

Screening and Characterization of Luminescent Bacterial Strain

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Abstract: Several samples of sea water and sea organisms were collected for the isolation of luminescent bacterial strain. The samples were collected from Clifton, Hawksbay, fisheri at Landhi and Jetti at Korangi, Karachi, Pakistan, the sea organisms such as Crab, Prawn, Fish, Eel, Fish Gut, Electric Fish were collected. Three different media were used for enrichment of samples that is BOSS, Luminescent Agar (LA) and nutrient broth. A total of twenty one different bacterial strains were selected for further studies on the basis of different colonial morphologies. They were designated as DGU101 to DGU116, DGU221-DGU224 and DGU227. These strains were checked for luminescent and tolerance against antibiotics and heavy metal salts. Only one strain isolated from sea water (Clifton beach) designated as DGU227 showed luminescent on Luminescent Agar (LA) medium. It also exhibited multiple metal and antibiotic resistances. The two large plasmids were isolated from DGU227 which were more than 10 kb in size. The luminescence, growth, quorum sensing phenomenon of bioluminescent bacteria is affected by the presence of metal and antibiotics in environment, which act as inducer, and substrate in activating and enhancing these natural activities. Mostly Luminescent was observed after 24 hours of incubation it enhanced while growing in presence of antibiotic Ampicillin. The strain DGU227 was further identified by 16S rRNA gene analysis and exhibited 98% homology with *Vibrio chigassi* bacterial strain.

Keywords: Bacteria, luminescent, metal resistance, antibiotic resistance, plasmid, *Vibrio chigassi*.

INTRODUCTION

Bioluminescence is associated with the emission of light by living microorganisms and it plays a very important role in real-time process monitoring [1]. Luminescent bacteria emit light as result of a chemical reaction during which chemical energy is converted to light energy [2]. The majority of luminescent bacteria inhabitate the ocean. Two genera of marine bacteria, *Vibrio* and *Photobacterium*, are among the most abundant luminous bacteria. Their natural light emission is at a maximum near 490 nm, but mutants have been isolated or genetically produced which emit a variety of colors. Luminescent bacteria exist as symbiotic organisms carried within a larger organism, such as many deep sea organisms, including the Lantern Fish, the Angler fish certain jellyfish, certain clams and the Eel. The expression of genes related to bioluminescence is controlled by an operon called the lux operon [2]. Five closely linked structural genes are involved, lux A and lux B are responsible for the alpha and beta subunits of luciferase, and lux C, D, and E encode the fatty acid reductase complex required for the generation and recycling of fatty acid to aldehyde [3]. In addition, a lux I gene is part of the operon and is believed to control auto induction of lux gene expression [4]. Some species of luminescent bacteria possess quorum sensing, the ability to determine local

population by the concentration of chemical messengers. Species which have quorum sensing can turn on and off certain chemical pathways, commonly luminescence; in this way, once population levels reach a certain point the bacteria switch off light-production.

These luminous bacteria are very useful in the field of biotechnology. Luminous (*lux*) gene can be used as a reporter gene in the construction of biosensor. In present study marine bacterial strains were isolated and characterized.

MATERIALS AND METHODS

Sampling and Purification of Luminescent Bacterial Strains

In order to isolate Luminescent bacterial strains samples (sea organisms and sea water) were collected from Clifton, hawksbay, fisheri at Landhi & Jetti at Korangi, Karachi. Sea organisms which were collected are as follows Crab, Prawn, Fish, Eel, electric ray Fish. For enrichment of luminous bacteria's samples were inoculated in various growing media such as Boss (3%NaCl, 0.1%Glycerol, 1% Peptone (Bacto-Peptone), 0.3% Beef Extract, (pH was about 7.3). luminescent (LA) medium (1% NaCl, 0.5% Yeast Extract, 1% Peptone (Bacto-peptone) 1.5% Agar), and Nutrient broth supplemented with NaCl (23.0g), Na₂HPO₄ (15.5g), glycerol (10ml) per Litre deionized water [5]. The isolated colonies were purified by sub culturing.

Samples were inoculated in the sterilized 300 ml of LA, Nutrient broth and BOSS medium. Flasks were

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kept in incubator at 20°C for 24 hours. The enriched samples were serially diluted in saline (0.89%) for isolation of bacteria and spread on solidified agar plates and incubated for 24 hours at 20°C. Morphologically different colonies were selected and studied further.

Metal Salt Tolerance

1M stock solution of salts of Cd⁺², Ni⁺², Zn⁺², Cu⁺², Cr⁺² was used to make further dilutions of 1mM, 2mM and 3mM to check the MTC (maximum tolerable concentration). MTC's were checked in LA media because it cannot grow in minimal media and incubation was done for 24-48hrs under salt stress at 20°C.

Antibiotic Tolerance

Stock solutions of Amp (50mg/ml), Em (5mg/ml), Cm (34mg/ml) and Sm (50mg/ml) was prepared to check the antibiotic resistance. To check antibiotic tolerance LA media was used and incubated for 24-48hrs at 20°C.

Isolation of Plasmid and Genomic DNA

Plasmid DNA was isolated by a modified Birnboim and Dolly method [6] and by Gene JET Plasmid mini prep Kit (Fermentas Life sciences). The genomic DNA of marine bacterial strains was extracted by phenol chloroform method [7].

16S rRNA Gene Sequencing

The luminescent bacterial strain DGU227 was identified by using partial 16S ribosomal gene sequence homology. The universal set of primer was used as described by Badar *et al.*, [8]. Sequence was analyzed using blast algorithm and submitted to GeneBank.

RESULTS

Sample Collection, Enrichment and Purification of Bacterial Strains

Water samples and sea organisms for the isolation of luminous bacteria were collected from fishery at

Table 1: Maximum Tolerable Concentrations of Antibiotics

	Antibiotics µg/ml				
	Cm	Sm	Km	Amp	Em
DGU102	-	250	75	350	25
DGU103	-	275	75	425	50
DGU104	-	50	100	425	50
DGU105	-	275	50	425	50
DGU106	-	125	-	425	25
DGU107	-	50	50	425	50
DGU108	-	50	-	425	25
DGU109	150	175	175	425	100
DGU110	-	275	175	375	50
DGU111	-	50	175	375	-
DGU112	-	275	50	375	75
DGU113	150	175	150	25	75
DGU114	-	25	50	425	25
DGU115	-	50	75	425	25
DGU116	-	250	75	425	25
DGU220	-	200	-	200	50
DGU221	-	125	-	150	75
DGU222	-	100	-	200	75
DGU223	-	175	-	200	75
DGU227	-	275	-	15000	100

Table 2: Maximum Tolerable Concentration of Heavy Metals

strain codes	heavy metals (LA medium)		
	NiCl ₂	CuSO ₄	CdCl ₂
DGU220	3.0mM	1.5mM	1.5mM
DGU221	3.0mM	1.0mM	1.5mM
DGU222	3.0mM	1.5mM	1.5mM
DGU223	-	3.0mM	1.5mM
DGU227	1.0mM	2mM	1mM

Landhi & Jeti at Korangi, Hawks bay, Clifton beach, Cape mount Karachi. Several bacterial colonies were observed on all three selected growth media i.e., LA, BOSS and Nutrient broth. All the plates were observed in dark to check the luminescent only one bacterial colony that was grown on LA medium showed luminescent. On the basis of variable colonial morphology several bacterial colonies were purified. They were designated as DGU101-DGU116 and DGU201-DGU224. Luminous bacteria DGU227 was isolated from Sea water, some bacterial strains were isolated from Crab, Prawn, Fish, Eel, Fish Gut, and Electric Fish. DGU101, DGU109, DGU111 were isolated from Crab. DGU102, DGU107, DGU110, DGU114 were isolated from Prawns DGU103, DGU105. DGU112 were isolated from fishes. DGU104, DGU106, DGU113 were isolated from Eel DGU108, DGU116 were isolated from Electric Fish. DGU105 and DGU115 were isolated from Fish Gut whereas DGU227 isolated from sea water (Clifton beach). LA was found to be most effective medium for Luminescent bacteria rather than Boss and nutrient broth. The strain DGU227 showed best glowing on LA agar plates.

Colonial and Cellular Morphology

The colonial morphology of all the purified bacterial strains was observed on LA medium. Most of the colonies were circular few of them are irregular in shape, elevation was convex and flat, most of colonial margins were entire type; translucent and opaque were equally frequent, surface was smooth and shiny but only DGU111 surface was rough. All bacterial strains was non luminescent except DGU227 which showed luminescent on LA medium. The gram reaction of all the isolated bacterial strains was Gram negative.

Maximum Tolerance against Heavy Metals and Antibiotics

The tolerable concentrations of strains against Copper, Cadmium and Nickel have shown that only five

isolated strains were capable of growing at high concentration of heavy metals. These are DGU220, DGU221, DGU222, DGU223 and DGU227. MTCs of metal salts are given in Table 1. All the selected bacterial strains showed multiple antibiotic tolerances, the maximum tolerable concentrations are given in Table 2. In case of DGU227 luminescent bacterial strain showed luminescence in presence of nickel and zinc salts while it was faint in presence of cadmium salt whereas it is completely inhibited in presence of copper (see Figure 1), similarly when it grown only in presence of antibiotic ampicillin the luminescence was enhanced it was also observed that it showed highest tolerance i.e., greater than 15,000µg/ml (see Figures 2 and 3).

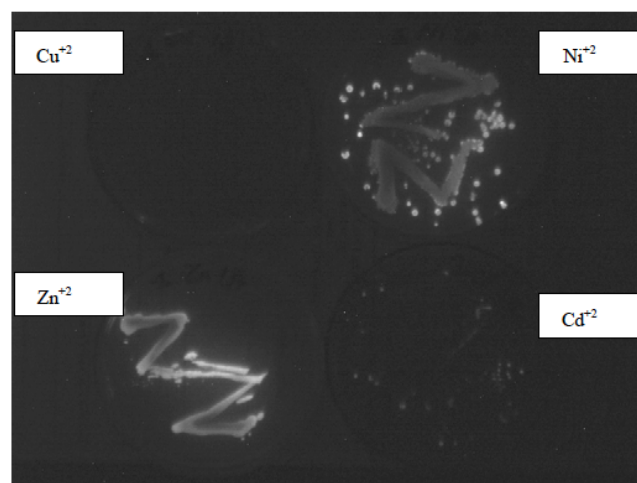


Figure 1: Luminescent bacterial strain grown in presence of metal salts. The luminescent bacterial strain DGU227 are grown in a medium supplemented with copper, nickel, zinc and cadmium salt. The glowing was sharped in nickel and zinc metal salts whereas it is affected in presence of cadmium and copper.

Isolation of Plasmid DNA

The Plasmid DNA was successfully isolated from bacterial strain DGU227 it carries two plasmid DNA having molecular weight more than 1kb (See Figure 3). Whereas rest of the bacterial strains isolated from marine have no plasmid DNA.

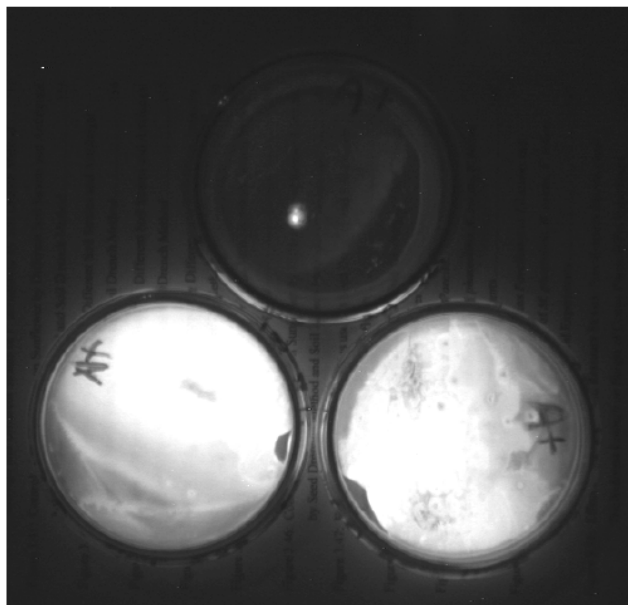


Figure 2: Growth of luminescence bacterial strain in presence of Antibiotic ampiciline. The upper one plate is of bacterial strain that are non luminescent whereas the lower two plates are of DGU227 showed strong luminescent in presence of antibiotic ampicilline 5000µg/ml.

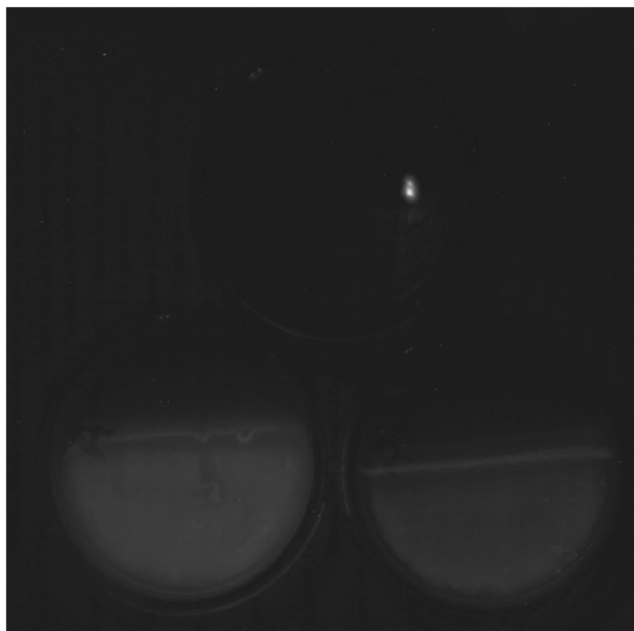


Figure 3: Growth of luminescence bacterial strain in absence of Antibiotic ampiciline. The upper one plate is of bacterial strain that are non luminescent whereas the lower two plates are of DGU227 showed low luminescent in absence of antibiotic ampicilline.

16S rRNA Gene Sequencing

The luminescent bacterial strain DGU227 was further identified by 16S rRNA gene analysis. The obtained PCR product of 16S rRNA gene was purified by using GeneJet gel extraction kit (Fermantas cat. No.

K0691) and commercially send for sequencing to CEMB (Centre for Excellence Molecular Biology, Lahore, Pakistan). The sequencing results showed 98% homology with *Vibrio chigassii* bacterial strain the sequence was submitted to GenBank having accession no: JF342691.

DISCUSSION

We have identified and characterized the luminescent bacterial strain isolated from sea water. The investigation of the Ocean bioluminescence showed the luminous bacteria to be among the most numerous unicellular inhabitants of sea water. Bioluminescence is one of the forms of a chemi-luminescent reaction, the final product of which is visible light [9].

Luminescent bacteria emit light as the result of a chemical reaction during which chemical energy is converted to light energy [10]. The majority of luminescent bacteria inhabit the ocean. Two genera of marine bacteria, *Vibrio* and *Photobacterium*, are among the most abundant luminous bacteria. Their natural light emission is at a maximum near 490 nm.

The luminescence ability is in strict dependence on the medium composition. Similarly in DGU227 the best luminescent was observed in LA medium while observed in dark that's why this medium was used for further studies. The luminescent bacteria do not produce light (or produce it very weakly) when their cells are in considerable dispersion (e.g. in the sea-water). In contrast, when many cells are in condensed suspensions (e.g. cultures growing in the liquid, microbiological medium) they produce light very efficiently.

It was observed that the luminescence of DGU227 is enhanced in presence of antibiotic ampicillin and also the growth was not inhibited by ampicillin as the concentration is increased in media up to 15,000µg/ml. It was observed by looking at Figure 2 that the agar plate which did not contain ampicillin is not showing sharp luminescence of DGU227. This is apparent that ampicillin is acting and might be activating the lux operon as an inducer or providing substrate or ampicillin metabolite is affecting its autoinduction system of quorum sensing. The marine bacteria have been exposed to large amounts of heavy metals because of industrial waste contamination or due to natural salts present in marine. The contamination has taken place over several decades, so that the current

bacterial populations are well adapted to such high concentration of salts, environmental pollutants and become resistant. Some areas of the marine environment were found to demonstrate the presence of heavy metal resistance bacteria [11]. The MTC's of these isolated marine bacterial strains against heavy metal demonstrate the contamination of heavy metals in their environment.

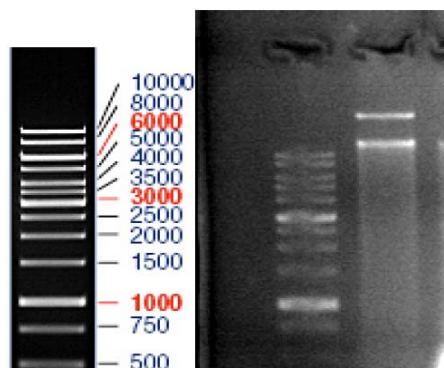


Figure 4: Isolation of Plasmid DNA from DGU227 luminescent bacterial strain. The two plasmids were extracted from DGU227, the left lane represent ladder.

The MTC's of DGU227 are taken by using LA media because it cannot grow on Tris minimal media, it need enriched environment to grow. And the luminescence hierarchy was observed to be $Zn^{+2} > Ni^{+2} > Cd^{+2} > Cu^{+2}$.

The bacterial luminescence lux gene has been widely applied as a reporter either in an inducible or constitutive manner. In the inducible manner, the reporter lux gene is fused to a promoter regulated by the concentration of a compound of interest. As a result, the concentration of the compound can be quantitatively analyzed by detecting the bioluminescence intensity. In the constitutive manner, the reporter gene is fused to promoters that are continuously expressed as long as the organism is alive and metabolically active. This kind of reporter is advanced for evaluating the total toxicity of contaminant. Both types of reporters have been shown to be useful for biosensor development [2].

DGU227 was found to be luminous among all other strains. Mostly Luminous was observed after 24 hours.

These luminous bacteria are very useful in the field of biotechnology. Lux gene can be used as a reporter gene in the construction of biosensor.

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