrio* sp.	*Vibrio* sp.	*Vibrio* sp.	*Vibrio* sp.	*Vibrio* sp.

Key: + ve = Positive, - ve = Negative

In the present study, marine water and sediment samples were collected from different points of marine water Nembe city sea shore areas. The bioluminescent bacteria distribution after isolation was found to have lower counts in sea water agar (Table 1) but higher counts in Photobacterium agar (Table 2) in both water and sediment samples, respectively. This revealed that the photobacterium agar supports the growth of bioluminescent bacteria more than the sea water agar medium. Also, a total of 10 isolates were confirmed to be luminescent bacteria (Table 3) and they belong to the Vibrionaceae family according to the key for identification in Bergey's Manual for Determinative Bacteriology. Adoki and Odukuma [12] reported that four bioluminescent bacteria (*Vibrio harveyi, V. fisheri, Photobacterium leiognathi* and *P. phosphoreum*) were isolated from the Bonny estuary in the Niger Delta, Nigeria. Kannahi and Sivasankari [13] reported that a total of ten bioluminescent bacterial strains were isolated and identified based on cultural, morphological and biochemical characteristics which belonged to the genera of *Vibrio* sp. and *Pseudomonas* sp. The observations made by these authors are similar to the results obtained in this study.

Table 4 Effect of heavy metals (1 mg/mL) on the luminescence of luminescent bacteria

Isolate code	Lead (cm)	Copper (cm)	Zinc (cm)	Cobalt (cm)	Cadmium (cm)
1	R	R	R	R	S(1.2)
2	R	R	R	R	R
3	R	S(1)	S(1.3)	R	S(1.7)
4	R	R	S(1.4)	S(2.2)	R
5	R	S(2.2)	R	S(1.2)	S(2.2)
6	R	S(2.0)	R	S(1.8)	R
7	R	S(1.2)	R	R	R
8	R	S(2.1)	R	S(1.2)	S(1.4)
9	R	S(2)	R	R	S(1.7)
10	R	S(1.6)	R	S(1.3)	R

Key: R- resistant; S – Susceptible. R = 0.0 – 0.9 cm; S = 1.0 cm >; Values in parentheses are zone of inhibition in mm

Table 5 Effect of heavy metals (2 mg/mL) on the luminescence of luminescent bacteria

Isolate code	Lead (cm)	Copper (cm)	Zinc (cm)	Cobalt (cm)	Cadmium (cm)
1	R	S(1)	R	R	S(1.5)
2	R	R	R	R	R
3	R	S(1.8)	S(2)	R	S(2.5)
4	R	R	S(2.3)	S(2)	R
5	R	S(2.5)	R	S(1.5)	S(2.5)
6	R	S(2.5)	R	S(1.8)	R
7	R	S(1.9)	R	R	R
8	R	S(2.9)	R	S(1.5)	S(2.0)
9	R	S(2.9)	R	R	S(1.7)
10	R	S(2)	R	S(1.5)	R

Key: R- resistant; S – Susceptible. R = 0.0 – 0.9 cm; S = 1.0 cm >; Values in parentheses are zone of inhibition in mm

These 10 bioluminescent bacteria were characterized for their tolerance to heavy metals and antibiotics. Copper, zinc, cobalt and cadmium metals at 1 mg/mL concentration inhibited the growth and luminescence of the all strains except strains 1, 2 and 7. Surprisingly, lead metal at the same concentration dd not inhibit any of the ten strains (Table 4). However, at 2 mg/mL concentration, similar trend was observed on the growth and luminescence of all the 10 strains (Table 5) implying that there were no significant differences on the growth and luminescence of all the strains by increasing metal concentrations. Ranjitha and Karthy [14] examined 57 strains of luminous bacteria for their natural patterns of heavy metal tolerance. The behaviors of these strains were not homogeneous with respect to all metals tested, even within the strains belonging to the same genus. At least 1 to 4 different MICs were detected for every metal except barium and cobalt. Previous study by Kumar [8] revealed similar findings and therefore upholds the observations made in this study.

Agricultural activities and human industrialization are mainly responsible for the release of heavy metals into the environment, especially the air and the water. The first step towards the effective management of water resources is the assessment of pollution levels. Biosensors for the detection of pollutants in the environment can complement analytical methods by distinguishing bioavailable from inert, unavailable forms of contaminants. A bioassay system for

detecting heavy metals in water using bioluminescent bacteria, *Vibrio harveyi* and *Vibrio fischeri* has been developed, which offers the advantages of simplicity and rapidity for screening heavy metals in water sources. Bioluminescence was found to be species specific and strain specific. Mercury, zinc and copper showed definite microbial toxicity and inhibition of bioluminescence. The inhibition range for each strain of a species was standardized and its reproducibility verified. The utility of the biosensors to detect heavy metals in tap water was demonstrated with samples supplemented with Hg (II) **[**15].

Table 6 Antibiotic susceptibility/resistance of luminescent bacterial strains (OPTU DISC GRAM -VE)

Isolate code	CPX (cm)	OFX (cm)	PEF (cm)	AU (cm)	S (cm)	CN (cm)	CEP (cm)	NA (cm)	SXT (cm)	PN (cm)
1	R	R	R	R	R	R	R	R	R	R
2	S(1.6)	R	R	R	R	R	R	R	R	R
3	R	R	R	R	R	R	R	R	R	R
4	R	S(1.6)	R	R	R	R	R	S(1.7)	R	R
5	R	R	R	R	R	R	R	R	R	R
6	R	R	R	R	R	R	R	R	R	R
7	R	R	S(0.5)	R	R	R	R	R	R	R
8	R	R	R	R	R	R	R	R	R	R
9	R	R	S(1.8)	R	R	R	R	R	S(2)	R
10	S(2.4)	R	R	R	R	R	R	R	R	R

Key: R- resistant; S – Susceptible. R = 0.0 – 0.9 cm; S = 1.0 cm >; Values in parentheses are zone of inhibition in mm

Table 7 Antibiotic susceptibility/resistance of luminescent bacterial strains (OPTU DISC GRAM +VE)

Isolate code	CPX (cm)	NB (cm)	CN (cm)	AML (cm)	S (cm)	RD (cm)	E (cm)	CH (cm)	APX (cm)	LEV (cm)
1	R	R	R	R	R	S(1.5)	R	R	R	R
2	R	R	R	R	R	R	R	R	R	R
3	R	R	R	R	R	R	R	R	R	R
4	R	R	R	R	R	R	R	R	R	R
5	S(2.0)	R	R	R	R	R	R	R	R	R
6	R	R	R	R	R	R	R	R	R	R
7	R	R	R	R	R	R	R	R	R	S(1.8)
8	R	R	R	R	R	R	R	R	R	R
9	R	R	R	R	R	R	R	R	R	S(2)
10	R	R	R	R	R	R	R	R	R	R

Key: R- resistant; S – Susceptible. R = 0.0 – 0.9 cm; S = 1.0 cm >; Values in parentheses are zone of inhibition in mm

In this study, all the 10 bioluminescent bacterial strains were also characterized for their antibiotic susceptibility against ten different Gram negative and positive antibiotics discs and the results are displayed on Tables 3 and 4, respectively. In Table 6 (Gram negative disc), isolates 2, 4, 7, 9 and 10 were sensitive to ciprofloxacin at 10 microgram concentration, oflaxacin at 20 microgram concentration, perfloxacin at 20 microgram concentration, nalidixic acid at 30 microgram concentration and septrin at 30 microgram concentration, respectively. All the isolates were resistant to augmentin at 30 microgram concentration, streptomycin at 30 microgram concentration, gentamycin at 10 microgram concentration, ceporex at 10 microgram concentration and ampiclox at 20 microgram concentration, respectively. Similarly, In Table

7 (Gram positive disc), isolates 1, 5, 7 and 9 were sensitive to reflaxine at 10 microgram concentration, ciprofloxacin at 10 microgram concentration and levofloxacin at 20 microgram concentration, respectively. All the isolates were resistant to taravid at 10 microgram concentration, amoxicillin at 20 microgram concentration, streptomycin at 30 microgram concentration, erythromycin at 20 microgram concentration, gentamycin at 10 microgram concentration, chloramphenicol at 30 microgram concentration and ampicillin at 30 microgram concentration, respectively. The results corroborate with the findings of Kumar [8] who reported that the selected six luminescent bacterial strains were also characterized for their antibiotic susceptibility against six different antibiotics. Strain AMET 1901 was found to be resistant to Amikacin at 30 microgram concentration. Strain AMET 1905 was found to be highly resistant to Amikacin at 30 microgram, nalidixic acid at 30 microgram and ciprofloxacin at 5 microgram concentrations. The rest of the strains were susceptible to all the tested six antibiotics at varied degrees. Langaoen et al. [16] reported that the obtained bioluminescent bacterial isolates designated here as strains UPB-01 to UPB-03, were isolated from *C. chanos*, and strains UPB-04 to UPB-07 isolated from *O. niloticus*, were resistant to ampicillin (10 µg). Furthermore, bacterial strains UPB-01, UPB-06, and UPB-07 were found to be multiple-drug resistant, UPB-01 was also resistant to tetracycline (30 µg) while UPB-06 and UPB-07 were also resistant to polymyxin B (300 µg).

4 Conclusion

The ten luminescent bacteria used in this study revealed their heavy metal resistance zones to only lead metal. All the other heavy metals tested showed inhibition to the tested strains. Also, all the tested isolates were sensitive to all Gram negative and positive antibiotics being tested except isolate strains 3, 6 and 8, respectively. Microbial diversity is a major resource for biotechnological products and processes. Thus, the strains isolated from the different sample types have good beneficial potentials such as heavy metal tolerance and antibiotic sensitivity. It is recommended that future study should be carried out to determine the molecular mechanisms behind heavy metals and antibiotics resistances and also further study on pathogenicity profile using quorum sensing testing should be carried out.

Compliance with ethical standards

Acknowledgments

We wish thank the Technical Staff of Microbiology Department, Chukwuemeka Odumegwu Ojukwu University, Uli Campus for the technical and material supports towards the completion of this study.

Disclosure of conflict of interest

The authors declare there is no conflict of interests with the publication of the manuscript.

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