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ORIGINAL ARTICLE **OPEN ACCESS**

Isolation and characterizationof bioluminescent bacteria *Photobacterium leiognathi* from Pony fish *Secutorruconius*

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ABSTRACT

The bioluminescent bacteria are highly diverse and abundant in the marine ecosystem and are commonly subsisted on the surface of several marine flora, fauna, open seawater, and in digestive tracts of marine fish. The present study aims to isolate the bright strain,Photobacterium sp. from marine fish and study its growth kinetics and physicochemical characteristics for over24 hours. The growth kinetic study revealed optimized temperature and pH as 25°C and 7.5 for bioluminescent bacterial sustainability, respectively. Also, the molecular and phylogenetic analysishave prophesied the isolated strain as Photobacterium leiognathi. Further, the strain was evaluated for primary pathogenicity and found to have no toxicity through hemolytic assay.The isolated strain,P. leiognathiwas also assessed for Heavy metal analysis and showed more sensitivity with Mn, Zn, Se, and Cu elements. Therefore, the study achieves that the bright bacterial isolate was isolated as P. leiognathi with no pathogenicity and thus could also be used as a marker for selected heavy metal identification due to their heavy metal sensitivity.

Keywords:*Photobacterium, Bacterial growth kinetics,Pathogenicity, Heavy metal sensing.*

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INTRODUCTION

Bioluminescence is a beautiful fragment of the ocean which is produced through the phenomenon of a natural living organism by the chemical reaction. As the sun sets on the horizon, glimpses of nature could observe the occurrence of glittering glow appearing in the ecosystem which could have been fascinated by its attractiveness, shown by the variety of organisms, it is called bioluminescence. In the dark environment, bioluminescence organisms are very easy to maintain and have evolved through brighter light lacking response by aflame a small number of fossil fuels, and thus are called living light.

Bioluminescence is one of the property can magnificent the benefits of ocean livelihood towards the human population and which could also add beauty to the sea [1]. Since 1600 A.D. this fascinating phenomenon has had an ancient history [2], and its mystery is well known [3]. Bioluminescent bacteria are highly diverse and abundant marine microorganisms. The bioluminescent-producing bacteria are also attached tothe surface of several marine flora and fauna. Bioluminescent bacteria are commonly present in marine water and normally prevail in the digestive tracts of marine fish. Some luminescent bacteria are symbiotic in marine fish and squid, they abide in light organs and sometimes dwell on the surface of the fish. The importance of bioluminescence in nature is to attract prey, deter predators, camouflage, and aid in hunting [4].The gross health of the microorganisms and the bioluminescence reaction is reflexed by the bioluminescent intensity with reflex actions and an expansive range of sensitive toxic substances. Additionally to this, marine microorganisms possess the proficiency to endure very low temperatures and high salinity [5].

In the past decades, the studies of bioassay on toxicants using bacteria as a sensing organism have been promoted because of their sensitivity, ease to use, cost-effective and fast responses for detection and evaluation of environmental toxicity [6], [7], and [8]. The significance ofthe application of luminous bacteria in different aspects has been studied [9]. This bioluminescenceis used for sensing and hygiene control for many industries such as fish and milk industries, moreover,bioluminescence-based assays apply for mapping pollution in the ecosystem and sensing pH. Metal ions, transmembrane potential, drug molecules, other metabolites, gene assays, the observation of protein-protein interactions. The bioluminescent bacteria act as biosensors for various marine environmental studies such as toxicity tests

and water purification [10]. And these bacteria monitor different environmental pollutants also, such asair, seawater, sediment, soil, surface water, groundwater, and tap water [8]. The biosensor is a methodical device that uses gratitude constituent habitually biomolecules, to the analyzed contaminant [11].

The biosensor is a methodical device that uses biological gratitude constituent habitual biomolecules, to an analyzed contaminant [11], [12]. Biosensors are cheap, rapid, selective, and usually specific. Based on several criteria, biosensors could be categorized as affinity and catalytic depending on the activities of the bio recognition elements [13]. They can be classified (based on bio recognition element used) as an enzyme, nucleic acid,cofactor, antibody receptors, organelle, cells, tissues, liposomes, molecular imprinted polymers, and microbial biosensors [13], [14] stated that the choice of biological response agent depends on the analyte, specificity, and storage environmental stability. Microbial biosensors immobilize the microorganisms on a transducer for the detection of analytes. Though numerous microorganisms could be used as microbial biosensors, only bacteria are ideal as they are major constituents of several environmental biotic community and they control most of the ecosystem functions in terms of biogeochemical cycling [15]. Whole-cell bacterial biosensor generally operates on the principle of bioluminescence and can be constitutively expressed (bioluminescent in the absence of the toxicant) or induced (bioluminescent in the existence of a specific substrate) (Table1). Numerous bacterial biosensors with anecdotal detection limits have been developed and successfully applied by [16]. The detection capacity of bioluminescent biosensors ranges from rather a negligible level to very high contaminant concentrations [11].

MATERIAL AND METHODS

Isolation of Bioluminescent bacteria

Ponyfish, *Secutorruconius* was collected from the Mudasalodai landing center. Live fish was collected and protected from sunlight. The fish was transferred to an icebox and carried to the laboratory for further analysis. They were washed with 1% sodium hypochlorite mixed with sterile seawater to remove loosely bounded organisms and dirt. The sample was swabbed below the scale lining the bioluminescent region of the fish and spread over a Zobell Marine Agar plate. The plates were prepared in triplicate and incubated at RT for 1 d in dark. All the techniques were performed in strict aseptic conditions and in complete darkness. A single luminescent colony was observed after the incubation period. The luminescent bacterium was isolated from the mother plate and sub-cultured until single colony formation in luminescent Agar.

Screening of Bioluminescent bacteria

Subsequently, isolated single colonies have undergone screening for the characterization of phenotype and genotype.The genotype characterization was carried out by 16S rRNA gene sequence analysis. The screening of the colonies was done through the gram staining and TCBS Agar test followed by starch agar test and the plenary biochemical test including Catalase, Oxidative, Nitrate reduction, Methyl red test, Indole formation, and Voges- Proskauer.

Hemolytic assay

Hemolytic activity is regarded as the preliminary test for pathogenicity. Blood agar plates were used to scrutinize bacteria separately for their proficiency to produce blood hemolysis. The blood agar media was prepared after cooling, chicken and lamb blood were added and mixed well and then poured into the plates. The blood was freshly assimilated freshly and ethylenediamine tetraacetic acid (EDTA) to prevent coagulation. Subsequently to solidification of a blood agar plate, the bacteria were deliberately streaked and then incubated at 35°C for the period of 24 hours. Followed by incubation the various activities were observed.

pH Optimization

The luminescent broth preparation was accomplished by various pH values arranged in ascending order from minimum to maximum values such as 2.5, 3.5, 4.5, 5.5, 6.5, 7, 7.5, 8.5.9.5, 10.5, and 11.5. The pH was adjusted in the broth considering both acid and base. This calibrated pH broth was then autoclaved, later from the cooled mediaone loop of bioluminescent bacteria from stationary culture was added and allowed to incubate for 24 hours. Then the highly intensive culture was observed through naked eyes in a dark room along with the OD values

Temperature Optimization

Further, LB broth was prepared and 10µl bacterial culture was added to it and then this was kept aside for incubation at contrasting temperatures with intervals of 5, up to 40°C. After the incubation absorbance was measured every two hours at 600 nm till it reached the death phase.

Growth curve

The growth curve depicts the accumulation of the population of cells in liquid culture and is obtained by the measurement of optical density (OD) of cell populations. The simplest method to support strength beginning growth curves is by approximation of the growth rate (i.e., Malthusian parameter) through the exponential growth phase, using the grade record of the growth curve.

The overnight culture was prepared by inoculating pure colony into 100ml of LB broth and it was incubated. The absorbance was measured at 600nm every two hours of an interval, till the broth reached the death phase.

Heavy metal sensitivity assay

Heavy metal sensitivity assay was performed to study its sensitivity as a natural biosensor to detect ten common metal ions such as mercury, cadmium, zinc, copper, selenium, cobalt, and magnesiumin the form of mercuric chloride, cadmium, coarse powder, zinc sulfate, selenium dioxide, cobalt sulfate, both magnesium carbonate, and magnesium sulfate. Concentrations of 10mM into 30µL, 60µL, and 90µL were tested, following the method as described by [17] with slight modifications. Stock solutions of 1M of all heavy metal were prepared by dissolving each of these in an appropriate volume of distilled water and agitated until complete homogenous solutions were achieved. Stock solutions were further diluted to 10mM concentration and used in the study.

Agar well diffusion method was performed by the metal sensitivity test followed by [18] slightly modified. NaCl (3%) was mixed with Mueller Hinton agar and poured in to plate and the overnight culture broth was swabbed on the entire surface of the plates. A sterilecork borer was used to make Wells was on these plates and added with three different concentrations of all theheavy metal solutions were and then impregnated into the wells.Plates are allowed to incubate 24 hours at room temperature and zones of formation were measured. In this method,a highly rapid and consistent heavy metal analysis process was involved. The nutrient broth was also used for the analysis of heavy metals based on time.

RESULT

Screening of Bioluminescent bacteria

A bioluminescent bacterium was isolated from a marine fish sample. The strain showed higher luminescence intensity in the luminescent agar as compared to the Zobell Marine Agar. The isolated bacteria were isolated on Luminescent agar and subcultured in the same media. The morphological appearance of these strains exposed different individuality related to Photobacterium. The bacterium exhibited green colonies on TCBS Agar and they were showed catalase-positive, Gram-negative, motile, short, rod shape, and similar in nature to colonies of photobacterium. The isolates were also found to exhibita small colony size with convex elevation. The isolated colonies were showedpositive oxidase, nitrate reduction, and methyl red test. Indole formation was not found, nevertheless, they were positive to Voges-Proskauer.

The genotypic characterization of isolated colonies through the 16S rRNA gene sequence expressed their group in separate clusters related to Photobacterium.16S rRNA gene sequence of the strains was found to show a sequence similarity range of 99% for *Photobacterium* species in the GenBank database. Phylogenetic analysis of 16s rRNA gene sequences of the strains wasobtained with the top three BLAST search hits and found unambiguous clustering in distinct groupsof*Photobacteriumleiognathi*.The same sequence of photobacterium strain was submitted to the GenBank database and published with the accession numberMW854409.

pH

Bioluminescence isnot significantly affected by pH in a liquid medium. However, the result was found optimum pHat the range between 6.5 to 8.5 (Graph 1) for the encouraging sustenance of luminescence by luminescent bacteria. Amusingly, all the isolates circumscribed exhibited substantial luminescence in broth with pH 11.

Temperature

The temperature has played an extremely critical role in bioluminescence. Although the bacteria grow well at 20 to 30°C the optimized temperature for bioluminescent bacteria was foundat 25°C (Graph 2).

Bacterial Growth

The isolated photobacterial growth was analyzed for 24 hours along with itsbioluminescence intensity variations.The study results showed that bioluminescent bacteria were found to attain theiroptimum level of growththat around16hoursof incubation and the maximum fluorescence intensity of the bacteria was observed within 8 hours from the inoculum. Further, the most favorable culturing time duration was also found as 8-16 hours, of incubation to ensure good quality signal of emission (Graph3).

Pathogenicity of photo bacteria

The consequences of hemolytic activity of photo bacteria were evaluated on chicken and goat blood agar. The assay result depicted that the photo bacteriahas no hemolytic property towards any blood cells like erythrocytes, platelets, and lymphocytes.

Photobacterium as a biosensor for heavy metals

These bioluminescent bacteria were subjected to check the toxicity of heavy metals. The sensitivity assay showed better metalresistance to cadmium and sensitivity to remaining heavy metal at all tested concentrations i.e., 10mM to 30µl, 60µl, and 90µl. Based on the inhibition zones, the sensitivity was observed for cadmium (Fig. 2) and the maximum zone of inhibition was observed for magnesium with 30mm, 35mm, and 40mm of zone formation for the concentration of 30, 60, and 90µl, respectively.

Fig-1 Isolation bioluminescent bacteria in dark condition

Fig -2 Isolation of bioluminescent bacteria in light

Fig -3 Colony formation TCBS Agar

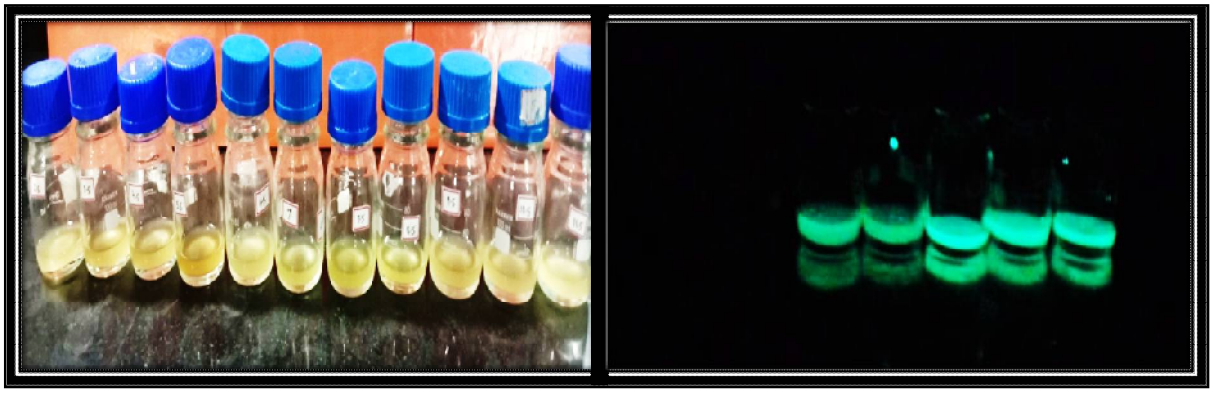
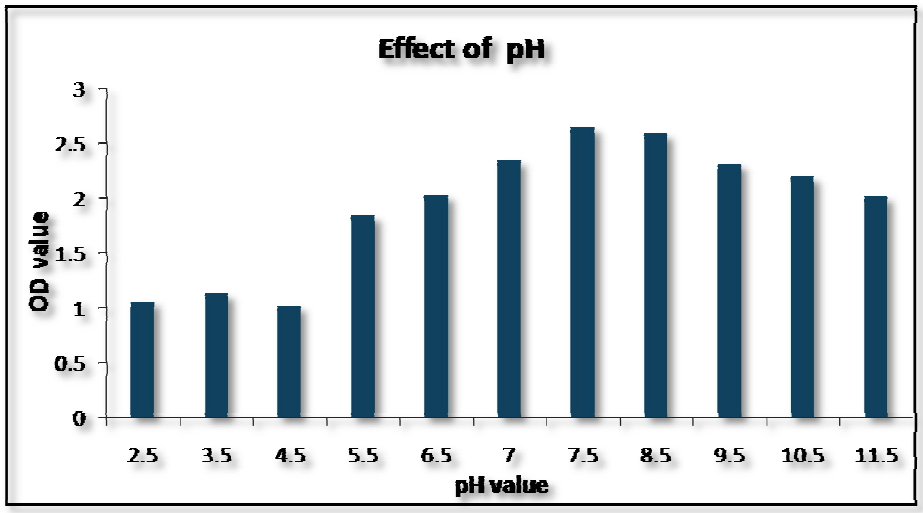
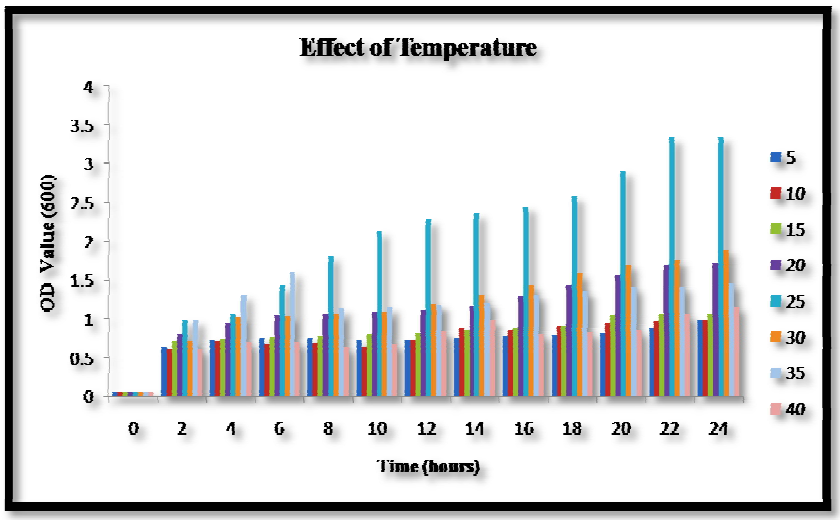


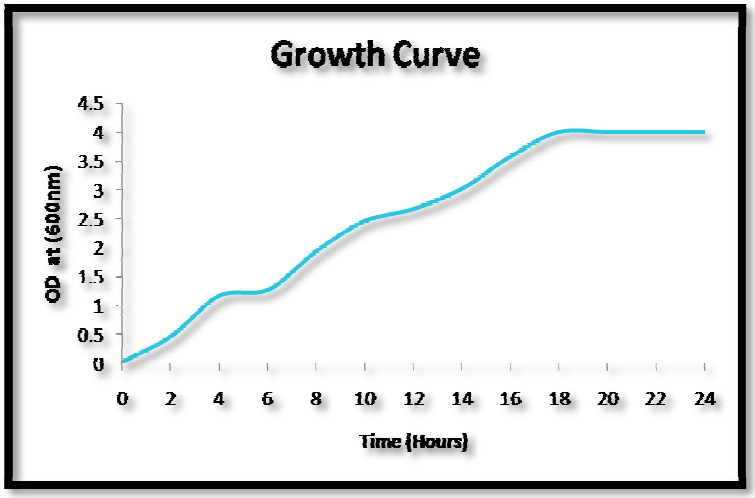
Fig -4 Optimized pH checking in light and dark condition



Graph 1. Optimized pH of bioluminescent bacteria



Graph 2. Optimized temperature of bioluminescent bacteria



Graph3. Growth curve of bioluminescent bacteria

Heavy metals	30 µl	60µl	90 µl	Negative
Manganese sulfate	30mm	35mm	40mm	-
Zinc sulphate	10mm	20mm	30mm	-
Copper sulphate	10mm	15mm	20mm	-
Cadmium coras	-	-	-	-
Mercury chloride	-	8mm	14mm	-
Cobalt sulfate	8mm	17mm	25mm	-
Magnesium carbonate	-	5mm	10mm	-
Selenium dioxide	15mm	24mm	30mm	-

Table 1. Zone of inhibition for heavy metal analysis

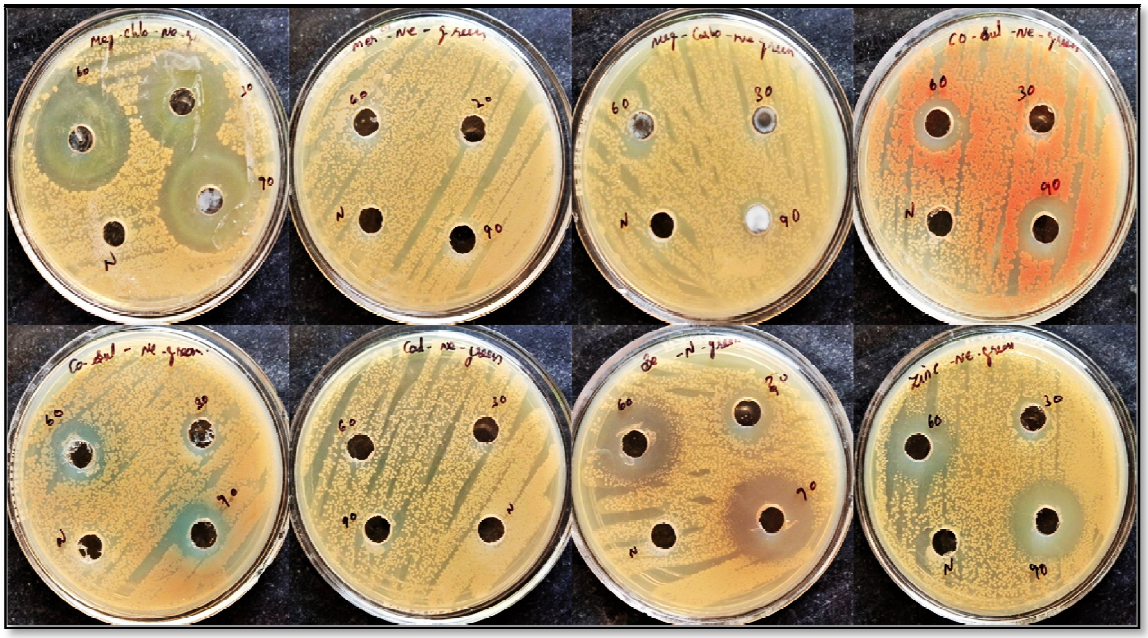


Fig -5: Heavy metal sensitivity assay

DISCUSSION

The bioluminescent bacteria were isolated from Ponyfish and characterized through biochemical assays.The bacterium was purified on luminescent agar and wasexhibited green-colored colonies on TCBS Agar.Naturally, photobacteriumwas Gram-negative, motile, short, rod-shapedsmall colonies with convex elevation. Hence, the luminescent bacteria isolated from pony fish also revealed the same characteristics along with catalytic, oxidase, andnitrate reduction properties[19]. In addition, photobacterial isolate has produced acetyl methyl carbinol from aerobic fermentation but failed to form Indole in the biochemical assay[20]. Therefore, the phenotypic characteristics and biochemical properties of bacterial isolate have revealed the characteristic features of photobacterium with better reliability.

The photobacterial isolate was also subjected to molecular level identification through 16s rRNA sequence similarity analysis and found to produce a better match percentage with *P. leiognathi*species. The same sequence was published in the GenBank with the accessionnumber MW854409. In accordance with[21], the isolates could be classification at species-level identification based on the 16S rRNA sequence resemblance asa minimum of 97% with the existing species.Hence, the isolate has showna cent percent sequence similarity with *P.leiognathi*, thus the culture was identified as*Photobacterium leiognathi* species. Moreover, the study reveals that *Photobacterium* speciescolonization and distribution might depend on the physiology of the host and environmental condition [22]. The preceding studies also elucidated extensive polymorphic characteristics ofluminous bacteriaat their genome level [23].

The pathogenic bacteriahave the abilityto lyse the entire cell of erythrocytes by hemolysin throughβ-hemolysin activity[24]. Further, the isolated strain does not producehemolysins,then it would not produce any toxicity to the healthy cells. The isolated photobacterial strain has not shown any hemolytic activity and is not involved in the infection process. Thus, the culture was found to have no toxic effect and was harmless to experiment with various applications. Since the bacterial strain was found to be nonpathogenic, it might alsobe havinga shared symbiotic relationship with the host organisms[25].

However, the pH and temperature have a crucial role in bioluminescence bacterial strain growth and the ability to produce luminescence in the broth medium. The present studyresult reveals thata pH range between 7.0 and 8.5 wasfound as optimum for the good sustenance and growth as well as luminescence character of bioluminescent bacteria. Supportively,[5] also recorded the same range of physical parameters for luminescence bacterial growth and their sustainability. Nevertheless, the study has also been proved that temperature significantly acts as[18]the most important parameter in bioluminescent bacterial growth and luminescence property**.** The bioluminescent bacteria could only grow atan optimized temperature between 24-25°C. Hence, the temperature variation drastically impacts the bacterial growth and luminescence character, which won't produce any fluorescence. Moreover, the growth study has elucidated that the optimum physicochemicalparameters could attainthe late logarithmic phase of P*hotobacterium leioghnathi* bacterial growth at around 16hours from the inoculums.

Photobacteriumis widely used in various fields such as medical, diagnosis, and molecular techniques due to its fluorescence property. Biosensing is another application of luminescence bacterial strains. The photobacterium could emit the fluorescence light which reacts with the sensitive heavy metal. Hence, the photobacteriumwas also evaluated for the ability of heavy metal sensitivity by rapid toxicity assay and found the better sensitivity activity for magnesium, zinc, copper, and cobalt metallic elements. Therefore, the photobacterial isolates can be used to evaluate the onsite metal pollution in the water. [26] also stated that the bioluminescent strains have been immensely recommended for onsite observation of heavy metal water pollution. In addition,the isolated *Photobacterium sp.* have showna better response for heavy metal pollutantslike Mercury, copper, and silver in seawater [27], [28], [29] have studied the heavy metal resistance property of luminous bacteria*V. campbellii* LZ5 was isolated from the meat of *Crassostreagigas* and found to show better metal resistanceproperties for cadmium and mercury metals. Therefore, the present study reveals that the isolated *Photobacterium leioghnathi*strainwas found to be sensitive enough for various toxic heavy metal ions and that could make the bacterium an excellent choice for the biomonitoring of heavy metal pollutants in the environment.

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