

Promising Biological Indicator of Heavy Metal Pollution: Bioluminescent Bacterial Strains Isolated and Characterized from Marine Niches of Goa, India

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Abstract In present study, several marine water samples collected from the North Goa Beaches, India for isolation of luminescent bacterial species. Isolates obtained labelled as DP1-5 and AB1-6. Molecular characterization including identification of a microbial culture using 16S rRNA gene based molecular technique and phylogenetic analysis confirmed that DP3 & AB1 isolates were *Vibrio harveyi*. All of the isolates demonstrated multiple metal resistances in terms of growth, with altered luminescence with variable metal concentration. Present investigations were an attempt towards exploring and reporting an updated diversity of bioluminescent bacterial species from various sites around the Goa, India which would be explored in future for constructing luminescence based biosensor for efficiently monitoring the level of hazardous metals in the environment.

Keywords Heavy metals pollution · Biosensor · *Vibrio harveyi* · Bioluminescent bacteria · 16S rRNA gene analysis · Bioassay · Monitoring

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Introduction

Luminescent bacteria are the most abundant and widely distributed amongst the light-emitting organisms. The mechanism of the bioluminescence reaction is catalyzed by *luciferase* which produces light by the oxidation of a light-emitting molecule called the *luciferin*. The luciferase enzyme coded by the *luxAB* genes catalyses the unique light-producing bioluminescence reaction. For this light reaction, dissolved oxygen, reduced flavine mononucleotide and a long chain aldehyde coded by *luxCDE* genes are vital substrate requirements [1, 2]. The DNA sequences coding the proteins in the luminescent system are termed as the '*lux* genes'. The 2 polypeptide chains comprising the luciferase enzyme are encoded by *luxAB* genes. The aldehyde required for the reaction is synthesized by an enzymatic complex coded by three different genes present in all bioluminescent bacteria, *luxCDE*. This aldehyde is derived from the fatty acid pathway and can be recycled after each reaction. The three proteins that form the enzymatic complex comprises of a reductase, a synthetase and a transferase. Other proteins involved in the reaction and are only present in certain species of bacteria. For example, *lumazine* and *yellow fluorescent protein (yfp)* are two different proteins present in *Photobacterium phosphoreum* and in some strains of *Vibrio fischeri* respectively. Both are called accessory proteins because they are not crucial for the basic reaction completion [3]. *Photobacterium phosphoreum* is also known to contain *LumP* gene which codes for lumazine protein [4, 5]. The biological significance of luminescence to these bacterial species is still unclear; and therefore bioluminescence continues to be a subject of exploration. Presently most interesting theme of research is the prospective use of bacterial bioluminescence trait for deciphering various basic and applied

problems. Hence understanding the biochemical and molecular basis of bioluminescence as well as effect of pollutants on it is of great importance. In view of the increasing ill-effects of environmental pollution, there is an utmost need for developing reliable tools for rapid assessment of the potential toxic and genotoxic effects of environmental samples. Bioluminescence can be used as a reliable reporter for the assessment or monitoring of various aquatic samples containing toxicants such as pesticides, PCBs, aromatic hydrocarbons, fuels, alkanes, alcohols and heavy metals because of its high degree of response, simple operation and low cost of performance [6–18]. Since marine luminous bacteria are ecologically resourceful, utilize several nutrients, occupy many niches in the marine environment and their bioluminescence being extremely sensitive to the toxicants, they are suitable bioassay candidate for detecting nano or pico molar concentrations of impurities in water bodies, pharmaceuticals and in the food industries [19]. These studies intend to investigate the physiological, biochemical as well as genetic diversity and relationships among bioluminescent bacterial strains isolated from various beaches of North Goa, India. Furthermore, current analysis is targeting to exemplify the potential use of newly isolated strains in construction of *heavy metal bioassay or biosensor* to monitoring the heavy metal contaminants in water samples.

Material and Methods

Water Sampling

Water samples collected from the several beaches of the Goa, India named as Dona Paulo, Miramar, Aguada in January 2013. The samples processed immediately in the laboratory for the bacterial isolation.

Isolation of Bioluminescent Bacteria

Collected samples were then spread plated on modified nutrient agar media (contains 25 % sea salt with 3 % glycerol) [20] and incubated in dark at 21 °C for 24 h as isolates shows enhanced luminescence when grown in dark. The luminescent colonies were picked maintained on modified nutrient agar at an interval of 4 days for continued luminosity & the isolates were stored at –20 °C with addition of 50 % glycerol. Gram and flagella staining were performed as per the standard procedure [21]. The morphology of the bacterial isolates was studied and confirmed using scanning electron microscopy. Motility was observed by using ‘hanging drop preparation method’ as per the standard protocol [22].

Biochemical Characterisation

In order to characterize the isolates for biochemical parameters a total of 14 biochemical assays [23] such as: Oxidase, Nitrate reduction, Gelatinase, Simmons citrate, Indole, Methyl Red, Voges-proskauer, Catalase, Urease, Cytochrome oxidase, Amylase, Gelatin liquefaction and Hydrogen sulphide production test were performed. For carbohydrate utilization test various sugars were used-glucose, lactose, mannitol, sucrose, maltose and pyruvate. All the controls (positive and negative) and the blank used for different biochemical test were incubated at respective optimum growth temperature (37 °C).

Bacterial Identification

In order to confirm the identity of the isolates genomic DNA was extracted using an Axyprep Bacterial Genomic DNA Miniprep Kit (Axygen). Further, fragment of 16S rRNA gene was amplified by Polymerase Chain Reaction (PCR) with the aid of universal primers for bacteria (27F, 5-AGAGTTTGTGATCMTGGCTCAG and 1492R, 5-TACG-GYTACCTTGTTACGACT-3). Amplification parameters for bacterial 16S rRNA gene were as follow: an initial denaturation of 94 °C for 3 min., 30 cycles of (94 °C 30 s, 52.7 °C 30 s, and 72 °C 1.30 min). DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The 16S rRNA gene sequence was used to carry out BLAST with the nrdatabase of NCBI genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 4 [24].

Antibiotic and Heavy Metal Sensitivity Profiling

4–5 colonies of isolated strains were inoculated with a loop to 5 ml Nutrient Broth aided with 50 % (v/v) sea salt & incubated at 21 °C for overnight to achieve light to moderate turbidity. By using standardized inoculum the entire agar surface of the plate was streaked with the swab & dried for 30 min. To study the antibiotic sensitivity profiling the Himedia made Icosa G-I-Minus antibiotic susceptibility testing discs was applied using aseptic technique and incubated at 21 °C for 16–18 h. The diameters of zones (millimetre) indicating growth inhibition was record using a calibrated zone scales. Heavy metal sensitivity profile was developed by standard well diffusion assay. In each of the inoculated plates well was created by using 6 mm cork borer and 100 µl of various

concentrations of heavy metals ranging between 0.05 and 0.50 mg/L was added. The diameters of zones (millimetre) showing growth inhibition was record using a calibrated zone scales.

Qualitative Estimation of Luminescence

Bacterial culture streaked on modified nutrient agar plates was partitioned in four compartments streaked & incubated at 21 °C. Partitioned culture plates were sprayed with three heavy metal solutions of 0.05, 0.25, 0.50 mg/L and incubated for 30 min at 21 °C. Measurement of qualitative (Visual) alterations in luminescence due to presence of respective heavy metal concentrations was made by observing these plates in completely dark room.

Results and Discussion

Isolation of Bioluminescent Bacteria

These bioluminescent isolates differ both in light emitting intensities and morphologies. Fine isolated bluish-green glowing colonies of the bacterial isolates were obtained.

Morphological Characterization

The characterisation with regard to size, shape, arrangement of cells and motile features, colonial characteristics viz., form, elevation, margin, colour, surface, density, consistency and presence of flagella have been proved to be fruitful in better understanding of the morphology of bacterial isolates using different staining procedures. The presence of flagella was also confirmed by the flaring outgrowth of the inoculated bacterial culture around the line of stab in SIM (Sulfite Indole Motility) medium. The SEM analysis provided an additional insight to the external morphology and appearance of the bacterial cells (Fig. 1a, b).

Biochemical Characterization

In the biochemical characterization samples differ in their ability to metabolize carbohydrates such as Maltose could be utilized by both of the isolates while Lactose is not utilized by any of the bioluminescent isolates. *Vibrio harveyi* strain *DP1* was unable to utilize citrate, while both isolates were found to be Indole positive. Enzymes such as catalase, urease, amylase were found to be absent for AB isolate while in contrast this gelatinase was actively present for *Vibrio harveyi* strain *ABI* isolate (Table. I in ESM). All strains gave a positive reaction to the oxidase test and dependent on NaCl for growth as well as luminescence production.

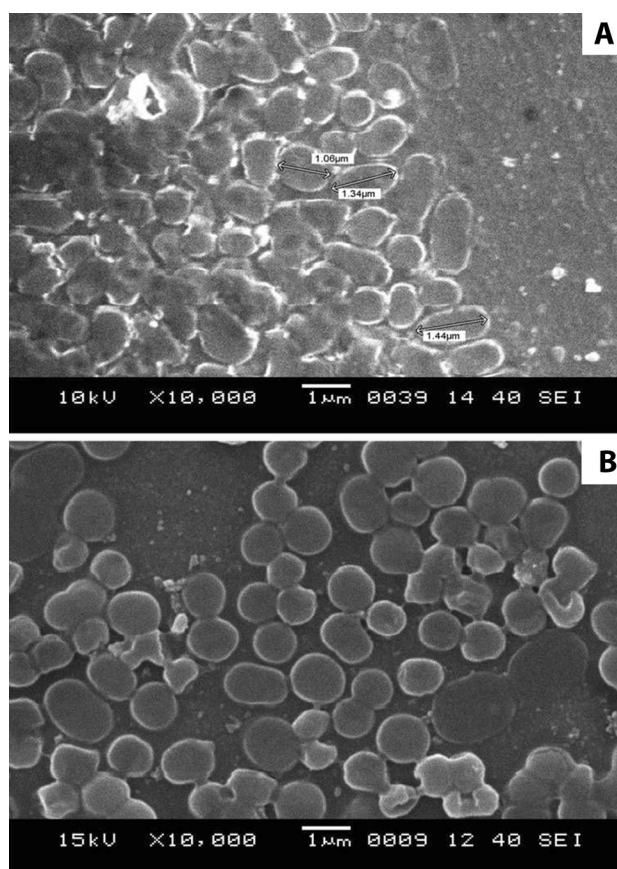


Fig. 1 SEM photograph of the bacterium isolated from, **a** Aguadha Beach Sample; **b** Dona Paulo Beach Sample. Sample **a** exhibit a basic morphology of elongated bacilli with 1–1.44 μm size, in contrast samples **b** shows a shorter bacilli morphology of 1 μm size

16S rRNA and Phylogenetic Analysis

The total genomic DNA was isolated by Axyprep Bacterial Genomic DNA miniprep kit (Genaxy). Fragment of 16S rRNA gene was amplified by PCR with 8F and 1492R primers from the above isolated DNA samples. A single discrete PCR amplicon band of 1438 and 1500 bp was resolved on Agarose Gel for AB & DP isolate respectively.

The PCR amplicon was purified to remove contaminants by QIAquick Gel Extraction Kit (QIAGEN) and respective consensus sequences of 1438 bp and 1500 bp 16S rDNA genes was generated by BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Based on BLAST and MEGA 4 the isolates regonised, AB as *Vibrio harveyi* [KF607036] and DP as *Vibrio harveyi* [KF607037] (Figs. 2, 3).

Antimicrobial Susceptibility of the Bacterial Isolate

Antibiotics susceptibility tests for each isolates were performed using disc diffusion method. Antibiotic impregnated

Fig. 2 Phylogenetic analysis of 16S rDNA gene amplified from Aguada Beach (AB) isolates and identified as *Vibrio harveyi* strain NB0901 with close homologs

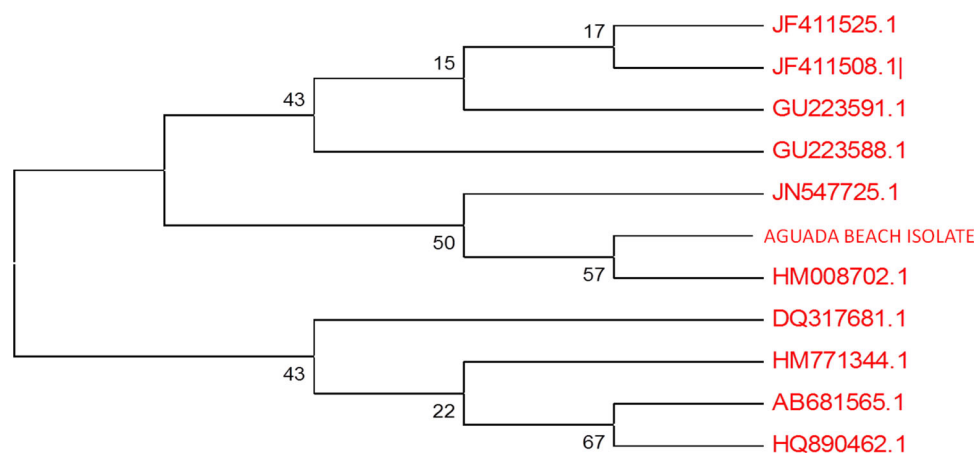


Fig. 3 Phylogenetic analysis of 16S rDNA gene amplified from Dona Paulo Beach (DP) isolates and identified as *Vibrio harveyi* strain NB0901 with close homologs

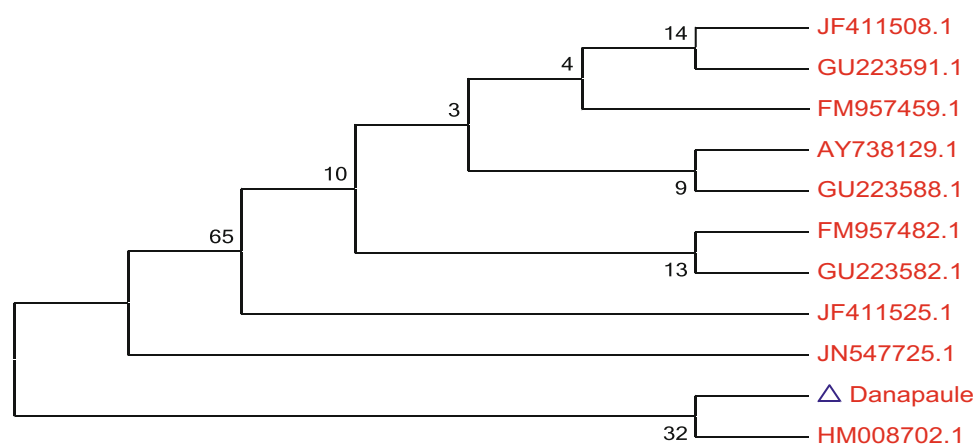
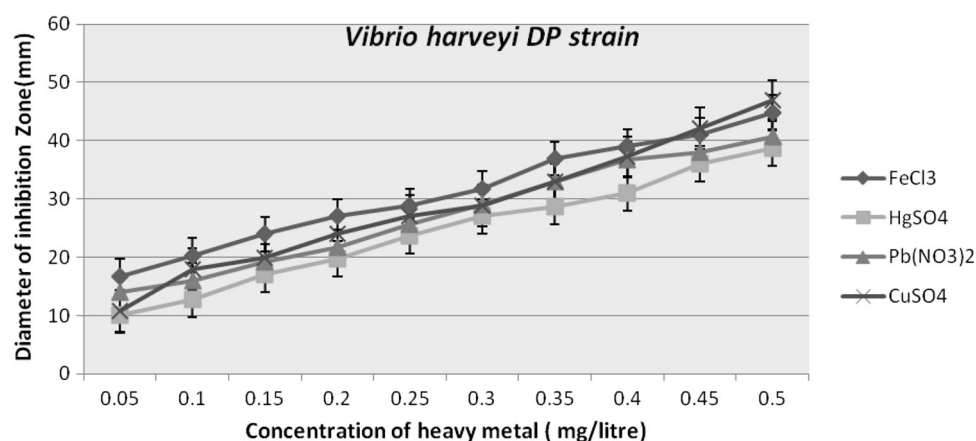


Fig. 4 Graph indicating heavy metal sensitivity profile of isolated *Vibrio harveyi* DP strain against various concentrations of heavy metals (0.05–0.50 mg/l)



paper discs (Himedia made Icosa G-I-Minus) were applied onto the agar surface, incubated overnight at 21 °C and then diameter of zone of inhibition (mm) around antibiotic discs to the nearest whole millimeter was recorded. Both bacterial isolate was observed to be susceptible to Kanamycin (30 µg), Gentamicin (10 µg) and Streptomycin (10 µg). For

Sparfloxacin (5 µg) and Ciprofloxacin (5 µg) DP isolate showed resistance while AB isolate was found to be sensitive and on other hand DP isolate indicated sensitivity towards Amikacin (30 µg) for which *Vibrio harveyi* strain AB1 isolate shown intermediate susceptibility. Few antibiotics such as Augmentin (30 µg), Cefpodoxime (10 µg) & Ticarcillin

Fig. 5 Graph indicating heavy metal sensitivity profile of isolated *Vibrio harveyi* AB strain against various concentrations of heavy metals (0.05–0.50 mg/l)

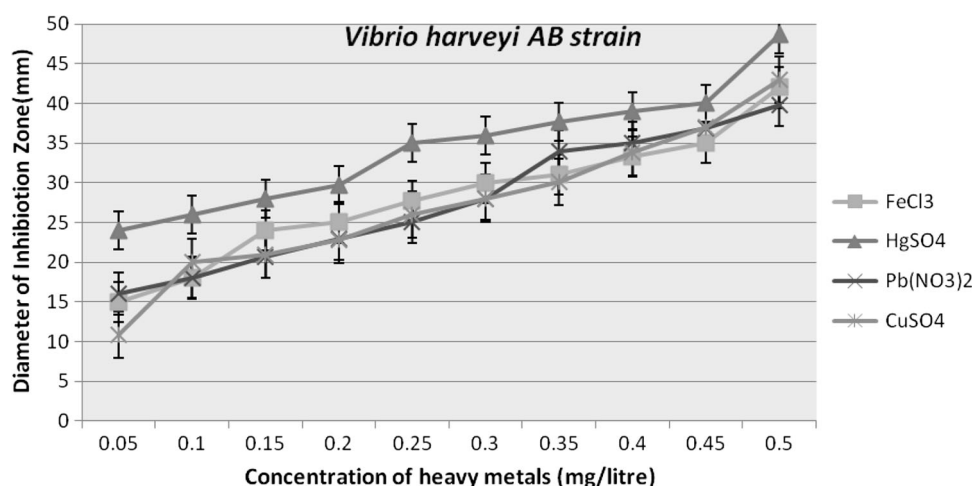
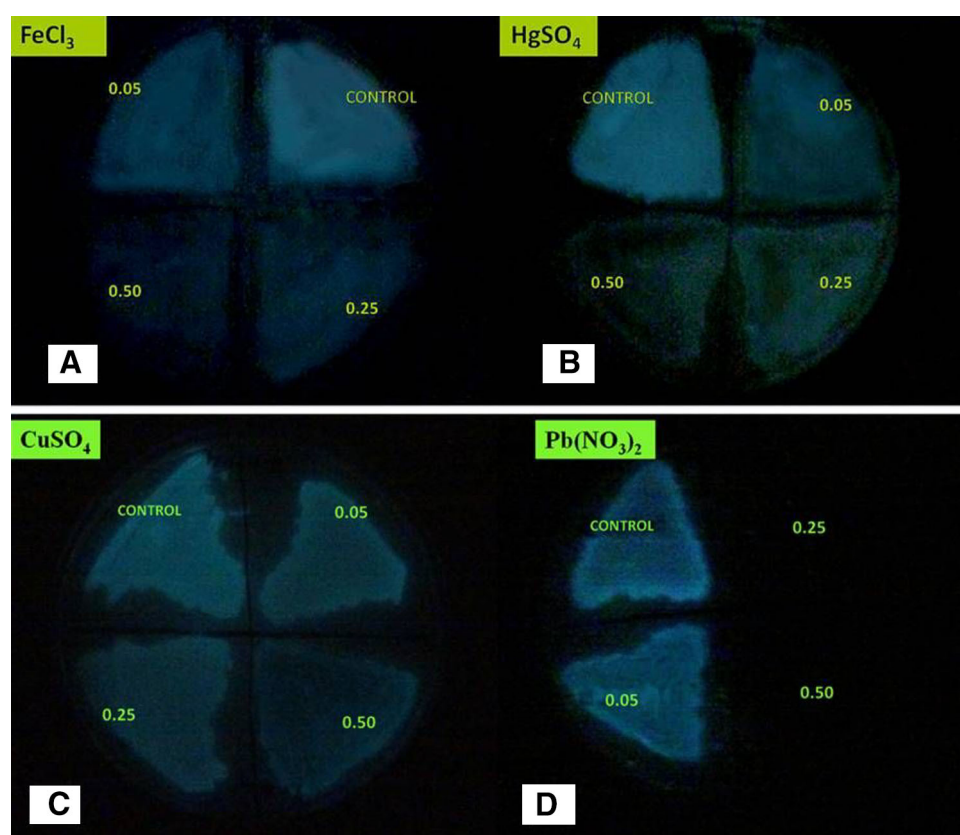


Fig. 6 a–d Photographic evidences indicating effect of increasing toxic concentrations (0.05, 0.25, 0.50 mg/L) of various heavy metals on test organism-*Vibrio harveyi* AB strain



(75 µg) showed no zone formation for both of the isolates (Table-II in ESM).

Effect of Different Heavy Metals on Bacterial Isolate

The effect of various concentrations of compounds containing heavy metals as Pb(NO₃)₂, CuSO₄, FeCl₃ and HgSO₄ was also seen on the growth and luminescence of *V. harveyi*. The incubated plates were observed for the diameter of the zone of growth and luminescence inhibition

around the heavy metal, in terms of millimetre (mm) (Table-III in ESM; Figs. 4, 5).

Visual Evidences for Luminescence Alteration due to Heavy Metal Presence

Both the strains were examined for luminescence alteration against various heavy metal concentrations and based on results *Vibrio harveyi* AB strain was considered for further

analysis. The change in the luminescence intensity of *Vibrio harveyi* AB strain were spray analyzed for the 0.05, 0.25 and 0.50 mg/litre of FeCl₃, HgSO₄, CuSO₄, Pb(NO₃)₂ (Fig. 6a–d). The results suggested that define change in the luminescence was recorded in Pb (NO₃)₂ at the 0.25 onwards with complete loss of luminescence. While at 0.05 mg/litre the luminescence remain unchanged compared to control. For FeCl₃ and HgSO₄ similar trend was apparent where luminescence decreased with increased metal concentration as compared to control. In contrast CuSO₄ treated sample showed very slight change in luminescence. Luminescent biosensor strain explored during present investigation is highly responsive to wide range of concentrations of FeCl₃, HgSO₄, CuSO₄ ions, except for Pb(NO₃)₂ and can be used to quantify their bioavailability in polluted water samples. Few recent studies reported recombinant GFP based bacterial biosensor responded to Pb²⁺ in the range of 50–400 µM [25]. Collectively, previous and present studies indicates the need for development of lead specific luminescence based recombinant bacterial strain which could provide a wide range for lead metal sensing in terms of luminescence changes.

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Conflict of interest All authors have none to declare.

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