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Light-emitting bacteria from the Caspian Sea, North of Iran

Mina Sadat Angaji[1], Leila Jabalameli[2*], Akram Tabatabaee Bafroee[1]

1. Department of Biology, East Tehran branch, Islamic Azad University, Tehran, Iran
2. Department of Microbiology, Faculty of Sciences, Karaj Branch, Islamic Azad University, Karaj, Iran

** Corresponding author's E-mail: laji85@yahoo.com*

ABSTRACT

Bioluminescence is the production and emission of light by living organisms. This phenomenon occurs widely in different organisms. In fact, light-emitting bacteria are the most abundant and widespread luminescent organisms found in marine, freshwater and terrestrial habitats. In this study, the sea water samples were collected from the Caspian Sea, North of Iran. Luminous colonies were observed after an overnight incubation at 25°C on nutrient sea water complete medium. One luminous bacterium isolated, identified and named as *Vibrio* sp. isolate Caspian based on biochemical tests and 16S rRNA gene sequencing. Besides, two genes involved in bioluminescence, *luxA* and *luxB* were sequenced. Light emission measurement was performed for this bacterium using luminometer, then compared with a control sample previously isolated from water samples in south of Iran. *Vibrio* sp. Caspian had a weak light emission in comparison with control sample. Sequencing result of 16S rRNA, *luxA*, and *luxB* genes showed that these sequences were highly similar to *V. vulnificus*. However, there were some difference in both nucleotide and amino acid sequences of luciferase. Phylogenetic analysis based on luciferase nucleotide sequences also showed high degree of relationship between the present tested *Vibrio* and other related species. In general, the present study revealed the capability of indigenous marine sources of Iran for providing bioluminescent bacteria with different characteristics which may result in finding new luminous systems with various applicable capacities.

Key words: Bioluminescence, Vibrio, luxA, luxB, Luminometer, 16S rRNA.

INTRODUCTION

Bioluminescent bacteria are widely distributed in the world and consist of a wide variety of species. Among the light-emitting species are bacteria, dinoflagellates, fungi, fish, insects, shrimp, and squid. This set of organisms includes terrestrial, freshwater, and marine species from almost 50% of the different phyla in the animal and plant kingdoms. In these organisms the enzymes that catalyze the bioluminescence reactions are called luciferases, and in many cases the substrates are defined as luciferin (Meighen 1991). The light-emitting reaction involves an intracellular, luciferase-catalyzed, oxidation of the reduced form of flavin mononucleotide ($FMNH_2$) and a long-chain aliphatic aldehyde, such as dodecanal, by molecular oxygen to create blue-green light according to the following reaction (Stewart & Williams 1992):

$FMNH_2 + RCHO + O_2 \rightarrow FMN + RCOOH + H_2O + light\ (490\ nm)$

Presently, just five genera of bacteria are recognized to naturally bioluminescence: *Vibrio*, *Photobacterium*, *Shewanella*, *Aliivibrio* and *Photorhabdus* (Hastings *et al.* 1985; Meighen 1994; Peat & Adams 2008). All reported species of bioluminescent bacteria affiliate to the class *Gammaproteobacteria.* Among the bioluminescent bacteria reported, 17 bioluminescent species were currently known. The luminous bacteria were also classified into three genera which are *Vibrio*, *Photobacterium* and *Xenorhabdus* (*Photorhabdus*), such that the species within the genus *Vibrio* and *Photobacterium* are usually inhabit marine environment, while *Xenorhabdus* belong to

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terrestrial environment (Yaser *et al.* 2014). These gram-negative, rod-shaped bacteria are chemoorganotrophic, nonsporulating, and motile by means of polar flagella. All species of the family *Vibrionaceae* are anaerobic, while the two species of the *Shewanella* are severe aerobic bacteria (Kita-Tsukamoto *et al.* 2006). Among *Vibrio* species, *V. vulnificus* is a cause of foodborne infections in human. It is a gram negative, halophilic bacterium frequently found in warm coastal waters. It is a deadly, human pathogen which is in charge of the most of seafood-associated deaths worldwide (Phillips & Satchell 2017).

In recent years the genes from the bioluminescence operons of a number of marine bacteria have been cloned, and the particular functions of the polypeptide products of the structural genes (luxA-E) have been recognized. The *luxA* and *luxB* genes code for the α (40-kDa) and β (36-kDa) subunits of luciferase, respectively. The *luxC, D*, and *E* genes code for the polypeptides of the fatty acid reductase system (reductase, acyl-transferase, and synthetase) responsible for synthesis of the fatty aldehyde substrate. Hybridization tests and mutant analyses have showed that the active site is placed primarily on the α subunit. However, the duty of specific amino acid chains on the α subunit implicated in or near the active site is still unidentified. The β subunit, however, is still vital for the light-emitting reaction, and some data implies that this subunit can influence the interaction of the enzyme with the reduced flavin (Meighen 1988).

In this study, the sea water samples from the Caspian Sea were collected and screened for isolating luminous bacteria. Bacterial identification, characterization, as well as light emission measurement of isolated luminous bacterium were examined.

MATERIALS AND METHODS

Sampling, collection, and isolation of luminous bacteria

Two samples were collected from subsurface (1 m) and bottom (13 m) of seawater within the 2 km of shore, in the Caspian Sea at 41.9350˚ N, 50.6689˚ E. Water samples were collected by submerging autoclaved bottles under water and then opening their caps. The bottles were sealed under water to keep the exact microbial population of selected water layers. 200 µL of the water samples were spread onto nutrient seawater complete (SWC) agar plates containing 3 mL glycerol, 1 g yeast extract, 3 g peptone, and 15 g agar per liter of 75% seawater (O'Grady & Wimpee 2008), and then incubating overnight at room temperature (25˚C).

The appearance of luminescent colonies was observed in a dark room after dark adapting for 5 min. One of the brightest bacterium was selected for more characterization and this isolate was restreaked onto SWC agar plates in order to gain purified colonies. For long-term storage, bacterial isolates were grown in SWC broth supplemented with 10% glycerol and kept at -72 ˚C.

Bacterial identification

Bacterial characterization was preliminary performed based on cellular and microscopic morphology, and biochemical tests. Gram staining was carried out as a common differentiating technique. Biochemical tests used in this study were as follows: carbohydrate fermentation includes galactose, maltose, manitol, sucrose, and arabinose, Simmons' citrate, MRVP (Methyl Red, Voges-Proskauer), indole, nitrate reduction, gelatinase, urease, oxidase, catalase, and TSI (Triple sugar iron agar).

Capability of growing on TCBS (Thiosulfate-citrate-bile salts-sucrose) agar medium was also tested to meet the nutritional requirements of *Vibrio* spp., as an important genus of light producing bacteria. All biochemical tests were performed by incubating bacteria at room temperature.

As a complement to confirm the identification of luminous strains, molecular analysis based on 16S rRNA gene sequencing was carried out. Characteristics of universal 16S rRNA primer pair are demonstrated in Table 1 (Jabalameli *et al.* 2015).

Genomic DNAs were extracted using laboratory kit, GeneAll, according to manufacturer instruction. Viability and presence of extracted genomic DNA was confirmed by 1% agarose gel electrophoresis. The final concentration of PCR reagents in each 25 µL reaction was as follow: 12 µL of Master Mix containing dNTPs, $MgCl_2$, Taq polymerase and buffer. 1 µL of template DNA and 0.5 µL of each forward and reverse primers were added to the master Mix. Total volume of PCR reaction mixture was brought to 25 µL. Also *luxA* and *luxB* genes encoding α and β subunits of luciferase, respectively were amplified separately with the nucleotide primer pairs, AF and AR for *luxA*, BF and BR for *luxB*.

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Characteristics of the used primers are also demonstrated in Table 1. These primer pairs were designed according to related nucleotide sequences in GeneBank database using GeneRunner software.

Table 1. Characteristics of the primers used for amplification of 16S rRNA, *luxA* and *luxB* genes.

Primers	**Target genes**	**Sequences (5'-3′)**	**Product sizes (bp)**	**Tm(°C)**
F	16SrRNA	AGAGTTTGATCATGGC	1500	58
R		AGAGTTTGATCATGGC		
AF	luxA	ATAAGGGGATCCTATGAAATTTGGAAACTTCCTTCTCAC	1068	56
AR		AAAAAGCTTTTACTGTTTTTCTTTGAGATATGGC		
BF	luxB	GAACATATGATGAAATTTGGATTATTCTTCCTCAAAAAAAT	975	55
BR		GTTTAAGGTACCCGAGTGGTATTTGACGATGTTG		

Purified PCR products of 16SrRNA, *luxA* and *luxB* were sent to Bioneer Company (Korea). The partial sequenced of the 16S rRNA gene (~1500 bp), *luxA* genes (~1060 bp) and *luxB* (~970 bp) were compiled with Chromas 2.0 software. Then they were deposited in GenBank and aligned with the16S rRNA sequences and luxAB sequences available in nucleotide database in NCBI, (National Center for Biotechnology Information), Available at: http://www.ncbi.nlm.nih.gov, using BLAST software, (Basic Local Alignment Search Tool).

Confirmed isolated luminous bacterium was submitted as a new indigenous species in GeneBank database via Bankit submission tool. Related amino acid sequences were also obtained using Expasy tool (http://web.expasy.org/translate/). Furthermore, nucleotide and amino acid sequences of alpha and beta subunits obtained in the present study were compared to the other related luminous bacteria, *V. vulnificus* strain ATCC 43382 (Urbanczyk *et al*. 2008). On the other hand, Aligned and edited nucleotide sequences of *luxA* and *luxB* were directed to phylogenetic tree using MEGA6 software package (NCBI Resource Coordinators 2017).

The phylogenetic analysis was conducted based on the different sets of aligned sequences of five other luminous bacteria (illustrated in Table 2). The evolutionary history was inferred using the Neighbor-Joining method (Gasteiger *et al*. 2003). The resultant tree topologies were evaluated using bootstrap analyses (Tamura *et al*. 2013) of the neighbor joining method base on 1000 replications.

Table 2. Strains used in phylogenetic analysis (DNA sequences of *luxA* and *luxB*).

Strains	**GeneBank accession nos.**	**References**
Vibrio sp. Persian 1	KF479406.1	(Jabalameli *et al*. 2015)
Vibrio vulnificus strain ATCC 43382	EU122289.1	(Urbanczyk *et al*. 2008)
Vibrio campbellii strain 151112C	CP025954.1	(Tsai 2018)
Vibrio sp. BCB494	EU192084.1	(O'Grady & Wimpee 2008)
Vibrio owensii strain 051011B	CP025796.1	(Tsai 2018)

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Light emission measurement

The isolated luminous bacterium was incubated in the SWC broth with a rotary shaker (120 rpm) at 25°C for an overnight. The light output was determined using a luminometer (Synergy4 Detection system, USA) and was reported as Relative Light Units (RLU/second). Light emission measurement of another luminous bacterium, *Vibrio* sp. Persian 1, isolated from southern marine habitats in Iran (Jabalameli *et al*. 2015) was determined in order to be compared with the present isolated bacterium.

RESULTS

Bacterial identification

Subsurface samples did not contain any luminous colonies. However, bright colonies from the bottom of the seawater (13 m in depth) were isolated and selected for further experiments. First step of bacterial characterization by gram staining revealed that all the isolates were gram negative rods, an expected finding provided by others (Baumann et al. 1983). Since the morphological characteristics of all isolated luminous bacteria were similar, one of the brightest colonies was selected for further examinations. The results of differential biochemical tests are shown in Table 3.

Table 3. Physiological characteristics of isolated luminous bacterium.

Test	Result
Maltose	+
Sucrose	–
Manitol	+
Galactose	+
Arabinose	–
Simmon Citrate	–
MR	–
VP	–
Indole	–
Gelatinase	–
Urease	–
Oxidase	+
Catalase	+
Nitrate	+
Movement	+
TSI	Alk/Acid*
Growth on TCBS	+
Agar	With yellow colonies

* Alkaline / Acid exhibits capability of glucose fermentation without any gas production.

The BLAST search of 16S rRNA gene sequences of isolated bacterium against sequences in nucleotide database revealed that the luminous isolate belongs to genus *Vibrio* and showed 96% identity with *V. vulnificus* strain ATCC 43382. This isolated bacterium was submitted to GenBank database as *Vibrio* sp. Caspian.

Fragments with the expected sizes were sequenced and the PCR products were electrophoresed on 1% W/V agarose gel (Fig. 1). Both fragments approved as bacterial luciferase subunits by performing DNA sequencing.

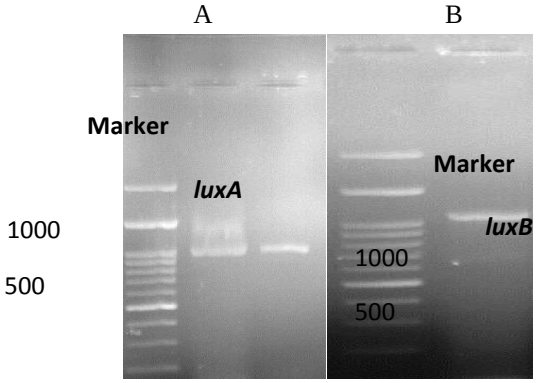

Fig.1. Gel electrophoresis of *luxA* (A) and *luxB* (B) genes.

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The BLAST search of *luxA* and *luxB* gene sequences of isolated bacteria against sequences in nucleotide database revealed that *luxA* and *luxB* belong to genus *Vibrio* and both of them submitted to GenBank database were considered as *Vibrio* sp. Caspian with the accession numbers of MK027741.1 and MK027742.1, respectively.
Furthermore, comparison between results obtained in the present study and sequences available in data bases showed that *luxA* and *luxB* from *Vibrio* sp. Caspian have maximum identity with *V. vulnificus* 43382 (GeneBank accession no. EU122289.1).
In order to perform a comparison between present sequences and previously-published sequences from the most identical bacterium, *V. vulnificus* 43382, the latter was considered as a reference. The consequence of nucleotide alterations on amino acid sequences were also revealed. Results showed that there are noticeable differences in both nucleotide and amino acid sequences of obtained genes in the present study and reference sequences, with most identity. Results exhibited that, 6 out of 62 changes observed in *luxA* nucleotides resulted in amino acid alterations, while among 93 nucleotide substitutions in *luxB* sequence, only 10 mutations led to amino acid changes.
In addition to reference sequences, five other nucleotide and amino acid sequences (Table A) were used for constructing phylogenetic trees. Because of the similar inferred patterns of phylogeny obtained from nucleotide and amino acid sequence analyses, with only very slight differences, Fig. 2 illustrates the phylogeny based on the nucleotide sequences of *luxA* and *luxB* genes.

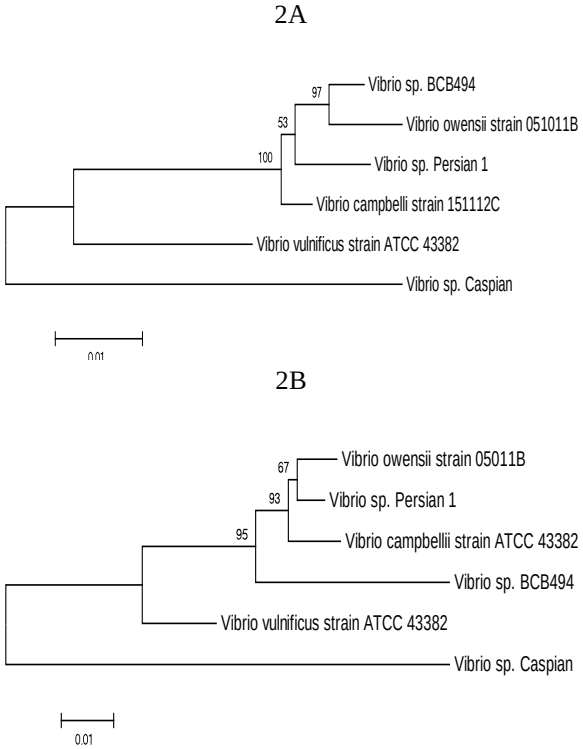

Fig. 2. Unrooted neighbor – joining trees based on *luxA* (2A) and *luxB* (2B) nucleotide sequences.

The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches (Felsenstein 1985). The trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic trees. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980) and are in the units of the number of base substitutions per site. The analysis involved 6 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 976 and 872 positions in the final datasets of 2A and 2B, respectively. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.* 2013).

Light emission measurement

Measurement of the amount of light produced by isolated bacteria, and control sample (*Vibrio* sp. Persian 1) is shown in Fig. 3. Maximum light emissions recorded from *Vibrio* sp. Caspian and *Vibrio* sp. Persian 1 cultures

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were at 460 and 480 nm, respectively. *Vibrio* sp. Caspian had a weak light emission in comparison with control sample. Furthermore, there was a significant difference between light intensities of the two examined bacteria.

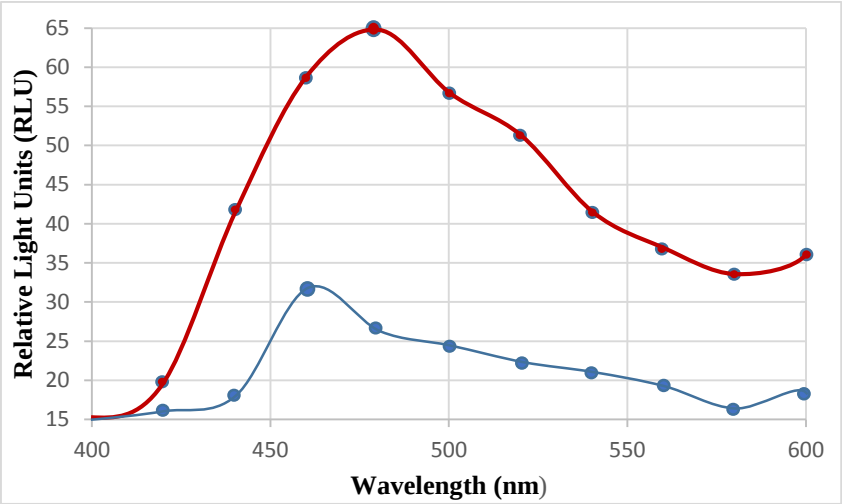

Fig. 3. Light output measurement of *Vibrio* sp. Persian 1 (red curve), and isolated sample (blue curve), as Relative Light Units i.e. counts per second at 400-600 nm of the culture**.**

DISCUSSION

Although many researchers have long been working on different aspects of bacterial bioluminescence ((Meighen 1988, Baldwin, 1995, Tanet, 2019), this fascinating phenomenon is still attracting. Studies on luminous bacteria as saprophytic (Davis & Sizemore 1982), symbiotic (Boettcher & Ruby 1990; McCann *et al*. 2003) and parasitic (Oliver 1986) forms have long been reported in several studies. In a recent study carried out by Mohseni *et al.* (2018), a luminous, gram negative bacterium with over 99% similarity to *V. campbellii* was isolated from the Caspian Sea. In the present study, we focused on isolating free-living bioluminescent bacteria, which can be isolated readily from sea water environments, highlighting the occurrence of bioluminescent bacteria in an indigenous marine habitat, Caspian Sea. Since in shallow sunlit regions of the marine habitats, biomass and organic compounds are required to support large accumulations of autotrophic, mixotrophic, and heterotrophic organisms (Haddock 2010). The southern part of the Caspian Sea was chosen for water sampling, which was performed in June. The air temperature was warm, and the salinity level was moderate. Hence, isolation of luminescent bacteria was expected. Since most species of marine luminous bacteria can be found in more than one habitat (Herring 2002), present luminous bacteria, isolated from the layers of sea water as free-living bacteria, might have established one of the possible relationships with other marine organisms in some stages of their lives. In a similar study performed by Omeroglu & Karaboz (2012), 20 strains of bioluminescent *V. gigantis* were collected from different ecological niches: sea water, sediment, and sea fish samples.

In the present study luminous bacterial isolates grew abundantly on SWC medium, in which sea water was used in order to support salts needed for bacterial growth. In an experiment performed by Budsberg *et al*. (2003) SWC was supplemented with inorganic salts such as NaCl, $MgCl_2$, $MgSO_4$, and KCl for bacterial growth. Due to the pliancy of biochemical tests performed on isolated bacteria, these traits could not literally and accurately lead us to exact identification of isolates. Accordingly, sequencing of 16S rRNA gene was also performed and results showed that the isolated luminous bacterium was belong to genus *Vibrio*, a widespread genus of bacteria in coastal waters which comprised over 130 species (Gradoville *et al.* 2018).

Since clear separation between the *Vibrio* species is achieved by sequencing analyses of the following seven genes, 16SrRNA, gyrB, recA, rpoA, topA, pyrH and mreB (Chimetto 2011), the characterized bacterium in this study was submitted in GenBank Database as *Vibrio* sp. Caspian (with the accession number of MK066461.1), though it exhibited maximum identity with *V. vulnificus* according to its 16SrRNA sequence.

In other words, nomination of isolated luminous bacterium was solely restricted to its genus, i.e. *Vibrio*, not to its species. *V. vulnificus* is a Gram-negative bacillus capable of causing severe to life-threatening infection in susceptible individuals and usually found worldwide in coastal or estuarine environments with water temperatures from 9 to 31°C (Horseman & Surani 2011). Genes encoding alpha and beta subunits of bacterial luciferase also

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displayed maximum sequence identity with *V. vulnificus*. Based on the phylogenetic analysis, illustrated in Fig. 1, high degree of relationship were observed between the examined sequences in the present study and other previously-determined sequences from different species of luminescent *Vibrios*. Comparison between the light emission measurements of the two tested bacteria, previously isolated *Vibrio* sp. Persian 1 and present isolated luminous *Vibrio* sp. Caspian revealed the much more capability of light production by the former bacterium. Observing little bioluminescence was also reported in broth culture of *V. vulnificus*, the most similar luminous bacterium with *Vibrio* sp. Caspian (Oliver 1986). Interestingly, as shown in Fig. 2, maximum light intensity from the *Vibrio* sp. Caspian culture was detected at about 460 nm, which varies from the reported wave length of produced light in bacterial luminescence reactions, i.e. 490 nm (Matheson 1981). Particularly, Oliver (1986) reported maximum emission of *V. vulnificus* luminescence at 483 nm. These data exhibit that other factors also have the potential to affect diffusion of light in luminous bacteria isolated from the same environment, with similar features, and belong to the same genus. In other words, amounts of light emission from different isolates belong to same genus, *Vibrio*, is variable, though bioluminescence is under the control of a fundamental similar genetic system.

The present study showed that screening of the Caspian Sea for luminous bacteria, led to the isolation and identification of new bioluminescent bacteria, revealing the Caspian Sea, as an important ecosystem for these bacteria .Since this luminous bacterium is highly similar to *V. vulnificus*, an important and dangerous pathogenic bacterium, its capability for causing severe human disease may be an alarming possibility which results in much more caring while swimming, fishing, and handling sea food. However, more experiments on molecular, biochemical, and pathophysiological aspects of this bacterium is necessary to be performed.

CONCLUSION

In conclusion, the isolation of luminescent bacteria from the Caspian Sea, as an indigenous discovery, along with increasing rate of exploring new findings about different aspects of bioluminescence phenomenon may conduct us to work on novel strains with desirable and different functional characteristics in order to improve our understandings about bioluminescence process. This may hold a great promise for improvement of biotechnological and medical applications of this phenomenon.

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باکتری‌های نشر دهنده نور از دریای خزر، شمال ایران

مینا سادات انگجی[1]، لیلا جبل عاملی[2*]، اکرم طباطبایی بفرویی[1]

۱- گروه زیست شناسی، واحد تهران شرق، دانشگاه آزاد اسلامی تهران، تهران، ایران
۲- گروه میکروب شناسی، واحد تهران شرق، دانشگاه آزاد اسلامی تهران، تهران، ایران

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چکیده

بیولومینسانس تولید و انتشار نور توسط موجودات زنده است. این پدیده به طور گسترده‌ای در موجودات مختلف رخ می‌دهد. در حقیقت، باکتری‌هایی که نور را منتشر می‌کنند، فراوان‌ترین و گسترده‌ترین موجودات زنده در جوامع دریایی، آب شیرین و زیستگاه زمین هستند. در این مطالعه، نمونه‌های آب دریا از دریای خزر در شمال ایران جمع آوری شد. کلنی‌های نورزا پس از گرمخانه گذاری شبانه در دمای ۲۵ درجه سانتی‌گراد بر روی محیط کشت کامل آب دریا (SWC) مشاهده شدند. یک باکتری نورزا جدا و شناسایی شده و بر اساس آزمون بیوشیمیایی و توالی‌یابی ژنی 16S rRNA، به نام *Vibrio* sp. Caspian نام گذاری شد. علاوه بر این، دو ژن دخیل در نورزایی زیستی luxA و luxB نیز توالی‌یابی شدند. اندازه‌گیری انتشار نور برای این باکتری با استفاده از لومینومتر انجام شد و با نمونه شاهد جداسازی شده از نمونه‌های آب دریا در جنوب ایران مقایسه شد. *Vibrio* sp. Caspian در مقایسه با نمونه کنترل، دارای انتشار نور ضعیف بود. نتایج توالی یابی 16S rRNA ژن‌های luxA و luxB نشان داد که این توالی‌ها بسیار شبیه به *Vibrio vulnificus* بودند. با این حال، در هر دو توالی نوکلئوتید و اسید آمینه لوسیفراز تفاوت‌هایی وجود داشت. تجزیه و تحلیل تبارشناختی بر اساس توالی‌های نوکلئوتیدی لوسیفراز هم چنین سطح بالایی از ارتباط *Vibrio* جدا شده و دیگر گونه‌های مرتبط را نشان می‌دهد. به طور کلی، مطالعه حاضر نشان‌دهنده توانایی منابع دریایی بومی در ایران برای ارائه باکتری‌های نورزای زیستی با ویژگی‌های مختلف است که ممکن است به دنبال یافتن سیستم‌های نورزایی جدید با ظرفیت‌های مناسب باشد.

*مؤلف مسئول

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