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Probing of Potential Luminous Bacteria in Bay of Bengal and Its Enzyme Characterization[S]

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The present study dealt with the isolation, identification and enzyme characterization of potential luminous bacteria from water, sediment, squid, and cuttle fish samples of the Karaikal coast, Bay of Bengal, India during the study period September 2007 – August 2008. Bioluminescent strains were screened in SWC agar and identified using biochemical tests. As *Shewanella henadai* was found to be the most common and abundant species with maximum light emission [69,702,240 photons per second (pps)], the optimum ranges of various physicochemical parameters that enhance the luciferase activity in *Shewanella hanedai* were worked out. The maximum luciferase activity was observed at the temperature of 25°C (69,674,387 pps), pH of 8.0 (70,523,671 pps), salinity of 20 ppt (71,674,387 pps), incubation period of 16 h (69,895,714 pps), 4% peptone (70,895,152 pps) as nitrogen source, 0.9% glycerol (71,625,196 pps), and the ionic supplements of 0.3% $CaCO_3$ (73,991,591 pps), 0.3% K_2HPO_4 (73,919,915 pps), and 0.2% $MgSO_4$ (72,161,155 pps). *Shewanella hanedai* was cultured at optimum ranges for luciferase enzyme characterization. From the centrifuged supernatant, the proteins were precipitated with 60% ammonium sulfate, dialyzed, and purified using anion-exchange chromatography, and then luciferase was eluted with 500 mM phosphate of pH 7.0. The purified luciferase enzyme was subjected to SDS-PAGE and the molecular mass was determined as 78 kDa.

Key words: Luminous bacteria, *Shewanella hanedai,* luciferase, Karaikal coast

Bioluminescence is a biochemical process in which luciferin reacts with oxygen in the presence of the enzyme luciferase, resulting in the emission of light [30]. Luciferase is the generic name for any enzyme that catalyzes a reaction that results in the emission of light of sufficient intensity. All luciferases catalyze oxidative processes in which an intermediate (or product) is formed in an electronically excited state. Light is emitted when the excited state is converted to the ground state. Bioluminescence is having pronounced applications in the fields of the medical arena, diagnostics, and drug discovery and also used as biosensors for the detection of environmental pollution and toxicity. In this backdrop, an attempt has been made in the present study to characterize bacterial bioluminescence and the enzyme involved in the process.

Among the various luminescent organisms found in different habitats, bioluminescent bacteria are the most abundant and widespread. Their primary habitat is in the ocean, and they form free-living, symbiotic, saprophytic, or parasitic modes of life [14, 19]. *Vibrio fischeri* and *Photobacterium phosphoreum* are mostly symbiotic microorganisms that live in high densities in the specific organs of marine bioluminescent fishes [13] and squids [22]. Most of the members of the family Vibrionaceae are bioluminescent in nature. Brackish, coastal, and pelagic waters are uniquely inhabited by distinct *Vibrio* populations [28]. In addition, members of the Vibrionaceae occur naturally in the digestive tract and on the skin surface of marine animals [7]. In general, the genus *Vibrio* along with their close relatives such as *Photobacterium* are thought to be specially adapted to engage in pathogenic and benign host–microbe interactions. These symbiosis interactions probably have deep and ancient common ancestry, arising independently numerous times during the evolution of Vibrionaceae [20].

Several taxonomic studies have been carried out in vibrios and luminous bacteria in recent years. *Bergey's Manual of Determinative Bacteriology* [15] recognized only 11 luminous species, among which one species is fresh-water.

Special attention has directed to identify the predominant and potential bioluminescent strain from water, sediment, squid, and cuttlefish samples of the Karaikal coast, Bay of

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Bengal, India and it was identified as *Shewanella hanedai. Shewanella hanedai* is a Gram-negative, aerobic, heterotrophic marine luminescent bacterium with one polar flagellum. They are predominantly found in deep-sea organisms, but can also reside in soil and sedimentary habitats. In the symbiotic conditions, luciferase is synthesized in large quantities according to a quorum-sensing regulatory system called auto-induction, although oxygen is always present in low levels [8].

The optimal physicochemical conditions that result in higher luciferase production have been worked out. Luciferase production in optimal conditions was subjected for purification and the molecular mass was determined. The results of the present study will stand as baseline data for the application of luciferase as a reporter gene, biosensor, and bioindicators of pollution, *etc*. in the future.

Materials and Methods

Sample Collection

The surface water samples, sediment samples, and squid and cuttle fish samples were procured from the Karaikal coast (Lat.10°56'28.20"N, Long.79°51'11.74"E) during September 2007 to August 2008. Collections were made in random in such a way that samples were procured on a need basis

Water and sediment samples. Surface water samples were collected in pre-sterilized sample bottles, allowing enough air space inside so as to facilitate thorough mixing. Precautionary measures were taken to minimize the contamination.

Sediment samples were collected using a sterile spatula. The central portion of the collected sample was aseptically transferred into sterile polythene bags. Both the water and sediment samples were transferred to the laboratory in an ice box maintained at 4°C for further study.

Squid and cuttle fish collection. Specimens of squid and cuttle fish were also caught from Karaikal with the aid of trap nets set offshore. The nets were raised in early morning and the live specimens were transferred into plastic tanks containing seawater. The live squids and cuttle fish procured were subjected for the isolation of luminous bacteria within 2 h. When it was not possible, samples were kept at 4°C until use.

Isolation and Screening of Bioluminescent Bacteria

The bioluminescent bacteria were isolated from samples using seawater complete (SWC) agar as a selective medium. SWC agar consists of pancreatic digest of casein - 5 g, yeast extract - 3 g, agar - 20 g, glycerol - 3 ml, and 75% aged seawater - 1,000 ml [2]. Luminous bacteria were screened using the soft agar overlay method [22] after serially diluting the samples using 75% aged sea water as a diluent. According to this method, 1 ml of sample dilution was mixed with 4 ml of SWC agar medium maintained at 40°C and poured immediately onto the surface of an already precooled SWC agar plate with 15 ml of SWC agar. The plates were incubated at room temperature (28 ± 2°C) for 24 h, and the luminous colonies were examined in a dark chamber for the appearance of visible bioluminescence.

Light Measurement

Pure cultures were obtained using SWC agar medium. Pure strains were inoculated to 10 ml of sterilized SWC broth kept in 30 ml screw-cap tubes and incubated for 24 h in a rotary shaker and used for the light measurement. The assay of bacterial luciferase involves a direct rate determination. In this, the rate of formation of product (light) is measured as light intensity in photons per second. This provides an instantaneous determination of reaction velocity, since the number of quanta emitted per second equals the number of molecules reacting per second; that is, bioluminescence quantum yield. Light intensity can be conveniently measured with any kind of photomultiplier photometer [18]. In the present study, the photons were measured with the help of a luminometer (Hidex, multi label tester). A light-tight housing with a shutter between the luminometer and the vial or test tube holding the culture broth ensures the prevention of entry of light from outside.

Identification

Identification of bioluminescent cultures was done using a taxonomic battery [4, 15]. These included growth on gelatin agar with 3% and without sodium chloride, gelatinase, arginine dehydrolase, lysine decarboxylase, ornithine decarboxylase, yellow orange pigmentation on marine agar, utilization of lactose, maltose, and Voges Proskauer reaction. The strain identification using a taxonomic battery on the basis of 10 biochemical tests was applied for their discrimination power and ease of application.

Optimization of Physicochemical Parameters for Maximum Luciferase Enzyme Production

The strains of luminous bacteria that exhibited maximum photon emission were selected. They were subjected for further culture at different concentrations of various physicochemical parameters to unravel the optimal levels of these parameters for maximum luciferase production.

The characterization of the strain was done by adopting the search technique, by varying parameters one at a time. The experiments were conducted in 250 ml Erlenmeyer flasks using SWC broth medium and all the experiments were carried out in triplicate and the average values were calculated. The range of parameter achieved by one step was fixed in subsequent experiments.

Factors like temperature, pH, salinity, incubation period, nitrogen source, glycerol, and ionic supplements were tested in different ranges with the production of luciferase enzyme by adopting the search technique.

Temperature. The effect of temperature on luciferase production was tested over a range from 20°C to 35°C with an interval of 5°C.

pH. For the evaluation of optimum pH range, the culture medium was prepared in pH range from 3 to 11 with an interval of pH 2.

Salinity. Media with 5 to 50 ppt were prepared with the help of natural sea water and inoculated to test the effect of salinity, since the strain is of marine origin. The consideration of various salinity ranges was based on the average salinity of open sea water being 35 ppt, so maximum values up to 50 ppt and minimum values up to 5 ppt were taken to access how the luciferase production interferes with the salinity.

Incubation period. Incubation was carried out for a time period ranging from 4 to 24 h with a 4 h interval, and the luciferase enzyme production was estimated.

Nitrogen source. The effects of different nitrogen sources like peptone, yeast extract, beef extract were studied in concentrations of 1% to 10% with an interval of 1% and were tested for luciferase enzyme production.

Glycerol. The strains were cultured in medium with glycerol at varying concentrations of 0.1% to 1% with the interval of 0.1% and were tested for maximum luciferase enzyme production.

Ionic supplement. The effect of various mineral ions such as magnesium sulfate, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, calcium chloride, and ferrous sulfate prepared in 0.1%, 0.2%, 0.3%, 0.4%, and 0.5% concentrations were tested.

Characterization of Luciferase Enzyme by Molecular Weight Determination

Ammonium sulfate fractionation and dialysis. It is well known that the fractional saturation of ammonium sulfate at which a specific protein will precipitate is strongly dependent on the concentration of that protein, in addition to other factors, especially temperature and pH [6]. It is therefore not surprising that the extraordinarily high concentrations of luciferase in the culture require changes in the ammonium sulfate fractionation protocol. Published methods for the purification of bacterial luciferase insist for the collection of protein precipitation between 40% and 75% saturation of ammonium sulfate [12, 17]. Using the culture grown as described above, the amount of ammonium sulfate was standardized at 60% saturation, at 4°C and pH 7.0. To 100 ml of culture, 33.1 g of ammonium sulfate was slowly added and kept overnight for precipitation. The precipitated protein was centrifuged for 30 min at 3,000 rpm at 4°C. The supernatant was decanted and the collected pellet was dissolved in a minimal volume of 100 mM phosphate, pH 7.0. The solution was taken in a dialysis tube and dialyzed against 1 L of the same buffer at room temperature for 20 min. The buffer was changed twice, and a total dialysis was done for about 1 h. The dialyzed sample was further centrifuged at 3,000 rpm for 30 min and used for molecular weight determination in SDS-PAGE.

Ion-exchange chromatography. The dialysed luciferase enzyme sample was subjected for further purification using anion-exchange chromatography (DEAE cellulose column). After washing the column with 50 ml of 200 mM phosphate, pH 7.0, 0.5 mM DTT (dithiothreitol), 1 mM EDTA at 2 ml/min, the column was eluted at the same flow rate with a 300 ml linear gradient from 400 to 700 mM phosphate, pH 7.0, with 0.5 mM DTT and 1 mM EDTA. Absorbances were read at 280 nm and the bioluminescence activity of each fraction was determined for all the fractions collected, and those with activity were pooled [27].

SDS-PAGE. The molecular weight of luciferase was determined using SDS-PAGE [10]. A volume (30 µl) of SDS gel loading buffer and sample was taken in a 1.5 ml tube. It was heated at 60°C for 3 min. Assembles were fixed in an electrophoresis apparatus and 15 µl of sample and markers (2,400–40,000 kDa) were loaded in different wells. The gel was run at 50 V and stained with Coomassie brilliant blue.

RESULTS AND DISCUSSION

Water, sediment, squid, and cuttle fish samples collected at random for a period of one year (Sep 2007 – Aug 2008) from the Karaikal region were analyzed for the presence of luminescent bacteria. The collected samples were immediately processed and the skin layer and the light organ of the squid and cuttle fish were taken for the study and were plated on SWC agar at pH 7 and incubated at 30°C room temperature. Among the samples, luminescent bacterial density varied between the collections, especially in the water and sediment samples. The luminescent bacterial density was found to be between 1.9×10^2 and 3.0×10^4 CFU/ ml in the water sample, 1.8×10^2 and 3.2×10^3 CFU/g in the sediment sample, 7.3×10^3 and 1.47×10^4 CFU/g in the squid sample, and 3.5×10^3 and 9.0×10^4 CFU/g in cuttle fish samples.

The percentage of luminescent bacteria among total heterotrophic bacteria was also varied between the samples. In the case of water samples, the percentage of luminescent bacteria ranged 30–40%, whereas in sediments it was 5–20%. Surprisingly, in squid and cuttle fish samples, 50–80% of total heterotrophic bacterial population were luminescent.

The luminescent bacteria grown on SWC agar plate were isolated based on their intensity of glow and colony morphology. All the isolated 167 luminescent strains were stored in individual SWC agar slants for further analysis and the strains were identified by biochemical tests following the identification battery [4, 15]. There were six different bacterial species found throughout the study period (Sep 2007 to Aug 2008), namely *Shewanella hanedai, Vibrio splendidus, V. mediterranei, V. orientalis, V. fischeri*, and *V. harveyi.* Among these, *Shewanella hanedai* was found to occur in all the samples. Bergey's manual identified [1] only 11 bacteria; of these, *Shewanella hanedai, Photobacterium leiognathi, P. phosphoreum, Vibrio cholera, V. fischeri, V. harveyi, V. logei, V. mediterranei, V. orientalis*, and *V. splendidus*, all belong to the Vibrionaceae family, and *Photorhabdus luminescens* comes under the Enterobacteriaeae family. All are luminescent bacteria and our study showed the isolated bacteria comes under the study.

Another report, carried out in the Vellar estuary, Porto Novo, India nearer to the Karaikal coastal site, supports our research that luminescent bacterial species such as *Vibrio harveyi, V. fischeri*, and *Photobacterium leiognathi* were predominately present in sediment, water, and fish samples [21]. Yet another study isolated different luminous bacteria from both Hydrozoa and Bryozoa and they isolated *Vibrio fischeri* from *Aglaophenia tubiformis* and *Halopteris diaphana* [26].

The identified strains were inoculated into broth and tested for the intensity of bioluminescence. Light intensity produced by the enzyme luciferase was expressed in terms of photons emitted per time in seconds with the help of a luminometer and representing its unit as photons per second (pps). Among the strains, *Shewanella hanedai* emitted the maximum intensity of 69,702,240 pps, followed by *V.*

Table 1. Maximum number of photons emission by the identified luminescent strains.

Bioluminescent bacteria	Maximum number of photon emission
Shewanella hanedai	69,702,240 pps
Vibrio splendidus	55,912,600 pps
V. mediterranei	31,933,457 pps
V. orientalis	50,231,025 pps
V. fischeri	69,382,415 pps
V. harveyi	50,289,758 pps

Table 2. Diversity of identified luminescent bacteria in the collected sample.

Bioluminescent bacteria	Presence(+) or absence(-) of the strain in the sample			
	Water	Sediment	Squid	Cuttle fish
Shewanella hanedai	+	+	+	+
Vibrio splendidus	+	–	+	+
V. mediterranei	+	–	+	+
V. orientalis	–	–	+	+
V. fischeri	–	+	+	+
V. harveyi	–	+	+	+

Table 3. Percentage of 167 luminescent strains present in the collected samples.

Bioluminescent bacteria	Abundance of bioluminescent bacteria in SWC agar plate in percentage
Shewanella hanedai	49.8
Vibrio splendidus	24
V. mediterranei	18.6
V. orientalis	3
V. fischeri	1.8
V. harveyi	1.2
Others	1.6

fischeri (69,382,415 pps), *Vibrio splendidus* (55,912,600 pps), *V. orientalis* (50,231,025 pps), and *V. harveyi* (50,289,758 pps), whereas *V. mediterranei* exhibited the lowest (31,933,457 pps) (Table 1). Moreover, *Shewanella hanedai* was found to be present in all the samples (Table 2).

Shewanella hanedai was selected for luciferase study as it was found to be the most luminescent species and commonly present in all samples (Table 3). Based on the predominance of the species on the study area and its limited luciferase characterization when compared with *V. fischeri, S. hanedai* was taken for further study.

Optimization of Physicochemical Parameters for Maximum Luciferase Enzyme Production

Temperature. Among the various temperature ranges used to detect the optimum temperature for enzyme production, enzyme activity was highest at 25°C (69,674,387 pps) (Fig. 1), beyond which the enzyme showed immediate negative response. A similar result was observed that 20°C to 26°C was ideal for *Vibrio fischeri* for maximum luciferase activity [16]. Contradictory to this, some research recorded the maximum production of luciferase at 30°C [23].

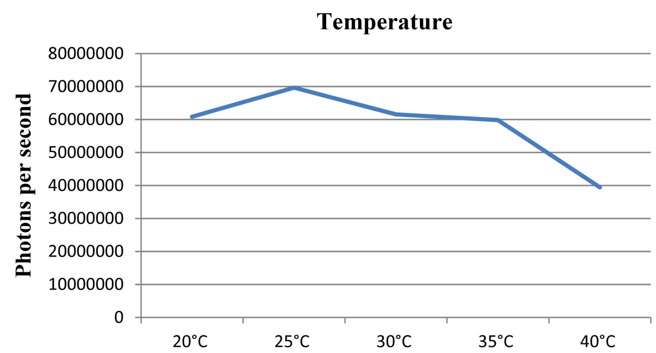

Fig. 1. Effect of different ranges of temperature on luciferase production.

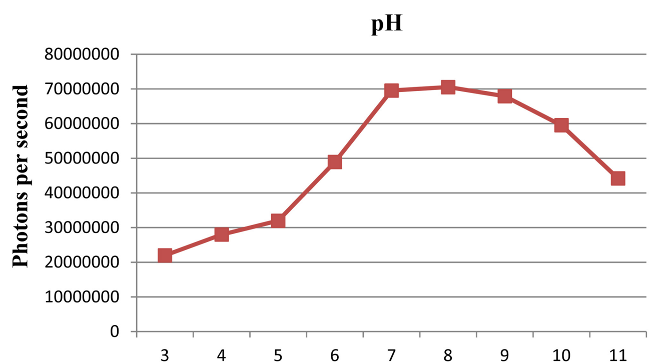

Fig. 2. Effect of different ranges of pH on luciferase production.

pH. pH values ranged 3–11 were studied for the detection of optimum pH for maximum enzyme activity. Enzyme activity was maximum at pH 8 (70,523,671 photons per second) (Fig. 2), which is almost similar to the pH of sea water in open sea, and showed a consistent luminescence between pH 7 and 9. Similar to this, an investigation evinced pH of 7.8 as the optimal pH for maximum production for *Vibrio fischeri* [24].

Salinity. Different salinity ranges from 5 to 50 ppt were employed to study their effect on enzyme production. Among these, enzyme activity was higher at 20 ppt (71,674,387 pps) (Fig. 3) and showed nearly negligible activity in 0 ppt. However, 25 ppt salinity was observed as being optimal for maximum production in the case of *Vibrio fischeri* [24].

Incubation period. Among the various incubation times maintained (4 to 24 h), maximum enzyme activity was observed from 16 h (69,895,714 pps) (Fig. 4) and it was

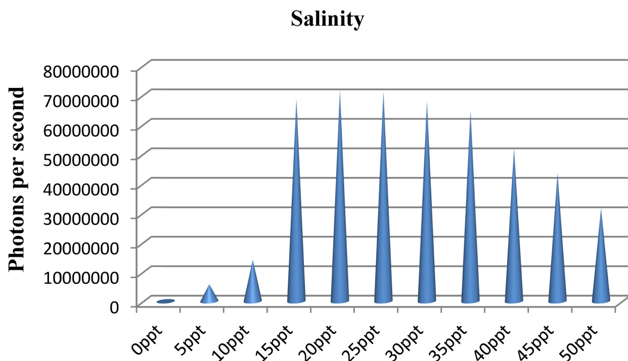

Fig. 3. Effect of different ranges of salinity on luciferase production.

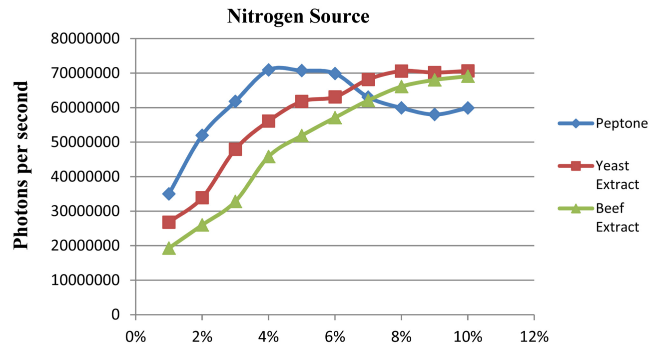

Fig. 5. Effect of different concentrations of various nitrogen sources on luciferase production.

maintained up to 36 h. The blooming of luminescence initiated from the log phase of culture and obtained its maximum at the end of the log phase, and maintained its ability up to the end of the stationery phase. However, an investigation observed the peak of luminescence at 19 h [3].

Nitrogen source. Various nitrogen sources such as peptone, yeast extract, and beef extracts were employed at different concentrations to study the effects on maximum luciferase enzyme activity. Among these, 4% peptone showed the maximum production of luciferase enzyme (70,895,152 pps) (Fig. 5). Beyond the limit of optimum nitrogen source, the luminescence maintained at a consistent range but the cells lost their luminescence ability very shortly and concentrated on cell growth.

Glycerol. Glycerol ranging in concentrations from 0.1% to 1% was employed to study its effect on enzyme production. The maximum enzyme activity was recorded from 0.9% (71,625,196 pps) (Fig. 6) and above this concentration it was maintained to 1%, as we studied, but 0.9% of glycerol was taken for production because of minimum volume with maximum enzyme activity. However, other researchers recorded glycerol at concentration of 0.3% to be ideal for maximum production of luciferase in the case of *Vibrio fischeri* [25].

Ionic supplements. Various ionic supplements were given at different concentrations and their effects on maximum luciferase enzyme production were studied (Fig. 7). Maximum luciferase activity was recorded in the case of magnesium sulfate at 0.2% (72,161,155 pps), followed by dipotassium hydrogen phosphate at 0.3% (73,919,915 pps) and calcium carbonate at 0.3% (73,991,591 pps). Potassium dihydrogen phosphate and ferrous sulfate showed no characteristic results in luciferase enzyme activity.

The results of the present investigation are strongly supported by several researchers [9, 29], where they estimated the influence of nutrient sources (particularly the nature and concentration of carbon and nitrogen sources) and environmental conditions (temperature, ionic strength, and pH) that may either stimulate or inhibit the luciferase enzyme production at various concentrations or ranges. It is important to conclude that, coupled with these parameters, the intrinsic physiological state of the bacterial cells also have pronounced effect on bioluminescence.

The above-mentioned ingredients that showed maximum enzyme production at optimum concentrations were used

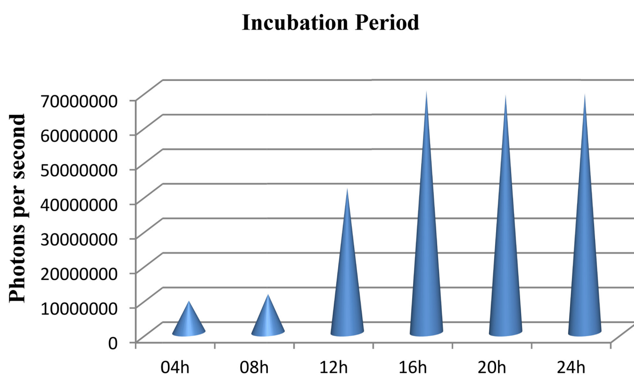

Fig. 4. Effect of different incubation periods on luciferase production.

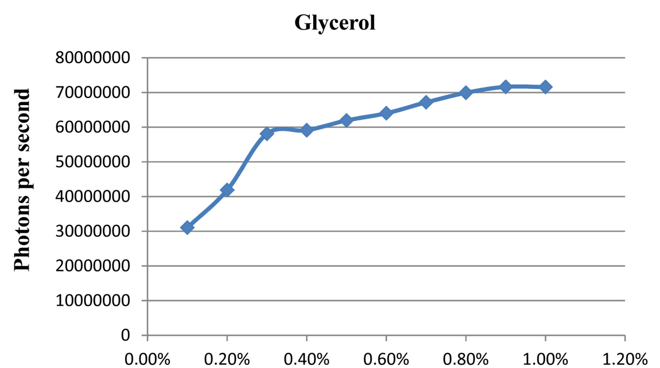

Fig. 6. Effect of different concentrations of glycerol on luciferase production.

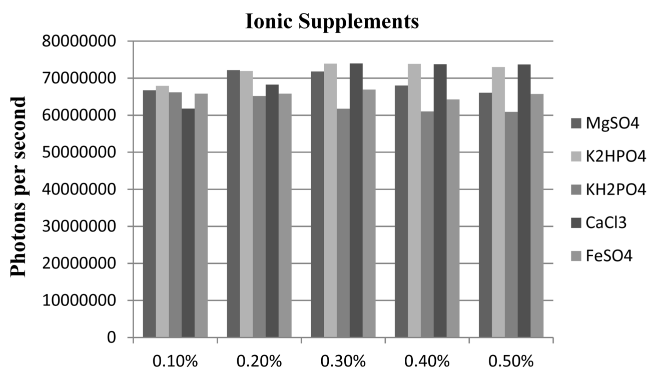

Fig. 7. Effects of different concentrations of various ionic supplements on luciferase production.

for the bulk production of *Shewanella hanedai* for further studies on the characterization of the luciferase enzyme.

Characterization of Enzyme

The bulk-produced *Shewanella hanedai* employing optimal ranges of various physicochemical and nutrient levels was subjected for purification of luciferase enzyme using ion-exchange chromatography and molecular weight determination using SDS PAGE.

The centrifuged culture supernatant was used as the enzyme source, from which the protein was precipitated at 60% saturation of ammonium sulfate and the protein concentrate dialyzed with dialysis membrane. In ion-exchange chromatography (DEAE cellulose), the most active fraction was eluted in 500 mM phosphate at pH 7.0 and the molecular mass of the luciferase enzyme was determined as 78 kDa by SDS-PAGE with standard and marker lanes. However, some researchers reported the molecular mass of bacterial luciferase as 80 kDa [11], followed by some reports that 76 kDa was the molecular mass for bacterial luciferase, flavin monooxygenase from *Vibrio harveyi* [5]. The purification was effective with 500 mM phosphate, pH 7, which was supported by a rapid protocol to separate luciferase subunits in 5 M urea-containing buffer using ion-exchange chromatography on Q Sepharose [5].

The present study evinced *Shewanella hanedai* to be the potential bioluminescent strain. The characterization of luciferase enzyme and the optimization of various physicochemical factors for maximum luciferase production and its activity stand as a ready reference for more elaborate work of this line in future. Discovering such species that produce luciferase with novel characteristics will be of great value to the enzyme industry for different applications, because most of the researches regarding bacterial luminescence were carried out on *Vibrio fischeri* and *V. harveyi* only. The present study also provides a commercially important species with its enzyme luciferase characterization, and may make inroads to recently advanced arenas such as *in vitro* and *in vivo* continuous monitoring of biological processes with clinical, diagnostic, and drug discovery applications, as well as the development of biosensors of compact size that enable the on-line and *in situ* monitoring of a large number of environmental parameters.

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