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Comprehensive depiction of novel heavy metal tolerant and EPS producing bioluminescent *Vibrio alginolyticus* PBR1 and *V. rotiferianus* PBL1 confined from marine organisms

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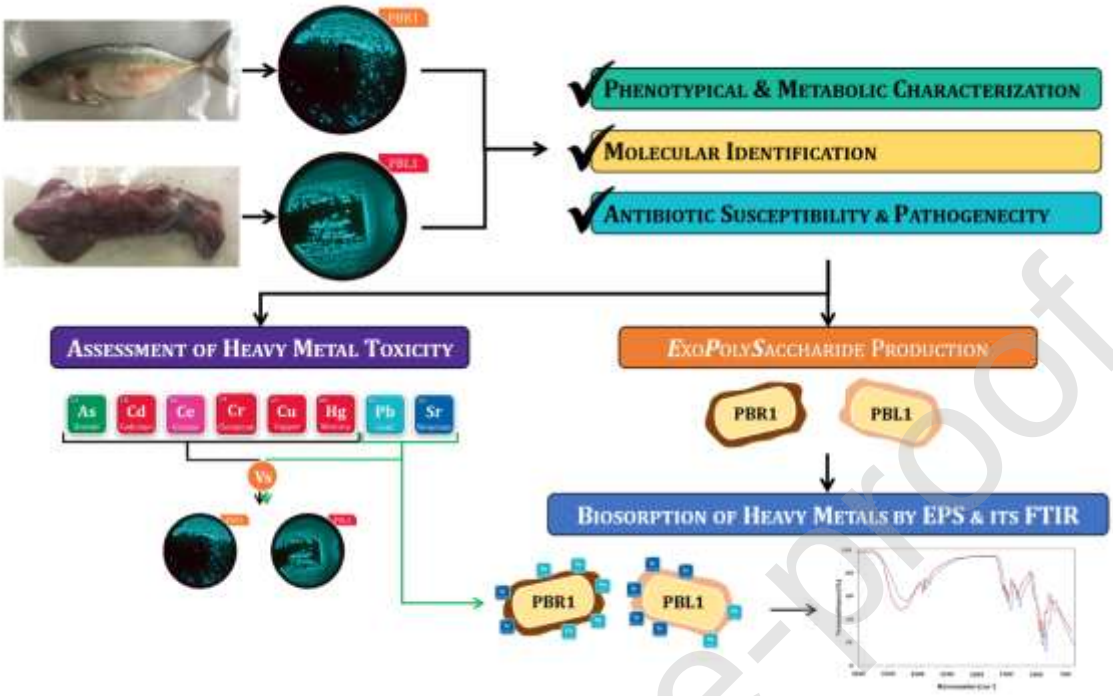
Graphical abstract



Highlights

- We isolated bioluminescent *Vibrio* spp. from marine organisms.
- Characterization of metal tolerance of the isolated strains.
- Assessing the impact of heavy metals on bioluminescence.
- Proposing use of these strains as biosensors to detect certain heavy metals.
- Assessing metal interaction with EPSs from these strains.

Abstract

The current study depicts the isolation of luminescent bacteria from fish and squid samples that were collected from Veraval fish harbour. From Indian mackerel, total 14 and from squid, total 23 bioluminescent bacteria were isolated using luminescence agar medium. Two bioluminescent bacteria with highest relative luminescent intensity PBR1 and PBL1 were selected. These two isolates were subjected to detailed biochemical characterization and were tested positive for 5 out of 13 biochemical tests. Furthermore, both PBR1 and PBL1 were able to ferment cellobiose, dextrose, fructose, galactose, maltose, mannose, sucrose and trehalose with acid production. Based on 16S rRNA partial gene sequence analysis, PBR1 was identified as *Vibrio alginolyticus* and PBL1 as *V. rotiferianus*. Antibiotic susceptibility test using paper-disc method showed that PBR1 and PBL1 were sensitive to chloramphenicol, ciprofloxacin, co-trimoxazole, gatifloxacin, levofloxacin, linezolid ad roxithromycin out of 18 antibiotics tested. Moreover, both strains were evaluated for their exopolysachharide (EPS) producing ability where PBR1 and PBL1 were able to yield 1.34 g% (w/v) and 2.45 g% (w/v) EPS respectively from 5 g% (v/v) sucrose concentration. Heavy metal toxicity assessment was carried out using agar well diffusion method with eight heavy metals and both the strains were sensitive to As(III), Cd(II), Ce(II), Cr(III), Cu(II), Hg(II) and while they showed resistance to Pb(II) and Sr(II). Based on these results, a study was conducted to demonstrate bio-removal of Pb and Sr by EPS of PBR1 and PBL1. Fourier transform infrared (FTIR) spectra revealed the functional groups of EPS involved in interaction with the heavy metals. Owing to the sensitivity for the remaining heavy metals, these bioluminescent bacteria can be used further for the development of luminescence-based biosensor.

Keywords: bioluminescence; *Vibrio* spp.; heavy metals; exopolysaccharides (EPS); biosensor; biosorption**.**

1. Introduction

Marine ecosystem naturally possess heavy metals in microquantities where few of these are essential owing to their biochemical and physiological role in living forms (World Health Organization, 1996; Ansari et al., 2004; Mertz, 2012; Azaman et al., 2015). However, the amount of heavy metals is increasing in marine ecosystems due to indiscriminate anthropogenic activities such as industrialization and technological advancements (Stern, 2010; Rajeshkumar et al., 2018). The progressive accumulation of heavy metals in ecosystems is highly toxic for the aquatic life and thus, create a serious global health concern due to their ecotoxic, non-degradable and persistent nature (Tak et al., 2013; Gaur et al., 2014; Dixit et al., 2015). Industrial discharges consisting toxic substances and heavy metals aid in the pollution of aquatic ecosystems which causes cytotoxic, carcinogenic and mutagenic effects in animals. The coastal line of Gujarat, a part of Arabian sea and the major harbour in western India is also affected by such anthropogenic activities (Mitra & Choudhury, 1993; Gbem et al., 2001; Woodling et al., 2001). Marine pollution with such heavy metals is gradually becoming a prominent problem which is impacting its habitant life forms as they are taken up by tissues of marine organisms (Intamat et al., 2016). Bioaccumulation of these metals in fishes and squids has also affected the adaptation of endogenous microbes that thrive in their tissues (Pettibone et al., 1996; Toroglu et al., 2009; Kumar et al., 2011; Amutha & Shamini, 2015). This manuscript is emphasizing the characterization of such microbes that have adapted to thrive in the tissues of such marine creatures dwelling in the marine environment contaminated with heavy metals. Human consumption of such marine life forms as food leads to health hazards by biomagnification process (Ali & Khan, 2019).

The state of Gujarat owns the longest coastline (1600 km) in India. It ranks first with respect to sea food production in the country (CMFRI, 2015). Veraval fish harbour, one of the largest fishing harbours in Asia, lies on southwest coast of Saurashtra-Gujarat coast. Veraval harbour sediments are known to possess high concentrations of heavy metals, as follows (µg/g): cadmium (Cd) - 0.628, cobalt (Co) – 8.39, chromium (Cr) – 171, copper (Cu) – 33, manganese (Mn) – 951, nickel (Ni) – 60.9, lead (Pb) – 220, and zinc (Zn) - 603 (Sundararajan et al., 2017). In Arabian sea, which is the primary source of sea food, the heavy metal content

was found to be as follows (µg/g): Cu – 90-130, Mn – 1034-1212, Ni – 66-87, and Zn – 109-204 (Borole et al., 1982). In these coastal regions, the studies examining toxic metal accumulation in marine water as well as organisms such as fishes, prawns, squids and crabs have revealed high concentrations of toxic heavy metals (Jebakumar et al., 2015; Sundararajan et al., 2017). Murthy et al. (2013) reported presence of heavy metals such as Cd, Ni, Pb, Cu and Hg that were ranging from 1.39 to 10.04 mg/L in fish and 0.97 to 50.76 mg/L in squid that are processed in Veraval. In oceanic squid and other cephalopods from Gujarat region have been reported to bioaccumulate cadmium above permissible limits (Murthy et al., 2008; Murthy et al., 2009). It is well reported that meek measurements of concentration of toxic pollutants along with established regulatory rules will not give an accurate measure of environmental noxiousness. Thus, attention has been paid to biosensors, biomarkers and biodetectors for detection of heavy metals in contaminated environments (Girotti et al., 2008). Over the past two decades many researchers and scientists have put great efforts to develop fast and sensitive bioassays to monitor and evaluate the presence of toxic material discharge. Some of these bioassays involve microorganisms such as bioluminescent bacteria which lives in close association with certain aquatic organisms (Parvez et al., 2006; Girotti et al., 2008).

Bioluminescent bacteria are simply light emitting bacteria, a result of biochemical reaction catalysed by an enzyme 'luciferase' (Hastings & Nealson, 1977). *Vibrio fischeri* is the most widely studied bioluminescent bacteria and it symbiotically thrives in tissues of certain marine lifeforms (Dunlap, 2009; Kumar, 2010; Tanet et al., 2019). The property of bacteria to produce luciferase is not only limited to *Vibrio fischeri* but is also found in the other species of *Vibrio* (CH & Mohanraju, 2019). At genomic level the light emitting process is regulated in a very strict manner by a cluster of genes known as *lux* operon. The *lux* operons of *Vibrio fischeri* and *Vibrio harveyi* have widely been studied and molecular mechanism and regulation aspects of *V. fischeri* are depicted in Fig. 1 (Hastings & Nealson, 1977; Willey et al., 2008; Lin & Meighen, 2009; Dunlap, 2014; Brodl et al., 2018) . At low cell density the autoinducers, Alcy-homoserine-lactone (AHL) that are being actively produced- diffuses into media, concentration of autoinducers increase as cells continue to grow, resulting in high level of autoinducers in the media. At high cell density AHL creates a regulatory response

leading to expression of *luxICDABE* genes and produces light (Willey et al., 2008). Toxic materials such as heavy metals, pesticides and industrial effluents can cause dramatic and measurable effect on the bacterial biochemical reactions responsible for light production. This phenomenon is exploited by researchers as bioluminescence inhibition assay (Hong et al., 2010; Mohseni et al., 2018). This occurrence of decrease in light upon exposure to toxic heavy metals provides an idea regarding the ecotoxic level and can be treated as biomarker of heavy metal (Thakre & Shanware, 2015).

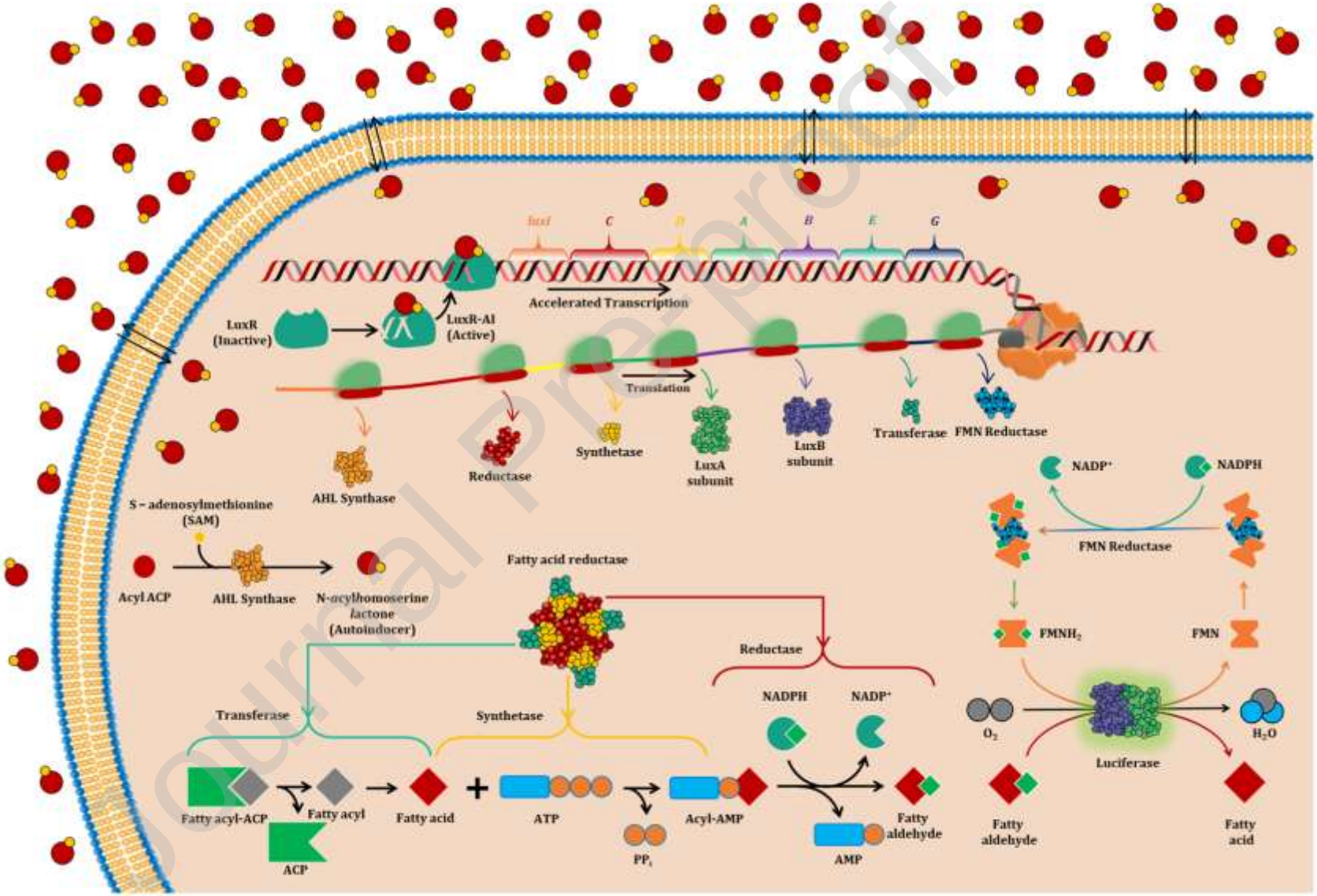


Figure 1: Molecular mechanism of bioluminescence in *Vibrio fischeri*.

Bioremediation techniques involving microorganisms have drawn researchers' attention due to their highly effective eco-friendly nature. Toxic metals in active form can affect the microbial population dynamics and indigenous microbial metabolism to adapt such toxic stress (Yin et al., 2019). One such adaptation acquired by microbes is to produce extracellular polymeric substances (EPS) against protective armour by blocking their (heavy metals) entry into the intracellular fluids (Wang et al., 2014). The adsorption of heavy metals in the matrix of EPSs by interaction of metal cations and negatively charged functional groups is a well-known phenomenon by which the microbes stay protected from heavy metals (Naik & Dubey, 2013; Nkoh et al., 2019). Evidently, marine bacteria produce varieties of EPSs which have unique affinity to bind to heavy metals (Nichols et al., 2005).

Under present study we isolated bioluminescent *Vibrio alginolyticus* PBR1 and *V. rotiferianus* PBL1 from the marine life forms flourishing in the water contaminated with heavy metals (Veraval fish harbour) (Jebakumar et al., 2015; Sundararajan et al., 2017). The marine samples chosen were Indian mackerel (*Rastrelliger kanagurta*), Ornate Threadfin bream (*Nemipterus japonicus*) and one Indian squid (*Loligo duvauceli*) which are known to have contaminated with heavy metals (Prafulla et al., 2001; Murthy et al., 2013; Azaman et al., 2015). The study focuses on resistance and sensitivity of the bioluminescent isolates, in terms of light and growth, to eight heavy metals. Hence, the present study is a key step towards development of biosensing tool employing bioluminescent bacteria for monitoring of ecotoxic pollutants. The strains under study also produce EPS, and property of their EPS to interact with heavy metals was studied as well. The data of heavy metal sensitivity and resistance is of a great help to develop bioassay based on bioluminescent bacteria, whereas, analysis of heavy metal interaction with EPS can help to develop a new strategy to bioremediate heavy metals. To the best of our knowledge this is the first report for such assays with EPS producing bioluminescent strains.

2. Materials and methods

2.1 Sampling site and sample collection

Veraval fish harbour is located on Saurashtra coast (Lat. 20° 54'N, Long. 70° 22'E). Being a largest fishing port, the local fish markets of Veraval possesses large range of fishes and other sea food. Three fresh marine organisms were collected from this fish market. Two fishes, Indian mackerel (*Rastrelliger kanagurta*), Ornate Threadfin bream (*Nemipterus japonicus*) and ~~one~~an Indian squid (*Loligo duvauceli*) were collected in a separate plastic bags previously surface sterilized with 95% (v/v) ethanol (Larson et al., 1991). The samples were stored at 4±2°C in ice box and brought to the laboratory for further processing.

2.2 Cultivation, screening and isolation of bioluminescent bacteria

The screening was carried out on three various media; Nutrient Agar (NA) (MM012, HiMedia, India), Luminescence Agar (LuA) (Malave-Orengo et al., 2010) and Sea Water Agar (SWA) (M592, HiMedia, India). Both kind of samples were dissected using standard methods (Brown & Kisiel, 2003; Gupta & Mullins, 2010). The micro-flora was collected using sterile cotton swabs (PW1136, HiMedia, India) from gut and intestine in case of fishes; while from stomach and ink sac of squid (Makemson & Hermosa Jr, 1999; Vishwakarma et al., 2013; Yaser et al., 2014; Kulkarni & Kulkarni, 2015; Zahaba et al., 2015; Parasar et al., 2016). The swabs were then streaked on respective media. Inoculated agar plates were incubated at 25±2°C for 18-24 h. The next day the plates were examined for luminescent glowing colonies in a dark room. Such colonies were further subcultured to obtain as pure culture on a suitable medium. Screening of the medium and luminescent colonies was done based on visual scoring luminescence intensity so produced by the bacteria (Malave-Orengo et al., 2010; Kannahi & Sivasankari, 2014; Kulkarni & Kulkarni, 2015). Selected isolates (PBR1 and PBL1) were further processed for characterization.

2.3 Activation, inoculation, incubation and preservation

To activate the selected cultures, a single loop-full of colonies was inoculated in 50 mL luminescence broths (LuB) respectively. The broths are then incubated at 25±2°C on an orbital shaker with 120±2 rpm for 18-24 h. In all the experiments involving broth studies, 0.1 % (v/v) aliquot of activated culture was used as inoculum and a loopful of activated cultures was streaked directly for the studies involving solid media. The cultures were

preserved on luminescence agar plates at 4±2°C and sub-cultured periodically at every 15 days. The measures mentioned above were kept unchanged unless otherwise specified.

2.4 Phenotypical and functional characterization

2.4.1 Colony and morphology characterization

The colony characteristics were recorded after growing the isolates on LuA for 24 h. Gram staining was performed using commercially available Gram-Staining Kit (K001, HiMedia, India).

2.4.2 Biochemical characterization

Detailed characterization was performed to determine the phenotypical and functional characters of two selected isolates that helped in building up their metabolic profile. These include: (A) physiological and phenotypical characters: luminescence, motility, assessment of growth on various NaCl concentrations, and temperature; (B) ability to utilize different substrates as sole source of carbon and nitrogen viz. citrate, gelatin, starch, and tributyrin; (C) production of various enzymes: arginine dehydrolase, lysin decarboxylase, ornithin decarboxylase, catalase oxidase, nitrate reductase, and urease; (D) other tests such as: production of mixed acid (MR-VP test) as well as production of indole from tryptone. These tests were performed in accordance with Alsina's key and Bergey's Manual® of Systematic Bacteriology: volume 2 The Proteobacteria (Alsina & Blanch, 1994; Garrity et al., 2006) to identify and differentiate the isolates metabolically. Fermentative efficacy of the screened isolates was determined for 17 sugars. Briefly, readily available sugar discs (HiMedia, India) were used. The discs were added aseptically to Andrade Peptone Water Broth (APWB) medium (M885S, HiMedia, India) to achieve sugar concentration of 25 mg/L. A loopful of an activated culture was inoculated in to these APWB tubes each with different sugar followed by their incubation as mentioned in **section 2.3**.

2.5 Molecular Analysis

2.5.1 g-DNA: extraction, purification and quantification

Bacterial genomic DNA (g-DNA) was extracted and purified using the HiPurA Bacterial Genomic DNA Purification Kit (MB505, HiMedia, India). The protocol given with

the kit was modified as described by Shukla et al. (2019a). The extracted DNA was analysed using agarose gel electrophoresis to assure appropriate extraction and purification. These samples were further assessed for RNA or protein contamination (if any) by Nanodrop spectrophotometer (Implen Nanophotometer, Germany) by its A_{260}/A_{280} and A_{260}/A_{230} absorbance ratio respectively.

2.5.2 16S rRNA gene amplification from g-DNA:

For amplifying 16s rRNA gene from the extracted genomic DNA, forward and reverse primers were synthesized and used as follows: (i) PBR1 - Forward primer (18bp): [SRB-255] 5' GAGAGTTTGATCCTGGCT 3' and Reverse primer (21bp): [SRB-257] 5' ACGGCTACCTTGTTACGACTT 3', (ii) PBL1 - Forward primer (18bp): [SRB-255] and Reverse primer (18bp): [SRB-256] 5' CTACCTCTACCTTGTTAC 3' (Di Cello & Fani, 1996; Marín et al., 2012). To amplify 16s rRNA gene, 50 µL systems were prepared using PCR Master Mix (Thermo Scientific™ K0171, India) using extracted DNA as template. The gene amplification by polymerase chain reaction (PCR) was performed in Veriti Thermal Cycler (Applied Biosystems, India) under conditions described by Thermo Scientific™ (K0171, India): (i) initial denaturation at 95 °C for 5′, (ii) denaturation at 95 °C for 30 s (x35 cycles), (iii) annealing at 55 °C for 30 s (x35 cycles), (iv) extension at 72 °C for 1′ (x35 cycles) and (v) final extension at 72 °C for 5′ . The amplicons were resolved on 0.4 % (w/v) agarose gel. The bands were analyzed using commercially available standard DNA ladder (ThermoScientific™ GeneRuler 1 kb #SM1333, India) by UviTech Gel Documentation (FireReader V10, UK) system.

*2.5.3 16S rRNA s*equence analysis and identification of bacteria

The amplicons were sequenced using Sanger's dideoxy method at Eurofins Genomics (India). The obtained 16S rRNA gene sequences were analyzed for anomalies and assembled manually using Chromas v2.6.6 software (http://technelysium.com.au/wp/chromas/) (Oem et al., 2019). The sequence similarities were determined by means of EZBioCloud database and also by BLASTn search algorithm at NCBI BLAST (Zhang et al., 2000; Yoon et al., 2017). Based on maximum identity score the first 15 sequences were selected and retrieved which were used to perform multiple sequence alignments using EZEditor2 (Jeon

et al., 2014). The phylogenetic trees were constructed using Neighbour-Joining method with bootstrap value set to 1000 simulations in MEGA X (Kumar et al., 2018). The processed 16S rRNA partial gene sequences of both the strains (PBR1 and PBL1) were submitted to GenBank.

2.6 Assessment of susceptibility to antibiotics & pathogenicity

The ability of the isolates to resist various commercially available antibiotic impregnated discs, including both broad and narrow spectrum antibiotics was evaluated using disc-plate method (Loo et al., 1945; Wayne, 2008; Jorgensen & Turnidge, 2015). The zone diameter of growth inhibition developed after incubation was measured to represent the susceptibility or resistance of the cultures. Total 18 antibiotic used under study which were (i) aminoglycosides – amikacin (AK) and gentamicin (GM); (ii) β-lactam antibiotics – ampicillin (AS), cloxacillin (CX) and piperacillin (PC); (iii) quinolones and fluoroquinolones – ciprofloxacin (RC), levofloxacin (QB), ofloxacin (ZN) and gatifloxacin (GF); (iv) sulfonamides - co-trimoxazole (BA) and cephalosporins – cephalexin (PR), cefotaxime (CF) and ceftizoxime (CI); and (v) miscellaneous – lincomycin (LM), roxithromycin (AT), tetracycline (TE), linezolid (LZ) and chloramphenicol (CH). Hemolysin is a kind of cytotoxin (cytolytic toxin) which damages the host cytoplasmic membrane, results in cell lysis and death. These are soluble and extracellular proteins produced by variety of pathogenic organisms. Production of such hemolysins can be detected by using a blood agar plate (a nutrient rich medium containing 5% sterile blood) (Madigan et al., 2010). For this test, sheep blood agar plate (MP1301, HiMedia, India) was used. The cultures were inoculated as described earlier in **section 2.3**. The inoculated plate was incubated at 37±2°C for 24-48 h, the appearance of clear zone indicates hemolysis of erythrocytes surrounding the colonies indicating positive test results, while absence of zone of hydrolysis indicate negative results. For this assay, *Staphylococcus aureus* was used as positive control.

2.7 Production and extraction of exopolysaccharide (EPS)

The ability of the strains to produce EPS was determined on EPS agar medium (Atlas & Parks, 1997). The culture broth was supplemented with sucrose in a concentration of 5 g% (v/v) and inoculated with 3 % (v/v) inoculum. On 3rd and 6th day, 10 mL of culture broth

was withdrawn, and EPS was extracted by adding 30 mL of chilled acetone to these broths. The extracted EPS was recovered by centrifugation and quantified by drying to a constant weight (Andhare et al., 2017; Shukla et al., 2019b). Assessment of types of EPS viz. slime EPS and capsular EPS from broth was performed as per the assay described by Subramanian et al. (2010).

2.8 Heavy metal toxicity assessment

Toxicity of heavy metals on PBR1 and PBL1 were performed on LuA. Heavy metal toxicity profile was performed by standard agar well diffusion assay (Hassen et al., 1998; Tomova et al., 2015) where eight different heavy metals were used in varying concentrations ranging from 100 to 500 mg/L. The spread plate method was used to inoculate the culture suspension (Thakre & Shanware, 2015). In four quadrants of the Petri plate, wells were punched using cork-borer and the heavy metals suspensions (200 µL) was loaded. On overnight incubation, the plates were observed for clear zone of inhibition and change in luminescence pattern. The zone of inhibition and luminescence were recorded by the method reported by Parmar et al. (2020) and Shukla et al. (2020b) using Nikon® D5100™ camera (Nikon India Pvt. Ltd.) with following modifications: (i) Bulb mode, (ii) aperture – size of lens opening – f/5.6 (iii) ISO sensitivity of 2000 (iv) 20 s exposure time. Heavy metals used under study were: Arsenious oxide [As_2O_3], Cadmium nitrate tetrahydrate [$Cd(NO_3)\cdot 2H_2O$], Cerium nitrate tetrahydrate [$Ce(NO_3)_2\cdot 4H_2O$], Chromium nitrate nonahydrate [$Cr(NO3)_3\cdot 9H_2O$], Copper sulfate pentahydrate [$CuSO_4\cdot 5H_2O$], Lead nitrate [$Pb(NO_3)_2$], Strontium nitrate [$Sr(NO_3)_2$], and Mercuric chloride [$HgCl_2$].

2.9. EPS - metal binding assessment

Analytical grade nitrate salts of Pb and Sr were dissolved in deionized water to attain 1000 mg/L stock concentrations. EPS broths supplemented in concentration of 200 mg/L of Pb and Sr were prepared. Both strains PBR1 and PBL1 were allowed to grow in presence of Pb and Sr individually in different culture flasks (Iyer et al., 2004; Kiliç et al., 2015; Shukla et al., 2020a). After incubation period of 72 h, cells with intact EPS were harvested by centrifugation at 4000 rpm for 15' and washed with deionized water to remove residual cell-free culture broth. Extracted EPSs were dried to a constant weight and collected for further

process (Andhare et al., 2017; Shukla et al., 2019b). For comparison with control EPS, same procedure was followed to extract EPSs from the cultures grown in EPS medium devoid of metal.

Control EPS and EPS extracted from culture flask containing metals were analysed using Bruker Alpha FTIR spectrometer (Germany) equipped with a universal Attenuated Total Reflectance (ATR) sampling stage containing diamond crystal. All FTIR spectra were recorded within frequency range of 4000-400 cm^{-1}. Modification in the pattern of spectra was analysed to study the interactions of heavy metal with the functional group of EPS. The peak assessments of EPS for detecting their functional group was performed as per Beech et al. (1999), Pagnanelli et al. (2000) and Kazy et al. (2002).

3. Results

3.1 Screening and isolation of bioluminescent bacteria

From the gut and intestine of Indian mackerel, total 14 luminescent colonies were obtained. No bioluminescent colonies were obtained in case of Threadfin bream fish in any of the media. From the ink sac of Indian squid sample, 23 luminescent colonies were obtained. Luminescent colonies were clearly visible among many other non-luminescent colonies under dark conditions. Out of these luminescent colonies one showing the maximum blue-green light intensity from each kind of samples were subcultured on LuA medium. Two strains designated as PBR1 and PBL1 were isolated from Indian mackerel and Indian squid respectively (Fig. 2). Both the screened strains showed maximum luminescence on LuA medium.

Figure 2: Images showing luminescence expressed by (a) PBR1 and (b) PBL1 in dark room.

3.2 *Phenotypical and functional characterization*

3.2.1 Colony and morphological characterization

The colony characteristics of both the isolates were somewhat similar. The colonies were small, round, mucoid, convex, transparent, showed entire margin with smooth surface and were non-pigmented. However, on ageing colonies turned cream and translucent. Microscopically, both the isolates were gram negative short rods.

3.2.2 *Biochemical characterization*

Various biochemical tests and phenotypical traits established that the isolates PBR1 and PBL1 belonged to the genus *Vibrio*. Both the isolates were able to grow at 1.5 M NaCl concentration. However, PBR1 could also tolerate up to 2 M NaCl. PBR1 and PBL1 could flourish in temperature range of 20 to 40℃ Optimum growth and luminescence of both strains occurred at 25 ±2 ℃while, they could also grow at the temperature as high as 45 ℃. Interestingly, PBL1 was able to grow at 4℃ In terms of substrate utilization ability both

cultures were found to be quite similar as neither was able to utilize citrate, starch and tributyrin; but were able to utilize gelatine. In enzymatic profiling, both isolates showed positive results for lysin decarboxylase, catalase and oxidase. However, PBR1 was also able to produce ornithin decarboxylase. None of the strains were able to utilize arginine, nitrate and urea. In addition, production of acetylmethyl carbinol from glucose was not seen by both strains. Presence of tryptophanase enzyme was detected in PBR1 only (Table 1).

Evidently, both PBR1 and PBL1 showed identical reactions in terms of sugar fermentation. Out of 17 sugars tested, isolates were able to utilize and produce acid from cellobiose, dextrose, fructose, galactose, maltose, mannose, sucrose and trehalose. However, adonitol, arabinose, dulcitol, melibiose, raffinose, rhamnose, salicin, sorbitol and xylose were not metabolized.

3.3 Molecular Analysis

3.3.1 g-DNA: extraction, purification and quantification

A_{260} using spectroscopy estimated that total yield of purified g-DNA was 356 and 364 ng/L for isolate PBR1 and PBL1 respectively. First and second degree DNA purity was measured by absorption assessment by taking ratios at A_{260}/A_{280} and A_{260}/A_{230} respectively. Values for ratio A_{260}/A_{280} was calculated for PBR1 (1.3) and PBL1 (1.79) were less than 1.8 which confirms no contamination of Protein and RNA in the extracted g-DNA. The values obtained were between 2 and 2.1 for the ratio A_{260}/A_{230} and which suggests DNA free of phenol contamination.

3.3.2 16s ribosomal gene: PCR amplification, identification and phylogenetic analysis

Results of gel electrophoresis revealed that the approximate length of amplicons were of ~1000 bp for both the isolates. The identification was carried out based on top hit displayed by BLAST results as the method described by Ramesh and Mohanraju (2017). 16S rRNA gene sequence analysis showed that the isolate PBR1 and PBL1 belong to the genus *Vibrio* as their gene sequences showed maximum similarity with *Vibrio alginolyticus* ATCC 17749 (98.12%) and *Vibrio rotiferianus CAIM 577 LMG 21460* (100.0%) on performing BLASTn analysis with the existing sequences of same gene in GenBank. In accordance with

similarity of the hypervariable region of 16S rRNA gene sequence, the isolates PBR1 (Fig. 3a) and PBL1 (Fig. 3b) were identified as *Vibrio alginolyticus* PBR1 and *Vibrio rotiferianus* PBL1.

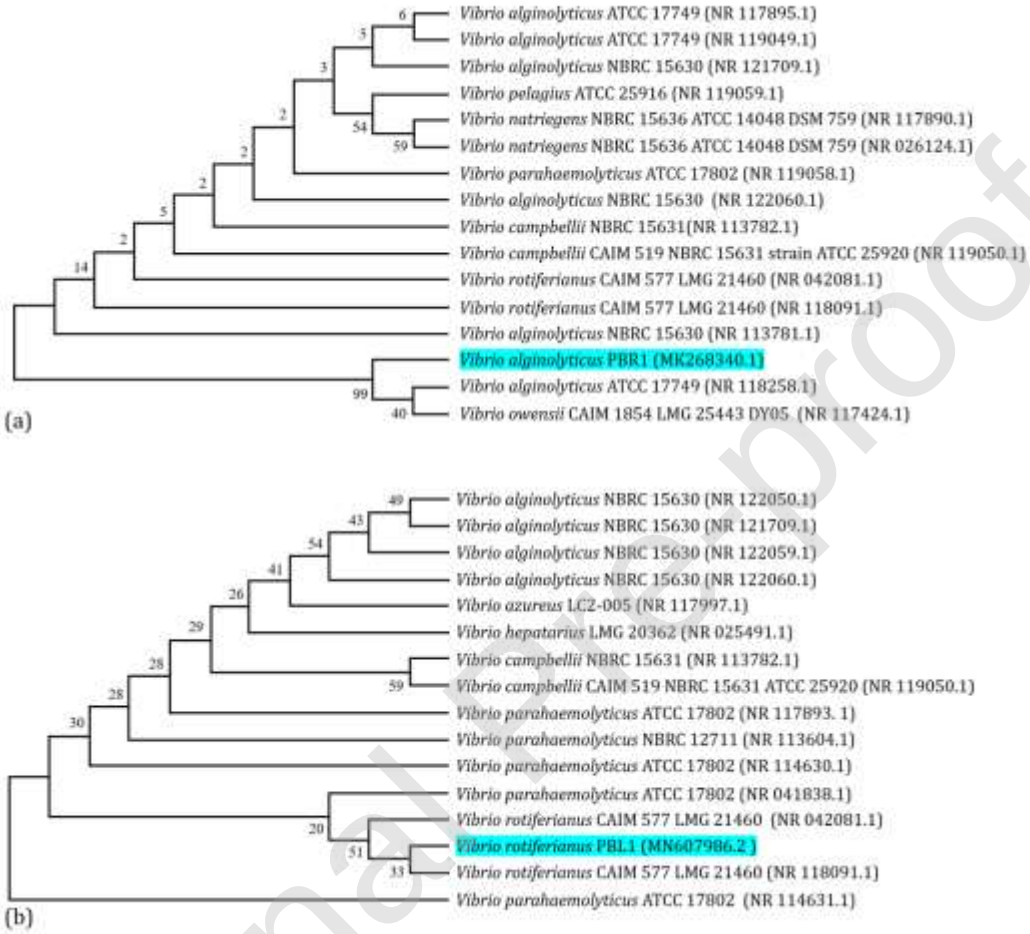


Figure 3: Phylogenetic dendrogram based on 16S rRNA gene sequences using Neighbor-Joining method showing the relatedness of (a) PBR1 and (b) PBL1 isolates (in bold) and close species. Digits at nodes stands for the level of bootstrap support values after 1000 replications.

3.4 Assessment of susceptibility to various antibiotics & pathogenicity

From the antibiotic sensitivity assay, it was found that both the strains were resistant to wide range of antibiotics including amikacin, ampicillin, cefotaxime, ceftizoxime, cephalexin, cloxacillin, gentamicin, lincomycin, ofloxacin, piperacillin, and tetracycline. However, chloramphenicol, ciprofloxacin, trimoxazole, gatifloxacin, levofloxacin, linezolid, and roxithromycin were able to inhibit the growth of both the isolates (Table 2). β-hemolysin test was performed to determine the pathogenicity of isolated bioluminescent strains. The results suggested the non-pathogenicity of both the strains as they failed to produce clear zone of hemolysin degradation. For the same assay clinical strain of *S. aureus,* used as positive control and showed clear zone of hemolysis surrounding the colonies (figures not shown). These results corroborate the non-pathogenic nature of both the bioluminescent strains.

3.5 EPS: Production and extraction

The EPS plate assay preliminarily determined ability, of both strains, to utilize sugars to produce EPS by typical ropiness of the mucoid colonies, after three days of incubation period. This positive result served basis for the quantitative broth study. The influence of incubation temperature on EPS production was carried out. Results revealed that both organisms were actively involved in EPS production from sucrose. After recovery and extraction, the maximum amount of EPS was obtained from PBR1 and PBL1 was 1.34 g% (w/v) and 2.45 g% (w/v) after 72 h of incubation at 25±2°C respectively from broth suggesting sugar to EPS conversion efficiency of 27% for PBR1 and 49% for PRL1 (Fig. 4). Further, increase in temperature and incubation period showed reduction in the EPS yield. Out of these two strains, PBL1 was found to be efficient EPS producer. The results suggested that for both strains, the optimum conditions for maximum EPS production to be 25±2°Cand an incubation period of 72 h. The nature of the EPSs produced by both strains was found to be capsular. Authors' here, for the first time report, an EPS producing *Vibrio alginolyticus* and *V. rotiferianus* to be bioluminescent. Given the versatile application of EPS in various fields,

these isolates can be utilized in remediation of heavy metals from aquatic environments and the luminescent nature of these microbes can be used to develop a light emitting biosensor.

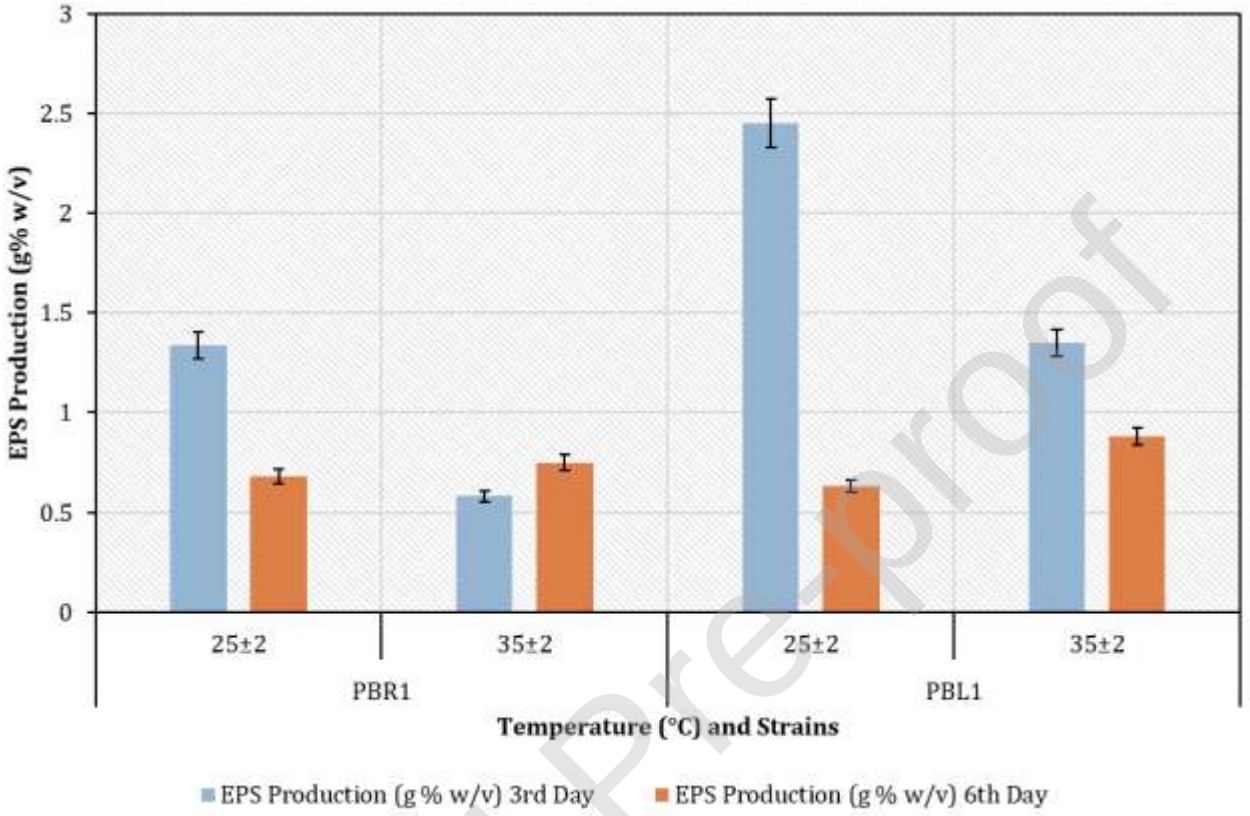


Figure 4: Graph showing production of EPS from PBR1 and PBL1.

3.7 Heavy metal tolerance assessment

Effect of various heavy metals with varying concentrations (100 to 500 mg/L) on growth and luminescence was also evaluated. Results demonstrated that out of 8 metal cations including As(III), Cd(II), Ce(II), Cr(III), Cu(II), Hg(II), Pb(II), and Sr(II) PBR1 and PBL1 both strains were highly resistant to Pb and Sr even at the concentration of 500 mg/L. By observing the trend of toxic effect of each heavy metal on growth of PBR1, degree of toxicity in the order of Cd>Hg>Cr>As>Ce>Cu suggesting Cd to be most toxic to both the strains (Fig. 5a and b). The organisms were able to grow and also showed luminescence in presence of 100 mg/L concentration of As, Ce, and Cu. When PBR1 was allowed to grow in the presence

of Hg and Cr with an increasing concentration of 100 to 500 mg/L. No inhibition of PBR1 was observed by As and Ce below 200 mg/L concentration hence, suggesting relative resistance to these metals as compared to Cd and Hg. No significant inhibitory effect of Cu on growth and luminescence of PBR1 was observed up to 300 mg/L. However, there was reduction in growth as well as luminescence above 400 mg/L concentration. Similar trends in growth and luminescence were seen in PBL1 upon exposure to different metals (Fig. 5b). Though there was significant difference in tolerance to As where PBR1 tolerated 100 mg/L concentration while PBL1 had failed to grow. Plates showing the effect of various heavy metal on PBL1 is shown in Fig. 6. During this study an effect of heavy metals on luminescence was documented where it findings suggested that Cr, Pb, and Sr had no effect on luminescence even though Cr has showed lethal effect on growth from 200 to 500 mg/L concentration [Fig. 6 (vii, viii, xiii-xvi)]. However, Cd and Cu showed narrow zone of moderate decrease in luminescence immediately after clear zone of growth inhibition [Fig. 6 (iii, iv, x)]. As, Ce and Hg showed clear zone of growth inhibition which was followed by narrow zone of luminescence stimulation. The intensity of light in these luminescence stimulation zones were in order of Ce>Hg>As [Fig. 6 (i-ii, v-vi, xi-xii)]. This indicates that at low concentration of these three metals stimulate the luminescence where their higher concentration of this metals has detrimental effect on growth and luminescence directly.

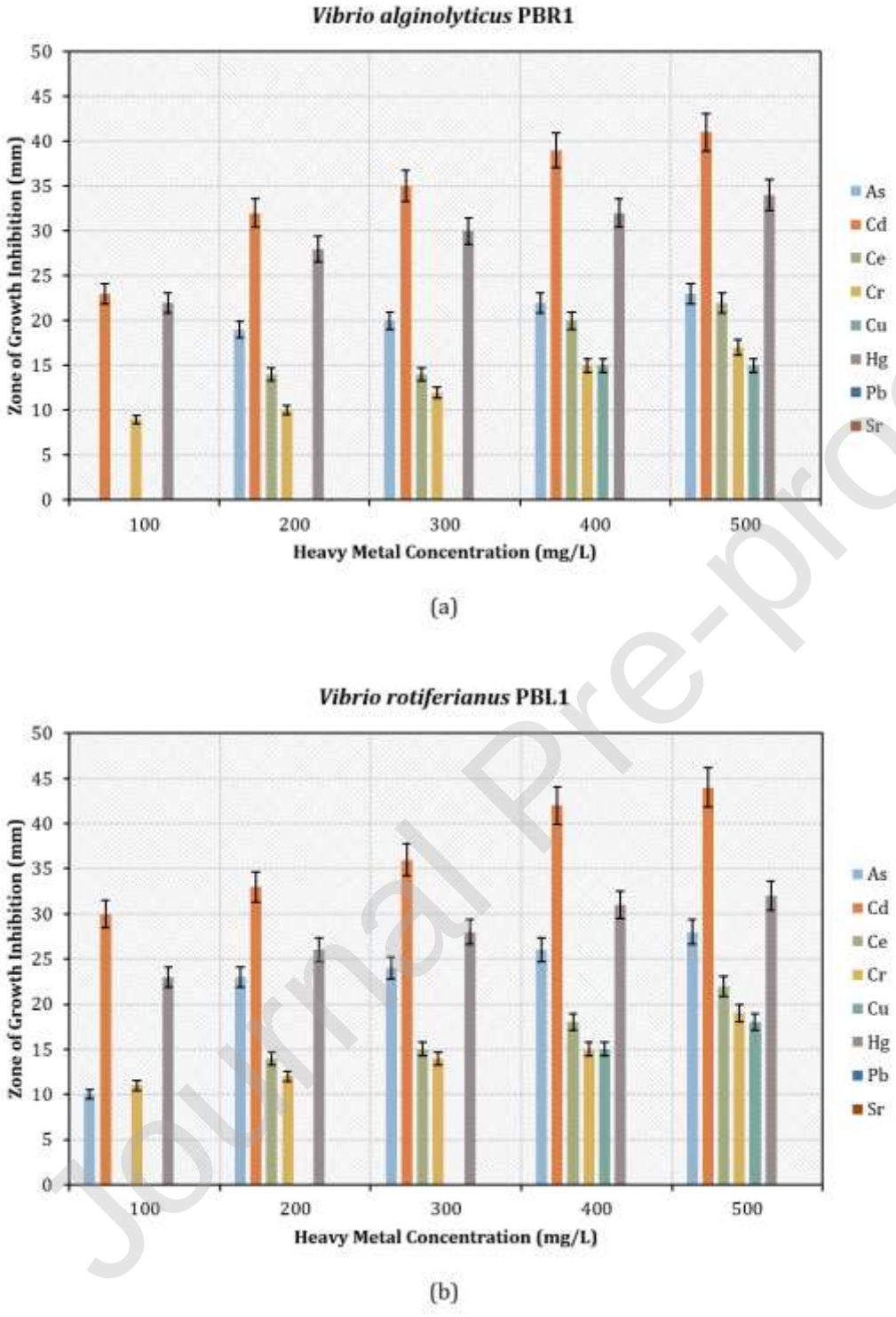


Figure 5: Graph showing profile of heavy metal toxicity of (a) *V. alginolyticus* PBR1 and (b) *V. rotiferianus* PBL1 against 100 to 500 mg/L concentration.

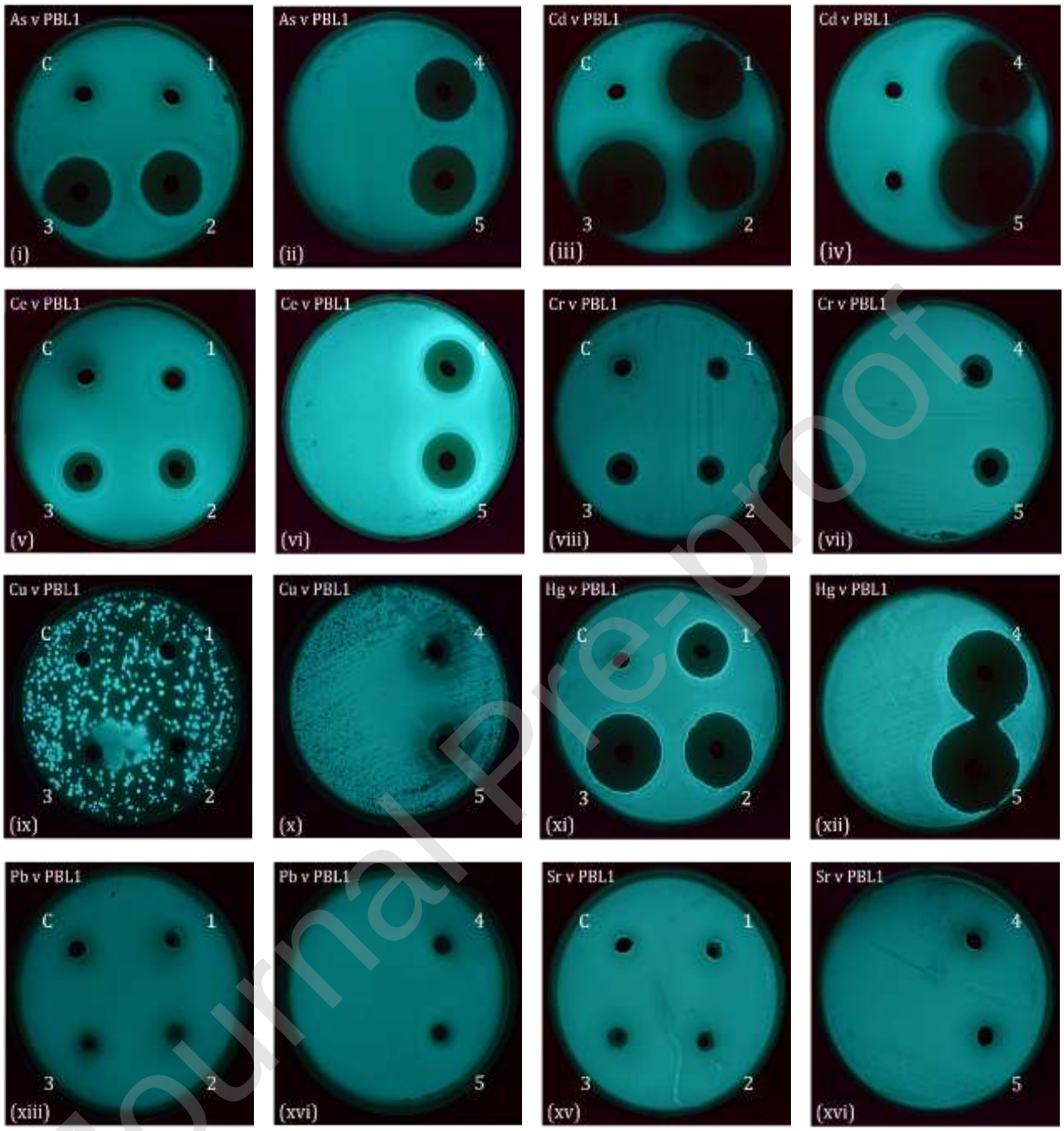


Figure 6: Images demonstrating effect of heavy metals on PBL1 strain: heavy metal concentration (mg/L): C, Control; 1, 100; 2, 200; 3, 300; 4, 400 and 5, 500.

3.8 FTIR analysis: EPS and Metal adsorption

Based on the heavy metal tolerance assay, both strains were able to thrive in presence of Pb and Sr, hence these metals were supplemented in EPS production medium. The EPSs extracted from heavy metal supplemented EPS medium were appropriately labelled as: EPS^{Pb} and EPS^{Sr} while the EPS extract extracted from the culture medium not supplemented with heavy metals was labelled EPS^{C}. The FTIR spectra of $EPS^{C,}$ EPS^{Pb} and EPS^{Sr} for both the strains were overlapped and are shown in Fig. 7. The peak assignments are as follows: 3400-3200 cm^{-1} represented the O-H vibration stretch or hydrogen bond, 3000-2800 cm^{-1} related to C-H stretch vibration, 1850-1600 cm^{-1} due to the stretch vibration of C=O bond, 1400-1370 cm^{-1} attributed to C=O bond and 900-1150 cm^{-1} for C-O-C bond. The comparison of FTIR spectra of EPS^{C} with EPS^{Pb} and EPS^{Sr} for both strains showed many shifts in wavenumbers. In EPS^{C} spectra of PBR1 and PBL1, region of 3500-3200 cm^{-1} shows the stretching of the N-H bond of amino groups which lies within region dominated by a strong and wide band of 3200-3600 cm^{-1} due to presence of O-H of hydroxyl groups. In the spectrum of PBR1-EPS^{Pb} and PBR1-EPS^{Sr}, the peak was shifted from 3268 cm^{-1} to 3267 cm^{-1} and 3234 cm^{-1} respectively. In case of PBL1-EPS^{Pb} and PBL1-EPS^{Sr}, the peak shifted significantly from 3252 cm^{-1} to 3278 cm^{-1} and 3284 cm^{-1} separately. This is attributed to the role of amino and hydroxyl groups in Pb and Sr binding. The EPS^{C} of PBR1 showed peak at 2923 cm^{-1} which belongs to region of complex absorption at 2900-3000 cm^{-1} indicating asymmetric stretching of C-H bond of CH_2 group associated with that of the CH_3 groups. In case of PBR1, the peak in this region was disappeared in spectra of EPS^{Pb} and EPS^{Sr} showing involvement of methyl groups in metal binding. In contrast, the peak in 2900-3000 cm^{-1} region was absent in the spectra PBL1-EPS^{C} indicating absence of methyl group. Bands related to proteins were found to be present in all spectra. The bands around 1635-1684,1624 and 1555-1564 cm^{-1} were attributed to these protein related bands presented C=O of amide I and N-H/C=O mixture of the amide II bonds. In case of PBR1, peak of EPS^{C} obtained at 1624 cm^{-1} shifted to the same 1640 cm^{-1} in spectra of EPS^{Pb} and EPS^{Sr}. While in case of PBL1-EPS^{Pb} and PBL1-EPS^{Sr}, peak of PBL1-EPS^{C} at 1641 cm^{-1} shifted slightly to 1640 cm^{-1}. Band obtained at 1556 cm^{-1} for PBR1-EPS^{C} shifted to 1553 cm^{-1} and 1554 cm^{-1} in spectra of PBR1-EPS^{Pb} and PBR1-EPS^{Sr} respectively in comparison of which band obtained in PBL1-EPS^{C} at 1564 cm^{-1} was found to

be absent after Pb and Sr exposure. A decrease of ~10-20 % in intensity was observed in these C=O and N-H stretching bands in case of PBR1-EPS^{Pb} and PBR1-EPS^{Sr} most likely shows some alterations in the secondary structure of cellular proteins and other alternate conformations as a result of metal binding. In EPS^{C} of both PBR1 and PBL1 spectra peaks obtained at 1402 cm^{-1} and 1408 cm^{-1} depicts the presence of carboxyl groups. Shifting was observed to 1408 cm^{-1} and 1407 cm^{-1} in spectra of PBR1-EPS^{Pb} and PBR1-EPS^{Sr} respectively. A shift in case of PBL1-EPS^{Pb} to 1405 cm^{-1} was also observed, leaving the peak of PBL1-EPS^{Sr} at 1408 cm^{-1} unchanged when compared to PBL1-EPS^{C}. Peak rang of 1000-1100 cm^{-1} shows presence of carboxyl (C=O) and phosphate (P=O) groups in biopolymer. In case of PBR1-EPS^{C} two peaks in this region was obtained, 1043 cm^{-1} and 1104 cm^{-1} out of which first peak was shifted to 1057 cm^{-1} and 1059 cm^{-1} in the spectra of PBR1-EPS^{Pb} and PBR1-EPS^{Sr} respectively. While second band (1104 cm^{-1}) was found to be absent in both spectra of EPS^{Pb} and EPS^{Sr} of PBR1. PBL1-EPS^{C} displayed peak at 1054 cm^{-1} which was shifted to 1057 cm^{-1} and 1065 cm^{-1} in the spectra of PBL1-EPS^{Pb} and PBL1-EPS^{Sr} in the same order. The detailed study of the FTIR spectra revealed that hydroxyl, carboxyl group of amide I and N-H/C=O groups of amide II, phosphate groups were involved in biosorption of heavy metals Pb and Sr from the metal supplemented EPS with and additionally, methyl group in case of PBR1-EPS.

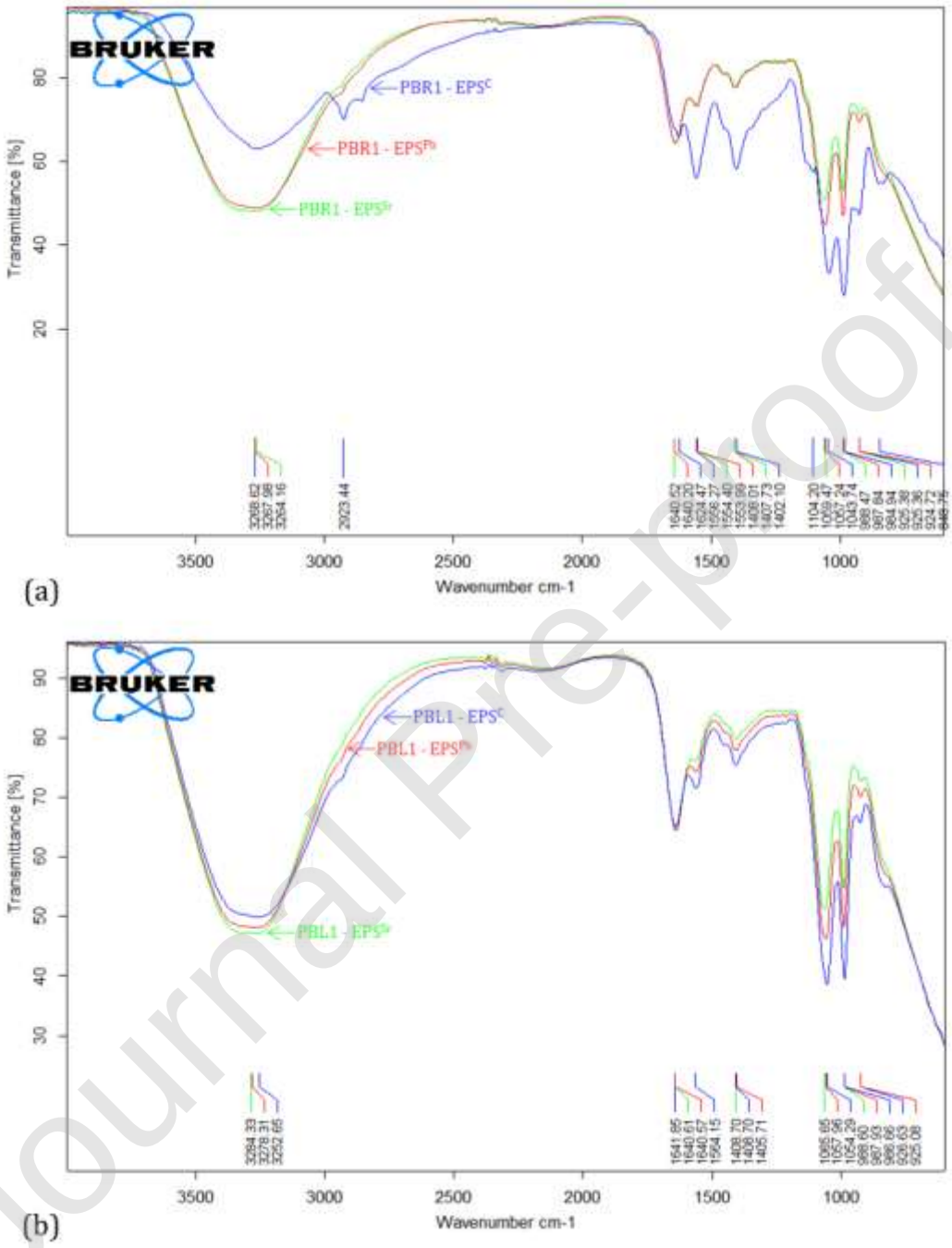


Figure 7: Fourier Transform IR spectra of (a) PBR1and (b) PBL1 EPSs: before (blue) and after lead (red) and strontium (green) adsorption.

4. Discussion

In this investigation, two bioluminescent bacterial strains, PBR1 and PBL1 were isolated from a fish Indian mackerel and an Indian squid. Bioluminescent bacterium *Kurthia gibsonii* have been isolated from Ornate Threadfin bream fish commonly known as Raja-Rani fish (Vishwakarma et al., 2013). On the contrary, in this presented research work no bioluminescent bacteria was obtained from the same fish sample which supports the results published by Parasar et al. (2016). Balan et al. (2013) Carried out a study where bioluminescent bacteria such as *Shewanella hanedai*, *V. splendidus*, *V. fischeri* and *V. harveyi* were isolated from squid and cuttle fish. Luminescent species of *Vibrio* genus are widely distributed among oceans and within its flora and fauna such as various marine plants, fishes, squids and other crustaceans in either planktonic form or in a close association with marine hosts respectively (Nawaz & Ahmed, 2011; CH & Mohanraju, 2019). Such symbiotic associations can be mutualistic, parasitic or sometimes saprophytic (Hastings & Nealson, 1977).

Conventional approaches of bacteriology can help to detect usual pathogens but such methods are cumbersome and erroneous sometime resulting in false identification and henceforth, molecular identification is preferred (Balcazar et al., 2007; Ransangan & Mustafa, 2009; CH & Mohanraju, 2019). For instance, a pathogenic *V. campbellii* was wrongly identified as *V. harveyi* multiple times (Gauger & Gómez-Chiarri, 2002; Owens & Busico-Salcedo, 2006; Lin et al., 2010). In order to eliminate such errors, in the presented study, both approaches were used to identify the isolated bioluminescent bacteria. Under present study, both isolates showed similarities in phenotypical and physiological characters with respect to gram reaction, motility, as well as an ability to grow on varying NaCl concentration and temperatures except luminescence when compared to genetically closely related species. According the key tests suggested by Alsina and Blanch (1994), results obtained for PBR1 showed that it is in the same cluster as *V. campbellii* and *V. fischeri* but in a different cluster from *V. harveyi* whereas, PBL1 indicated that it is in same cluster as *V. harveyi* but in different cluster from *V. fischeri.* In contrast to closely related non-luminescent species of *Vibrio*, PBLR1 and PBL1 displayed negative results for nitrate reductase.

16S rRNA sequencing has proven to be useful as a molecular tool to classify and identify the bacterial species (Ransangan & Mustafa, 2009; Cano-Gomez et al., 2015). Also to identify the bacterial strains at species level, 16S rRNA sequence similarity should be 97.0% or above (Thompson et al., 2004). Here, identification by molecular method using 16S rRNA gene sequencing suggested both the isolates belonging to *Vibrio* were different strains which would not have been detected using biochemical assays. Based on taxonomic analysis, these isolates were identified as *V. alginolyticus* PBR1 and *V. rotiferianus* PBL1. *Vibrio alginolyticus* strains previously been isolated from fishes and marine environment were found to be luminescent which supports the results obtained in presented work (Ramesh & Mohanraju, 2017; Langaoen et al., 2018). However, non-luminescent isolates of *V. alginolyticus* and *V. rotiferianus* have also been reported from various other sources (Gomez-Gil et al., 2003; Drouillard et al., 2015). This observation indicates that despite of close genetic similarities between the strains there could be major differences in phenotypic and physiological characteristics such as luminescence. Lal and Ransangan (2013) hypothesized that such variability may have resulted due to gene transfer which occurs in both ways within the same species as well as among the different genera. The horizontal gene transference of *lux* genes in nature from luminescent *V. harveyi* to non-luminescent *V. vulnificus* and *V. chagasii* is well demonstrated by Urbanczyk et al. (2014) out of which *V. vulnificus* biochemically had been placed in same group as *V. alginolyticus* according to A-L-O test key (Alsina & Blanch, 1994).

The information regarding the antibiotic sensitivity and resistance by *Vibrio* sp. holds importance since several strains have been reported to be pathogenic. Owing to this fact, it was inevitable to assess antibiotic sensitivity assay for the strains under study. Strains of *Vibrio* have found to gain resistance to ampicillin, tetracycline and gentamicin (Gomez-Gil et al., 2003; Langaoen et al., 2018). Our findings were in agreement to those reported by Zhang et al. (2019) suggesting our strains being resistant to above mentioned antibiotics. However, PBL1 showed sensitivity towards ciprofloxacin and chloramphenicol which corroborates the findings of Shanware et al. (2013). The luminescence of the *V. rotiferianus* was found to be enhanced when it was grown under ampicillin stress (Shanware et al., 2013) whereas in this study PBR1 and PBL1 failed to produce luminescence in presence of antibiotics. Here, we failed to explain the phenomenon behind such contrast findings. The genus *Vibrio*

contains more than 30 species out of which 12 *Vibrio* species are pathogenic to humans (Chakraborty et al., 1997). Few of *Vibrio alginolyticus* strains are previously reported to be pathogenic (Fernández-Bravo et al., 2019; Peng et al., 2019). Therefore, hemolysin is a known putative factor in numerous pathogenic bacteria. It is an exotoxin that lyses membranes of red blood cells along with excretion of haemoglobin and it is most widely distributed among various pathogenic species of *Vibrio* genera (Shinoda, 1999; Jia et al., 2010). Certain strains of *Vibrio alginolyticus* are known to be opportunistic pathogen for human as well as for aquaculture (Peng et al., 2019; Yu et al., 2019). A study carried out by Ruwandeepika et al. (2012) also suggested that the *Harveyi* clade of *Vibrio* genus includes both pathogenic as well as non-pathogenic bacteria with the inclusion of *V. alginolyticus* into pathogenic group. Therefore, to determine the pathogenicity of isolated *V. alginolyticus* PBR1, β hemolysin test was carried out which resulted in favour of non-pathogenicity of both the strains. It is noted that *Vibrio alginolyticus* isolated from deep water lacked *tdh* and *trh* genes coding for thermostable direct hemolysin (TDH) and/or TDH-related hemolysin (TRH) indicating non-pathogenicity of the strain (Drouillard et al., 2015).

EPSs are well known for their applications in food, cosmetics, textile industries, petroleum, agriculture and bioremediation and medical fields. In bioremediation, where toxic pollutants are removed by means of biological compound or agent, EPSs have proved to be potent remediating agent for removal of toxic heavy metals (Gupta & Diwan, 2017; Shukla et al., 2019b). There are several reports on production and extraction of EPS from *V. alginolyticus* isolated from various sources (Rui et al., 2008; Ye et al., 2008; Drouillard et al., 2015). Bramhachari and Dubey (2006) reported for the first time, the EPS production by bioluminescent *V. harveyi*. However, we could not find a single report on EPS production by bioluminescent *V. alginolyticus* as well as *V. rotiferianus*. EPS produced by non-luminescent *V. alginolyticus* was 0.1 g% (w/v) (Muralidharan & Jayachandran, 2003) whereas, our luminescent *V. alginolyticus PBR1* and *Vibrio rotiferianus* PBL1 produced significantly greater volumes of EPS. Several reports suggest EPS producing *Vibrio* species including *Vibrio alginolyticus* to lack the luminescence capability (Muralidharan & Jayachandran, 2003; Bramhachari et al., 2007; Kavita et al., 2013; Drouillard et al., 2015). However, Bramhachari and Dubey (2006) first reported one such luminescent *Vibrio harveyi* isolated from the

coastal regions of Goa, India to produce exopolysaccharides. We, for the first time, report an EPS producing *Vibrio alginolyticus* and *V. rotiferianus* to be bioluminescent. Given the versatile application of EPS in various fields, these isolates can be utilized in remediation of heavy metals from aquatic environments and the luminescent nature of these microbes can be used to develop a light emitting biosensor.

Before applying the strains for bioremediation development strategy as well as for the production of biosensors, the strains under study had to be examined for its behaviour against several heavy metals. Shanware et al. (2013) reported different results where *V. rotiferianus* was inhibited various heavy metals in toxic order of Hg>Co>Cu>Fe>Pb>Zn. They have demonstrated that *V. rotiferianus* was very sensitive to concentration as low as 50 mg/L of these metals. However, we observed the sensitivity of heavy metals against PBR1 and PBL1 in the order of Cd>Hg>Cr>As>Ce>Cu suggesting Cd to be most toxic and showed resistance to heavy metals at two-fold concentration when compared to the reported results. Such property may be attributed to epigenetic adaptation. Another study reported by Thakre and Shanware (2015) two strains of *V. harveyi* that were also very sensitive to heavy metals in terms of growth and luminescence. The toxicity orders of heavy metals were found to be Hg>Pb>Fe>Cu and Fe>Pb>Cu>Hg for *V. harveyi* strains AB and DP respectively. These studies indicated that bioluminescent *V. harveyi* strains were sensitive to even 0.05 mg/L concentration of above-mentioned heavy metals and also, they demonstrated the decrease in luminescence of the strains with increasing the heavy metal concentration. Interestingly, in this study, both *V. alginolyticus* PBR1 and *V. rotiferianus* PBL1 were found to be completely resistant to Pb and Sr in terms of both growth and luminescence. Ranjitha and Karthy (2012) conducted a study where toxicity of different heavy metal was tested against various luminous bacteria by microtiter plate method. The toxicity order was found to be Hg>Zn>Cu>Ag>Co>Ba. In addition, Ranjitha and Karthy (2012) concluded that the resistance to heavy metals may have resulted from the presence of naturally occurring plasmids. Heavy metals are known to have an oligodynamic effect on microorganisms (Tien et al., 2008; Ahn et al., 2009) where clear zone of growth inhibition is obtained surrounding the well having heavy metal solution or a heavy metal coin after incubation period which then followed by narrower zone of metal stimulated growth and normal growth occurs in remainder of agar

surface (Salle & Salle, 1954). We also observed oligodynamic effect for Ce, As and Hg however, other metals did not show such phenomenon and Cd even reduced the luminescence. Another study reported by Balachandar et al. (2010) suggested that bioluminescent bacteria isolated form marine organisms can act as biosensor for heavy metal monitoring. Hence, we claim that a biosensor can be developed that can emit light in detection of As, Ce and Hg while light sequestering upon Cd detection. The detailed compiled data representing tolerance to heavy metals of various *Vibrio* species along with their EPS producing ability is provided in the supplementary material file.

The FTIR Spectrum revealed presence various functional groups such as hydroxyl group, methyl group, protein related amide I and amide II groups. A diverse variety of bacterial genera such as *Bacillus*, *Pseudomonas*, *Azotobacter, Shewnella* have been well documented for metal adsorption properties (Martinez et al., 2002; Borrok & Fein, 2005; Gauri et al., 2011; Yuan et al., 2019). Similar groups were found in EPS produced by *Vibrio harveyi* VB23 (Bramhachari & Dubey, 2006). Wang et al. (2014) reported the binding of EPS with heavy metals tend to modify the bending and stretching of various functional groups of polysaccharides. Metals tend to carry positive charge which interacts with negatively charged functional groups of EPS. The groups such as -OH, -COOH, -NH, -C=O, -CHO have been reported to interact with the metal ions. Moreover, EPS as well as other biopolymers have cross-linked mesh like structures that traps the metal, changing the overall charge of this large macromolecule (Pandya et al., 2015). Here we found the results that abides the previous findings suggesting binding of heavy metals to the EPS which were revealed using FTIR. The Use of FTIR has been extensive in studying interactions of metals with biopolymers like EPS. Kazy et al. (2002) have shown the interaction of radionuclides with EPS by the use of FTIR spectra. The shifting of peaks, as well as change in the intensity of the peak area suggest increase or decrease in the functional groups contents directly engaging with the metal (Yue et al., 2015).

5. Conclusions

It can be concluded that *Vibrio alginolyticus* PBR1 and *V. rotiferianus* PBL1 isolated from an Indian mackerel and squid, could produce exopolysaccharides and ~~even~~ bioluminescence. Pathogenicity of the strains were also studied where we proved the strains to be non-pathogenic and also reported sensitivity of antibiotics. The strains were screened for heavy metal sensitivity and resistance where we found resistance towards Pb and Sr. For certain metals the oligodynamic phenomenon was also observed. These findings could pave a path towards developing luminescence based biosensor. Both the strains under study could produce EPS and their characterization was performed for its heavy metal binding property. Such EPS metal binding study could help us in devising a sustainable bioremediation strategy.

6. Conflict of interest

All authors have none to declare.

7. Acknowledgments

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References

Ahn, S.-J., Lee, S.-J., Kook, J.-K., & Lim, B.-S. (2009). Experimental antimicrobial orthodontic adhesives using nanofillers and silver nanoparticles. *Dental Materials, 25*(2), 206-213. doi:10.1016/j.dental.2008.06.002

Ali, H., & Khan, E. (2019). Trophic transfer, bioaccumulation, and biomagnification of non-essential hazardous heavy metals and metalloids in food chains/webs—Concepts and implications for wildlife and human health. *Human and Ecological Risk Assessment: An International Journal, 25*(6), 1353-1376. doi:10.1080/10807039.2018.1469398

Alsina, M., & Blanch, A. R. (1994). A set of keys for biochemical identification of environmental Vibrio species. *Journal of Applied Bacteriology, 76*(1), 79-85. doi:10.1111/j.1365-2672.1994.tb04419.x

Amutha, K., & Shamini, S. (2015). Metal tolerance of bioluminescent bacteria isolated from marine organisms. *Indian Journal of Geo-Marine Sciences, 14*(11), 1799-1806.

Andhare, P., Goswami, D., Delattre, C., Pierre, G., Michaud, P., & Pathak, H. (2017). Edifying the strategy for the finest extraction of succinoglycan from Rhizobium radiobacter strain CAS. *Applied Biological Chemistry, 60*(3), 339-348. doi:10.1007/s13765-017-0286-8

Ansari, T., Marr, I., & Tariq, N. (2004). Heavy metals in marine pollution perspective-a mini review. *Journal of Applied Sciences, 4*(1), 1-20. doi:10.3923/jas.2004.1.20

Atlas, R. M., & Parks, L. C. (1997). *Handbook of microbiological media* (2nd ed.): Boca Raton: CRC Press

Azaman, F., Juahir, H., Yunus, K., Azid, A., Kamarudin, M. K. A., Toriman, M. E., . . . Saudi, A. S. M. (2015). Heavy metal in fish: Analysis and human health-a review. *Jurnal Teknologi, 77*(1). doi:10.11113/jt.v77.4182

Balachandar, R., Jayakumar, M., Thangaraja, A., Bharathiraja, B., Gurumoorthy, P., & Bharathy, S. D. (2010). Use of luminous bacteria as a biosensor for monitoring of heavy metals in marine water. *International Journal of Chemical Sciences, 8*(3), 1553-1566.

Balan, S. S., Raffi, S., & Jayalakshmi, S. (2013). Probing of potential luminous bacteria in Bay of Bengal and its enzyme characterization. *J. Microbiol. Biotechnol, 23*(6), 811-817. doi:10.4014/jmb.1206.06020

Balcazar, J. L., Vendrell, D., de Blas, I., Ruiz-Zarzuela, I., Girones, O., & Muzquiz, J. L. (2007). Quantitative detection of Aeromonas salmonicida in fish tissue by real-time PCR using self-quenched, fluorogenic primers. *Journal of Medical Microbiology, 56*(3), 323-328. doi:10.1099/jmm.0.46647-0

Beech, I., Hanjagsit, L., Kalaji, M., Neal, A. L., & Zinkevich, V. (1999). Chemical and structural characterization of exopolymers produced by Pseudomonas sp. NCIMB 2021 in continuous culture. *Microbiology, 145*(6), 1491-1497. doi:10.1099/13500872-145-6-1491

Borole, D., Sarin, M., & Somayajulu, B. (1982). Composition of Narbada and Tapti estuarine particles and adjacent Arabian Sea sediments. *Indian Journal of Geo-Marine Sciences, 11*(1). doi: http://nopr.niscair.res.in/handle/123456789/38918

Borrok, D. M., & Fein, J. B. (2005). The impact of ionic strength on the adsorption of protons, Pb, Cd, and Sr onto the surfaces of Gram negative bacteria: testing non-electrostatic, diffuse, and triple-layer models. *Journal of Colloid and Interface Science, 286*(1), 110-126. doi:10.1016/j.jcis.2005.01.015Get

Bramhachari, P., & Dubey, S. (2006). Isolation and characterization of exopolysaccharide produced by Vibrio harveyi strain VB23. *Letters in Applied Microbiology, 43*(5), 571-577. doi:10.1111/j.1472-765X.2006.01967.x

Bramhachari, P., Kishor, P. K., Ramadevi, R., Rao, B. R., & Dubey, S. K. (2007). Isolation and characterization of mucous exopolysaccharide (EPS) produced by Vibrio furnissii strain VB0S3. *Journal of Microbiology and Biotechnology*, *17*(1).

Brodl, E., Winkler, A., & Macheroux, P. (2018). Molecular Mechanism of Bacterial Bioluminescence. *Computational and Structural Biotechnology Journal, 16*. doi:10.1016/j.csbj.2018.11.003

Brown, C., & Kisiel, J. (2003). Squid Dissection From Pen to Ink. *Science Activities, 40*(1), 16-22. doi:10.1080/00368120309601108

Cano-Gomez, A., Høj, L., Owens, L., Baillie, B. K., & Andreakis, N. (2015). A multiplex PCR-based protocol for identification and quantification of Vibrio harveyi-related species. *Aquaculture, 437*, 195-200. doi:10.1016/j.aquaculture.2014.10.050

CH, R., & Mohanraju, R. (2019). A Review on Ecology, Pathogenicity, Genetics and Applications of Bioluminescent Bacteria. *Journal of Terrestrial and Marine Research, 3*, 1-32. doi:10.32610/JTMR.2019.v03i02.001

Chakraborty, S., Nair, G. B., & Shinoda, S. (1997). Pathogenic vibrios in the natural aquatic environment. *Reviews on Environmental Health, 12*(2), 63-80. doi:10.1515/REVEH.1997.12.2.63

CMFRI, K. (2015). CMFRI Annual Report 2014-2015.

Di Cello, F., & Fani, R. (1996). A molecular strategy for the study of natural bacterial communities by PCR-based techniques. *Minerva Biotecnologica, 8*(3), 126-134.

Dixit, R., Malaviya, D., Pandiyan, K., Singh, U. B., Sahu, A., Shukla, R., . . . Lade, H. (2015). Bioremediation of heavy metals from soil and aquatic environment: an overview of principles and criteria of fundamental processes. *Sustainability, 7*(2), 2189-2212. doi:10.3390/su7022189

Drouillard, S., Jeacomine, I., Buon, L., Boisset, C., Courtois, A., Thollas, B., . . . Helbert, W. (2015). Structure of an amino acid-decorated exopolysaccharide secreted by a Vibrio alginolyticus strain. *Marine Drugs, 13*(11), 6723-6739. doi:10.3390/md13116723

Dunlap, P. (2009). Bioluminescence microbiology. In M. Schachter (Ed.), *Encyclopedia of Microbiology* (Third ed., pp. 45-61). Oxford, UK: Elsevier.

Dunlap, P. (2014). Biochemistry and genetics of bacterial bioluminescence *Bioluminescence: Fundamentals and Applications in Biotechnology-Volume 1* (pp. 37-64): Springer.

Fernández-Bravo, A., Ballester, F., Pujol, I., Gomez-Bertomeu, F., & Martí, C. (2019). Vibrio alginolyticus Infections: Report of Two Cases from Spain with Literature Review. *J Med Microb Diagn, 8*(298), 2161-0703.1000298.

Garrity, G., Staley, J. T., Boone, D. R., De Vos, P., Goodfellow, M., Rainey, F. A., . . . Schleifer, K.-H. (2006). *Bergey's Manual® of systematic bacteriology: volume two: the proteobacteria*: Springer Science & Business Media

Gauger, E. J., & Gómez-Chiarri, M. (2002). 16S ribosomal DNA sequencing confirms the synonymy of Vibrio harveyi and V. carchariae. *Diseases of Aquatic Organisms, 52*(1), 39-46. doi:10.3354/dao052039

Gaur, N., Flora, G., Yadav, M., & Tiwari, A. (2014). A review with recent advancements on bioremediation-based abolition of heavy metals. *Environmental Science: Processes & Impacts, 16*(2), 180-193. doi:10.1039/c3em00491k

Gauri, S. S., Archanaa, S., Mondal, K. C., Pati, B. R., Mandal, S. M., & Dey, S. (2011). Removal of arsenic from aqueous solution using pottery granules coated with cyst of Azotobacter

and portland cement: Characterization, kinetics and modeling. *Bioresource Technology, 102*(10), 6308-6312. doi:10.1016/j.biortech.2011.02.037

Gbem, T., Balogun, J., Lawal, F., & Annune, P. (2001). Trace metal accumulation in Clarias gariepinus (Teugels) exposed to sublethal levels of tannery effluent. *Science of the Total Environment, 271*(1-3), 1-9. doi:10.1016/S0048-9697(00)00773-7

Girotti, S., Ferri, E. N., Fumo, M. G., & Maiolini, E. (2008). Monitoring of environmental pollutants by bioluminescent bacteria. *Analytica Chimica Acta, 608*(1), 2-29. doi:10.1016/j.aca.2007.12.008

Gomez-Gil, B., Thompson, F. L., Thompson, C. C., & Swings, J. (2003). Vibrio rotiferianus sp. nov., isolated from cultures of the rotifer Brachionus plicatilis. *International Journal of Systematic and Evolutionary Microbiology, 53*(Pt 1), 239-243. doi:10.1099/ijs.0.02430-0

Gupta, P., & Diwan, B. (2017). Bacterial Exopolysaccharide mediated heavy metal removal: A Review on biosynthesis, mechanism and remediation strategies. *Biotechnol Rep (Amst), 13*, 58-71. doi:10.1016/j.btre.2016.12.006

Gupta, T., & Mullins, M. C. (2010). Dissection of organs from the adult zebrafish. *J Vis Exp*(37), e1717. doi:10.3791/1717

Hassen, A., Saidi, N., Cherif, M., & Boudabous, A. (1998). Resistance of environmental bacteria to heavy metals. *Bioresource Technology, 64*(1), 7-15. doi:10.1016/S0960-8524(97)00161-2

Hastings, J. W., & Nealson, K. H. (1977). Bacterial bioluminescence. *Annual Review of Microbiology, 31*(1), 549-595. doi:10.1146/annurev.mi.31.100177.003001

Hong, Y., Chen, Z., Zhang, B., & Zhai, Q. (2010). Isolation of Photobacterium sp. LuB-1 and its application in rapid assays for chemical toxicants in water. *Letters in Applied Microbiology, 51*(3), 308-312. doi:10.1111/j.1472-765X.2010.02896.x

Intamat, S., Phoonaploy, U., Sriuttha, M., Tengjaroenkul, B., & Neeratanaphan, L. (2016). Heavy metal accumulation in aquatic animals around the gold mine area of Loei province, Thailand. *Human and Ecological Risk Assessment: An International Journal, 22*(6), 1418-1432. doi:10.1080/10807039.2016.1187062

Iyer, A., Mody, K., & Jha, B. (2004). Accumulation of hexavalent chromium by an exopolysaccharide producing marine Enterobacter cloaceae. *Marine Pollution Bulletin, 49*(11-12), 974-977. doi:10.1016/j.marpolbul.2004.06.023

Jebakumar, J. P. P., Nandhagopal, G., Sundradarajan, S., Karuppasamy, M., & Ragumaran, S. (2015). Eminence of heavy metal accumulation in fishes and crustaceans from the Gulf of Khambhat, India. *Current Science, 109*(3), 409-412. doi: https://www.jstor.org/stable/24906093

Jeon, Y.-S., Lee, K., Park, S.-C., Kim, B.-S., Cho, Y.-J., Ha, S.-M., & Chun, J. (2014). EzEditor: a versatile sequence alignment editor for both rRNA-and protein-coding genes. *International Journal of Systematic and Evolutionary Microbiology, 64*(2), 689-691. doi:10.1099/ijs.0.059360-0

Jia, A., Woo, N. Y., & Zhang, X. H. (2010). Expression, purification, and characterization of thermolabile hemolysin (TLH) from Vibrio alginolyticus. *Diseases of Aquatic Organisms, 90*(2), 121-127. doi:10.3354/dao02225

Jorgensen, J. H., & Turnidge, J. D. (2015). Susceptibility test methods: dilution and disk diffusion methods *Manual of Clinical Microbiology, Eleventh Edition* (pp. 1253-1273): American Society of Microbiology.

Kannahi, M., & Sivasankari, S. (2014). Isolation and Identification of Bioluminescent Bacteria from Marine Water at Nagapattinam Sea Shore Area. *International Journal of Pharmaceutical Science Review and Research, 59*, 346-351.

Kavita, K., Mishra, A., & Jha, B. (2013). Extracellular polymeric substances from two biofilm forming Vibrio species: characterization and applications. *Carbohydrate Polymers, 94*(2), 882-888. doi:10.1016/j.carbpol.2013.02.010

Kazy, S. K., Sar, P., Singh, S., Sen, A. K., & D'souza, S. (2002). Extracellular polysaccharides of a copper-sensitive and a copper-resistant Pseudomonas aeruginosa strain: synthesis, chemical nature and copper binding. *World Journal of Microbiology and Biotechnology, 18*(6), 583-588. doi:10.1023/A:1016354713289

Kiliç, N. K., Kürkçü, G., Kumruoğlu, D., & Dönmez, G. (2015). EPS production and bioremoval of heavy metals by mixed and pure bacterial cultures isolated from Ankara Stream. *Water Science and Technology, 72*(9), 1488-1494. doi:10.2166/wst.2015.365

Kulkarni, V. S., & Kulkarni, B. S. (2015). Isolation of Bioluminescent Bacteria and Their Application in Toxicity Testing of Chromium in Water. *Int. J. Curr. Microbiol. App. Sci, 4*(10), 23-32.

Kumar, A. R. (2010). *Isolation of luminescent bacteria from bay of Bengal and their molecular characterization.* University of Borås.

Kumar, P. A., Joseph, B., & Patterson, J. (2011). Antibiotic and heavy metal resistance profile of pathogens isolated from infected fish in Tuticorin, south-east coast of India. *Indian Journal of Fisheries, 58*(2), 121-125.

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Molecular Biology and Evolution, 35*(6), 1547-1549. doi:10.1093/molbev/msy096

Lal, M. T. B. M., & Ransangan, J. (2013). Taxonomic classification of Vibrio harveyi using 16S rDNA and atpA gene sequencing method. *DNA, 5*, 3.

Langaoen, A. F., Manzano, V. J. V., Requilman, E. M. R., Tabardillo, J. M., Maningas, M. B. B., & Calugay, R. J. (2018). Antibiotic-resistant bioluminescent vibrios from Philippine aquacultured Chanos chanos and Oreochromis niloticus. *Aquaculture, Aquarium, Conservation & Legislation, 11*(2), 505-515.

Larson, E., Morton, H., & Block, S. (1991). Disinfection, sterilization, and preservation. *Alcohols. Ed. Block SS. Lea and Febiger, Philadelphia*, 91.

Lin, B., Wang, Z., Malanoski, A. P., O'Grady, E. A., Wimpee, C. F., Vuddhakul, V., . . . Vora, G. J. (2010). Comparative genomic analyses identify the Vibrio harveyi genome sequenced strains BAA- 1116 and HY01 as Vibrio campbellii. *Environmental Microbiology Reports, 2*(1), 81-89. doi:10.1111/j.1758-2229.2009.00100.x

Lin, L. Y., & Meighen, E. A. (2009). *Bacterial Bioluminescence*. Retrieved from http://photobiology.info/Lin.html

Loo, Y., Skell, P., Thornberry, H., Ehrlich, J., McGuire, J., Savage, G., & Sylvester, J. (1945). Assay of streptomycin by the paper-disc plate method. *Journal of Bacteriology, 50*(6), 701.

Madigan, M. T., Clark, D. P., Stahl, D., & Martinko, J. M. (2010). *Brock biology of microorganisms 13th edition*: Benjamin Cummings

Makemson, J. C., & Hermosa Jr, G. V. (1999). Luminous bacteria cultured from fish guts in the Gulf of Oman. *Luminescence: the journal of biological and chemical luminescence, 14*(3), 161-168. doi:10.1002/(SICI)1522-7243(199905/06)14:3<161::AID-BIO538>3.0.CO;2-A

Malave-Orengo, J., Rubio-Marrero, E., & Rios-Velazquez, C. (2010). Isolation and characterization of bioluminescent bacteria from marine environments of Puerto Rico. *Technology and Education Topics in Applied Microbiology and Microbial Biotechnology, 1*(2), 103-108.

Marín, M., Garcia-Lechuz, J., Alonso, P., Villanueva, M., Alcala, L., Gimeno, M., . . . Bouza, E. (2012). Role of universal 16S rRNA gene PCR and sequencing in diagnosis of prosthetic joint infection. *Journal of Clinical Microbiology, 50*(3), 583-589. doi:10.1128/JCM.00170-11

Martinez, R. E., Smith, D. S., Kulczycki, E., & Ferris, F. G. (2002). Determination of intrinsic bacterial surface acidity constants using a Donnan shell model and a continuous pKa distribution method. *Journal of Colloid and Interface Science, 253*(1), 130-139. doi:10.1006/jcis.2002.8541

Mertz, W. (2012). *Trace elements in human and animal nutrition* (Vol. 2): Elsevier

Mitra, A., & Choudhury, A. (1993). Trace metals in macrobenthic molluscs of the Hooghly estuary, India. *Marine Pollution Bulletin, 26*(9), 521-522.

Mohseni, M., Abbaszadeh, J., Maghool, S.-S., & Chaichi, M.-J. (2018). Heavy metals detection using biosensor cells of a novel marine luminescent bacterium Vibrio sp. MM1 isolated from the Caspian Sea. *Ecotoxicology and Environmental Safety, 148*, 555-560. doi:10.1016/j.ecoenv.2017.11.002

Muralidharan, J., & Jayachandran, S. (2003). Physicochemical analyses of the exopolysaccharides produced by a marine biofouling bacterium, Vibrio alginolyticus. *Process Biochemistry, 38*(6), 841-847. doi:10.1016/S0032-9592(02)00021-3

Murthy, L., Mohan, C., Ravishankar, C., & Badonia, R. (2013). Biochemical quality and heavy metal content of fish meal and squid meal produced in Veraval, Gujarat. *Indian Journal of Fisheries, 60*(3), 113-117. doi: http://hdl.handle.net/123456789/1718

Murthy, L. N., Panda, S. K., Khasim, D. I., & Badonia, R. (2009). Monitoring of cadmium accumulation in cephalopods processed in Gujarat Cost. *Asian fisheries science, 22*, 319-330. doi: http://hdl.handle.net/123456789/1105

Murthy, L. N., Panda, S. K., Madhu, V., Ashokan, P., Ghosh, S., Das, S., & Badonia, R. (2008). Cadmium in the purple back flying squid sthenoteuthis oualanienisis (Lesson 1830) along north west coast of India. *Journal of the Marine Biological Association of India, 50*(2), 191-195.

Naik, M. M., & Dubey, S. K. (2013). Lead resistant bacteria: lead resistance mechanisms, their applications in lead bioremediation and biomonitoring. *Ecotoxicology and Environmental Safety, 98*, 1-7. doi:10.1016/j.ecoenv.2013.09.039

Nawaz, A., & Ahmed, N. (2011). Isolation and characterization of indigenous luminescent marine bacteria from Karachi coast. *Academic Research International, 1*(2), 74.

Nichols, C. M., Lardiere, S. G., Bowman, J. P., Nichols, P. D., J, A. E. G., & Guezennec, J. (2005). Chemical characterization of exopolysaccharides from Antarctic marine bacteria. *Microbial Ecology, 49*(4), 578-589. doi:10.1007/s00248-004-0093-8

Nkoh, J. N., Lu, H. L., Pan, X. Y., Dong, G., Kamran, M. A., & Xu, R. K. (2019). Effects of extracellular polymeric substances of Pseudomonas fluorescens, citrate, and oxalate on Pb sorption by an acidic Ultisol. *Ecotoxicology and Environmental Safety, 171*, 790-797. doi:10.1016/j.ecoenv.2019.01.043

Oem, J. K., Lee, S. Y., Kim, Y. S., Na, E. J., & Choi, K. S. (2019). Genetic characteristics and analysis of a novel rotavirus G3P[22] identified in diarrheic feces of Korean rabbit. *Infection, Genetics and Evolution, 73*, 368-377. doi:10.1016/j.meegid.2019.06.003

Organization, W. H. (1996). *Trace elements in human nutrition and health*

Owens, L., & Busico-Salcedo, N. (2006). Vibrio harveyi: pretty problems in paradise *The biology of vibrios* (pp. 266-280): American Society of Microbiology.

Pagnanelli, F., Petrangeli Papini, M., Toro, L., Trifoni, M., & Vegliò, F. (2000). Biosorption of metal ions on Arthrobacter sp.: biomass characterization and biosorption modeling. *Environmental Science & Technology, 34*(13), 2773-2778. doi:10.1021/es991271g

Pandya, V. M., Kortz, U., & Joshi, S. A. (2015). Encapsulation and stabilization of polyoxometalates in self-assembled supramolecular hydrogels. *Dalton Trans, 44*(1), 58-61. doi:10.1039/c4dt01372g

Parasar, G., Gavali, N., & Patil, T. (2016). Isolation and characterisation of bioluminescent bacteria. *International Journal for Science and Research in Technology, 2*(5), 40-42. doi:10.15242/IJAAEE.C0215136

Parmar, P., Shukla, A., Rao, P., Saraf, M., Patel, B., & Goswami, D. (2020). The Rise of Gingerol as Anti-QS Molecule: Darkest Episode in the LuxR-Mediated Bioluminescence Saga. *Bioorganic Chemistry, 99*, 103823. doi:10.1016/j.bioorg.2020.103823

Parvez, S., Venkataraman, C., & Mukherji, S. (2006). A review on advantages of implementing luminescence inhibition test (Vibrio fischeri) for acute toxicity prediction of chemicals. *Environment International, 32*(2), 265-268. doi:10.1016/j.envint.2005.08.022

Peng, T., Kan, J., Lun, J., & Hu, Z. (2019). Identification of novel sRNAs involved in oxidative stress response in the fish pathogen Vibrio alginolyticus by transcriptome analysis. *Journal of Fish Diseases, 42*(2), 277-291. doi:10.1111/jfd.12926

Pettibone, G., Mear, J., & Sampsell, B. (1996). Incidence of antibiotic and metal resistance and plasmid Carriage in Aeromonas isolated from brown bullhead (latalurus nebulosus). *Letters in Applied Microbiology, 23*(4), 234-240. doi:10.1111/j.1472-765X.1996.tb00073.x

Prafulla, V., Francis, L., & Lakshmanan, P. (2001). Concentrations of trace metals in the squids, Loligo duvauceli and Doryteuthis sibogae caught from the southwest coast of India. *Journal of Aquatic Science, 2*(2), 5-10. doi:10.12691/jas-2-2-1

Rajeshkumar, S., Liu, Y., Zhang, X., Ravikumar, B., Bai, G., & Li, X. (2018). Studies on seasonal pollution of heavy metals in water, sediment, fish and oyster from the Meiliang Bay of Taihu Lake in China. *Chemosphere, 191*, 626-638. doi:10.1016/j.chemosphere.2017.10.078

Ramesh, C., & Mohanraju, R. (2017). Genetic diversity of bioluminescent bacteria in diverse marine niches. *Indian Journal of Geo-Marine Sciences, 46*(10), 2054-2062.

Ranjitha, P., & Karthy, E. (2012). Detection of heavy metal resistance bioluminescence bacteria using microplate bioassay method. *Journal of Environmental Science & Engineering, 54*(1), 43-49.

Ransangan, J., & Mustafa, S. (2009). Identification of Vibrio harveyi isolated from diseased Asian seabass Lates calcarifer by use of 16S ribosomal DNA sequencing. *Journal of Aquatic Animal Health, 21*(3), 150-155. doi:10.1577/H09-002.1

Rui, H., Liu, Q., Ma, Y., Wang, Q., & Zhang, Y. (2008). Roles of LuxR in regulating extracellular alkaline serine protease A, extracellular polysaccharide and mobility of Vibrio

alginolyticus. *FEMS Microbiology Letters, 285*(2), 155-162. doi:10.1111/j.1574-6968.2008.01185.x

Ruwandeepika, H. A. D., Jayaweera, T. S. P., Bhowmick, P. P., Karunasagar, I., Bossier, P., & Defoirdt, T. (2012). Pathogenesis, virulence factors and virulence regulation of vibrios belonging to the Harveyi clade. *Reviews in Aquaculture, 4*(2), 59-74. doi:10.1111/j.1753-5131.2012.01061.x

Salle, A. J., & Salle, A. (1954). Fundamental principles of bacteriology: McGraw-Hill.

Shanware, A., Thakre, N., & Pande, S. (2013). Isolation and characterization of novel marine luminescent bacteria from Diu beach, India. *Journal of Pharmacy Research, 7*(6), 529-533. doi:10.1016/j.jopr.2013.05.019

Shinoda, S. (1999). Protein toxins produced by pathogenic vibrios. *Journal of Natural Toxins, 8*(2), 259-269. doi: http://www.ncbi.nlm.nih.gov/pubmed/10410336

Shukla, A., Mehta, K., Parmar, J., Pandya, J., & Saraf, M. (2019b). Depicting the Exemplary Knowledge of Microbial Exopolysaccharides in a Nutshell. *European Polymer Journal*. doi:10.1016/j.eurpolymj.2019.07.044

Shukla, A., Parmar, P., Goswami, D., Patel, B., & Saraf, M. (2020a). Characterization of Novel Thorium Tolerant Ochrobactrum intermedium AM7 In Consort with Assessing its EPS-Thorium Binding. *Journal of Hazardous Materials*, 122047. doi:10.1016/j.jhazmat.2020.122047

Shukla, A., Parmar, P., Rao, P., Goswami, D., & Saraf, M. (2020b). Twin Peaks: Presenting the Antagonistic Molecular Interplay of Curcumin with LasR and LuxR Quorum Sensing Pathways. *Current Microbiology*. doi:10.1007/s00284-020-01997-2

Shukla, A., Parmar, P., Saraf, M., & Patel, B. (2019a). Isolation and screening of bacteria from radionuclide containing soil for bioremediation of contaminated sites. *Environmental Sustainability*, 1-10. doi:10.1007/s42398-019-00068-y

Stern, B. R. (2010). Essentiality and toxicity in copper health risk assessment: overview, update and regulatory considerations. *Journal of Toxicology and Environmental Health. Part A, 73*(2), 114-127. doi:10.1080/15287390903337100

Subramanian, S. B., Yan, S., Tyagi, R. D., & Surampalli, R. (2010). Extracellular polymeric substances (EPS) producing bacterial strains of municipal wastewater sludge: isolation, molecular identification, EPS characterization and performance for sludge settling and dewatering. *Water Research, 44*(7), 2253-2266. doi:10.1016/j.watres.2009.12.046

Sundararajan, S., Khadanga, M. K., Kumar, J., Raghumaran, S., Vijaya, R., & Jena, B. K. (2017). Ecological risk assessment of trace metal accumulation in sediments of Veraval Harbor, Gujarat, Arabian Sea. *Marine Pollution Bulletin, 114*(1), 592-601. doi:10.1016/j.marpolbul.2016.09.016

Tak, H., Ahmad, F., & Babalola, O. (2013). Advances in the application of plant growth-promoting rhizobacteria in phytoremediation of heavy metals. *Reviews of Environmental Contamination and Toxicology*. doi:10.1007/978-1-4614-5577-6_2

Tanet, L., Tamburini, C., Baumas, C., Garel, M., Simon, G., & Casalot, L. (2019). Bacterial bioluminescence: light emission in photobacterium phosphoreum is not under quorum-sensing control. *Frontiers in microbiology, 10*, 365. doi:10.3389/fmicb.2019.00365

Thakre, N. A., & Shanware, A. S. (2015). Promising biological indicator of heavy metal pollution: bioluminescent bacterial strains isolated and characterized from marine niches of Goa, India. *Indian journal of microbiology, 55*(3), 327-332.

Thompson, F. L., Iida, T., & Swings, J. (2004). Biodiversity of vibrios. *Microbiology and Molecular Biology Reviews, 68*(3), 403-431, table of contents. doi:10.1128/MMBR.68.3.403-431.2004

Tien, D.-C., Tseng, K.-H., Liao, C.-Y., Huang, J.-C., & Tsung, T.-T. (2008). Discovery of ionic silver in silver nanoparticle suspension fabricated by arc discharge method. *Journal of Alloys and Compounds, 463*(1-2), 408-411. doi:10.1016/j.jallcom.2007.09.048

Tomova, I., Stoilova-Disheva, M., Lazarkevich, I., & Vasileva-Tonkova, E. (2015). Antimicrobial activity and resistance to heavy metals and antibiotics of heterotrophic bacteria isolated from sediment and soil samples collected from two Antarctic islands. *Frontiers in Life Science, 8*(4), 348-357. doi:10.1080/21553769.2015.1044130

Toroglu, S., Toroglu, E., Dincer, S., Kara, C., & Kertmen, M. (2009). Resistances of antibiotics and heavy metals in Enterobacteriaceae spp. isolated from gills and intestines of Achanthobrama marmid (Heckel, 1843) from Sir Dam lake Turkey. *Journal of Environmental Biology, 30*(1), 23-31.

Urbanczyk, H., Ogura, Y., & Hayashi, T. (2014). Contrasting inter-and intraspecies recombination patterns in the "Harveyi clade" vibrio collected over large spatial and temporal scales. *Genome Biology and Evolution, 7*(1), 71-80. doi:10.1093/gbe/evu269

Vishwakarma, A., Mathur, K., & Suneetha, V. (2013). Screening and tentative identification of bioluminescence producing bacteria in fish. *International Journal of Fisheries and Aquatic Studies, 1*(6), 225-231.

Wang, J., Li, Q., Li, M. M., Chen, T. H., Zhou, Y. F., & Yue, Z. B. (2014). Competitive adsorption of heavy metal by extracellular polymeric substances (EPS) extracted from sulfate reducing bacteria. *Bioresource Technology, 163*, 374-376. doi:10.1016/j.biortech.2014.04.073

Wayne, P. (2008). Performance Standards For Antimicrobial Susceptibility Testing, Ninth Informational Supplement. *NCCLS DOCUMENT*.

Willey, J., Sherwood, L., & Woolverton, C. (2008). Prescott, Harley, and Klein's Microbiology. 7th. *New York, NY, 10020*, 1088.

Woodling, J., Brinkman, S., & Horn, B. (2001). Nonuniform accumulation of cadmium and copper in kidneys of wild brown trout (Salmo trutta) populations. *Archives of Environmental Contamination and Toxicology, 40*(3), 381-385. doi:10.1007/s002440010186

Yaser, N. A., Abdullah, M. F. F., Aris, A. M., & Zainudin, I. I. (2014). Isolation and identification of bioluminescent bacteria in squid and water of Malaysia. *Int. J. Adv. Agric. Environ. Eng, 1*, 225-228.

Ye, J., Ma, Y., Liu, Q., Zhao, D. L., Wang, Q. Y., & Zhang, Y. X. (2008). Regulation of Vibrio alginolyticus virulence by the LuxS quorum-sensing system. *Journal of Fish Diseases, 31*(3), 161-169. doi:10.1111/j.1365-2761.2007.00882.x

Yin, K., Wang, Q., Lv, M., & Chen, L. (2019). Microorganism remediation strategies towards heavy metals. *Chemical Engineering Journal, 360*, 1553-1563. doi:10.1016/j.cej.2018.10.226

Yoon, S. H., Ha, S. M., Kwon, S., Lim, J., Kim, Y., Seo, H., & Chun, J. (2017). Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. *International Journal of Systematic and Evolutionary Microbiology, 67*(5), 1613-1617. doi:10.1099/ijsem.0.001755

Yu, Q., Liu, M., Su, H., Xiao, H., Wu, S., Qin, X., . . . Li, P. (2019). Selection and characterization of ssDNA aptamers specifically recognizing pathogenic Vibrio alginolyticus. *Journal of Fish Diseases, 42*(6), 851-858. doi:10.1111/jfd.12985

Yuan, W., Cheng, J., Huang, H., Xiong, S., Gao, J., Zhang, J., & Feng, S. (2019). Optimization of cadmium biosorption by Shewanella putrefaciens using a Box-Behnken design. *Ecotoxicology and Environmental Safety*, *175*, 138-147. doi:10.1016/j.ecoenv.2019.03.057

Yue, Z. B., Li, Q., Li, C. C., Chen, T. H., & Wang, J. (2015). Component analysis and heavy metal adsorption ability of extracellular polymeric substances (EPS) from sulfate reducing bacteria. *Bioresource Technology*, *194*, 399-402. doi:10.1016/j.biortech.2015.07.042

Zahaba, M., Halmi, M. I. E., Ahmad, S. A., Shukor, M. Y., & Syed, M. A. (2015). Isolation and characterization of luminescent bacterium for sludge biodegradation. *Journal of Environmental Biology*, *36*(6), 1255.

Zhang, Z., Schwartz, S., Wagner, L., & Miller, W. (2000). A greedy algorithm for aligning DNA sequences. *Journal of Computational Biology*, *7*(1-2), 203-214. doi:10.1089/10665270050081478

Zhang, Z., Yu, Y. x., Jiang, Y., Wang, Y. g., Liao, M. j., Rong, X. j., . . . Chen, J. (2019). First report of isolation and complete genome of Vibrio rotiferianus strain SSVR1601 from cage- cultured black rockfish (Sebastes schlegelii) associated with skin ulcer. *Journal of Fish Diseases*, *42*(5), 623-630.

Table 1: Various characteristics of PBR1 and PBL1 compared with closest related species

Sr. No.	Characteristics	Bacteria					
		PBR1	**PBL1**	***V. harveyi***	***V. parahaemolyticus***	***V. natriegens***	***V. campbellii***
A	***Physiological & Phenotypical*:***						
01	Luminescence	+	+	+	-	-	-
02	Gram reaction	-	-	-	-	-	-
03	Motility	+	+	+	+	-	+
04	Growth on NaCl (% w/v)						
	0	-	-	-	-	-	-
	3	+	+	+	+	+	+
	6	+	+	+	+	D	D
	8	+	-	V	+	+	V
	10	-	-	-	-	-	-
05	Growth at Temperature (℃)						
	4	-	+	-	-	-	-
	20	+	+	ND	+	+	+
	30	+	+	+	+	+	+
	35	+	+	+	+	+	+
	40	+	+	D	+	+	-
B	***Substrate Utilization*:***						
06	Citrate	-	-	V	+	-	V
07	Gelatin	+	+	+	+	+	+
08	Starch	-	-	+	ND	ND	ND
09	Tributyrin	-	-	+	ND	ND	ND
C	***Enzyme Production*:***						
10	Arginine dehydrolase	-	-	-	-	-	-
11	Lysin decarboxylase	+	+	V	+	-	D
12	Ornithin decarboxylase	-	+	V	+	-	-
13	Catalase	+	+		+	+	+
14	Oxidase	+	+	+	+	-	+
15	Nitrate reductase	-	-	+	+	+	+
16	Urease	-	-	V	V	V	-
D	***Others*:***						
17	Voges-Praskauer	-	-	-	-	-	-
18	Indole	+	-	+	+	-	+
E	***Sugar Fermentation (Acid Production)†:***						
19	Adonitol	-	-	-	-		
20	Arabinose	-	-	D	+	D	-
21	Cellobiose	+	+	+	-	+	D
22	Dextrose	+	+	V	+	ND	ND
23	Dulcitol	-	-	-	-	ND	ND
24	Fructose	+	+			ND	ND
25	Galactose	+	+	D	+	D	-
26	Malibiose	-	-	-	-	+	-
27	Maltose	+	+	+	+	ND	ND
28	Mannose	+	+	+	+	+	D
29	Raffinose	-	-	-	-	ND	ND
30	Rhamnose	-	-	-	-	+	-
31	Salicin	-	-	D	-	D	-
32	Sorbitol	-	-	-	-	ND	-
33	Sucrose	+	+	-	-	+	-
34	Trehalose	+	+	+	+	+	+
35	Xylose	-	-	-	-	-	-

+, positive; -, negative; V; variable between strains; D, delayed positive; ND, no data.

*(Alsina & Blanch, 1994), †(Garrity et al., 2006)

Table 2: Effect of various antibiotics on isolated strains PBR1 and PBL1

R, resistant; S, susceptible3

Sr. No.	Antibiotic	Strength (mcg)	Zone diameter (mm)		Resistant/Susceptible	
			PBR1	PBL1	PBR1	PBL1
01.	*Amikacin*	30	-	-	R	R
02.	*Ampicillin*	20	-	-	R	R
03.	*Cefotaxime*	30	-	-	R	R
04.	*Ceftizoxime*	30	-	-	R	R
05.	*Cephalexin*	30	-	-	R	R
06.	*Chloramphenicol*	30	40	22	S	S
07.	*Ciprofloxacin*	5	21	13	S	S
08.	*Cloxacillin*	1	-	-	R	R
09.	*Co-trimoxazole*	25	33	19	S	S
10.	*Gatifloxacin*	10	10	8	S	S
11.	*Gentamicin*	10	-	-	R	R
12.	*Levofloxacin*	5	15	10	S	S
13.	*Lincomycin*	2	-	-	R	R
14.	*Linezolid*	30	25	25	S	S
15.	*Ofloxacin*	5	-	-	R	R
16.	*Piperacillin*	100	-	-	R	R
17.	*Roxythromycin*	15	14	8	S	S
18.	*Tetracycline*	30	-	-	R	R