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Non-species specific composition of bioluminescent bacteria in non-bioluminescent squid *Sepioteuthis lessoniana* (Lesson, 1830)

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The present study investigated luminous bacterial species composition from non-bioluminescent big fin reef squid, *Sepioteuthis lessoniana* for the first time and revealed a new insight on the occurrence of five distinct luminous bacterial species such as *Vibrio harveyi*, *V. campbellii*, *V. vulnificus*, *V. alginolyticus* and *Photobacterium damselae* indicating the association of non-species specific luminous bacterial composition in *S. lessoniana*. Fourteen potential luminescent bacterial strains were isolated from *S. lessoniana* and identified based on morphological and biochemical tests. Majority of the isolates had produced industrially important extracellular enzymes- protease, chitinase and lipases. Antibiogram assay revealed that majority of the isolates were sensitive to various antibiotics and resistant to Ampicillin and Penicillin. While isolates CS1S and CS5S were sensitive to Ampicillin and Penicillin; and CS4G was sensitive to Penicillin. The present study is the first report to demonstrate luminous bacterial species diversity and extracellular enzyme production capabilities, and their antibiogram profile from big fin reef squid *S. lessoniana*.

[**Keywords**: Antibiotic Assay, Extracellular enzymes, Luminous bacteria, Non-species specificity, *Sepioteuthis lessoniana*]

Introduction

Bioluminescence is a natural light emitting phenomenon in numerous luminous organisms such as bacteria, fungi, firefly, fish, insects and squid which are found in different environments such as aerial, estuarine, freshwater, marine, and terrestrial milieus. Luminescence is produced upon a chemical reaction where the oxidation of a light-emitting organic molecule-generically called the luciferins-in conjunction with a catalysing enzyme, either a luciferase or a photoprotein[1-4]. Considerably, marine luminous organisms utilise bioluminescence for a variety of vital functions such as communication, diurnal migration, prey-predator interactions, reproduction and flow of food material through the food web[2]. Bioluminescence is generally higher in deep-living and planktonic organisms than in benthic and shallow organisms[3].

Luminous bacteria are the most widespread and abundant luminous microorganisms in nature. Marine luminous bacteria are represented in four bacterial genera: *Vibrios*, *Aliivibrio*, *Photobacterium* and *Shewanella*[3,4]. The functions of bioluminescence in bacteria may be either indirect or direct. In the indirect category, as shown by luminous symbionts in light organs, the light emission may be of benefit to the host as it is used as a behavioural cue for intraspecific signalling or interspecific interactions (prey attraction, predator avoidance, etc.). The host in turn provides a niche and/ or nutrient for the bacterium as in the case of Ceratioidea[1]. In direct cases-parasitic or commensal associations there are no such advantages to an associated organism involved. However, the light emitted may be an advantage to the bacteria by attracting predators which directly helps in the propagation of bacteria[4]. The bacterial luminescence reaction, catalysed by luciferase (an α-β heterodimer), involves the oxidation of reduced flavin mononucleotide ($FMNH_2$) and a long chain aliphatic aldehyde with the excess free energy being liberated in the form of light. The light emission continues until all the luciferin reserves in the cell are oxidized. The substrates of this reaction are coded on the *lux* operon present in all bioluminescent bacteria[4].

Bacteria does not show luminescence until they attain sufficient densities to initiate quorum sensing (QS), whereby there is a release of signalling molecules by bacterial cells into the extracellular environment and a response to the accumulation of the signalling molecule through changes in transcription and hence phenotype like bioluminescence[5]. The Gram-negative bacteria

produce N-acyl homoserine lactones (AHLs) that initiate QS. These AHLs are produced by the AHL synthetases[5,6]. As the intracellular concentration of the AHL molecules reaches a certain threshold limit, they bind to the receptors on the cell surface[7]. These active complexes in turn signal the promoter "*lux* operon" and induce the transcription of the QS genes responsible for bioluminescence.

The lux operon is the functional unit of genes coding for the components for the luminescence reaction. Five structural genes, *lux CDABE*, are required for light emission. The *lux A* and *lux B* genes code for α and β subunits of luciferase, a heterodimer involved as core genes in expression of luminescence[1]. The *lux C*, *lux D* and *lux E* code for the three components of the acid reductase complex that are required for the conversion of fatty acids into the long chain aldehyde required for the luminescent reaction[8]. In *A. fischeri*, *lux* I gene codes for auto inducers which bind to the receptor proteins, secreted by *lux R* and both *lux R* and *lux I* act as regulatory genes[4].

Recently, bioluminescent bacteria are in the limelight for their application as indicators or biosensors in use of living test organisms to measure the potential biological impact or toxicity of water or soil samples[4,9]. One such study was carried out where immobilized luminescent bacteria were used to check the toxicity of certain toxic chemicals[10]. Additionally, assessment of waste water metal toxicity was also successfully using luminous bacteria[9]. Clinical applications of luciferase are principally based on the measurement of ATP as biotic marker and have allowed a variety of enzymes, substrates, and cofactors to be assayed[11]. Bioluminescent bacteria, *V. harveyi*, *A. fischeri*, *Photobacterium leognathi* and *P. phosphreum*, isolated from the Boony estuary in the Niger Delta, have been found to utilize petroleum hydrocarbons as their sole carbon source[12], thus demonstrating their potential in bioremediation. Bioluminescence also serves as an early indicator of seafood spoilage[13]. These bacteria have been also used for monitoring environmental pollutants, both organic and inorganic[14] and are commercially available as environment monitoring bioluminescent assays. Luminescent vibrios are also known to produce a wide array of extracellular enzymes like proteases, chitinases, alginases, lipases, gelatinases etc. which have various industrial applications[4]. The present study was focused on the isolation, identification and ability to produce extracellular enzymes by luminous bacteria from non-luminous squid *Sepioteuthis lessoniana*.

Materials and Methods

Specimen collection and identification

Live squid specimens were collected from Chatham Jetty area (Fig. 1) during high tide with the

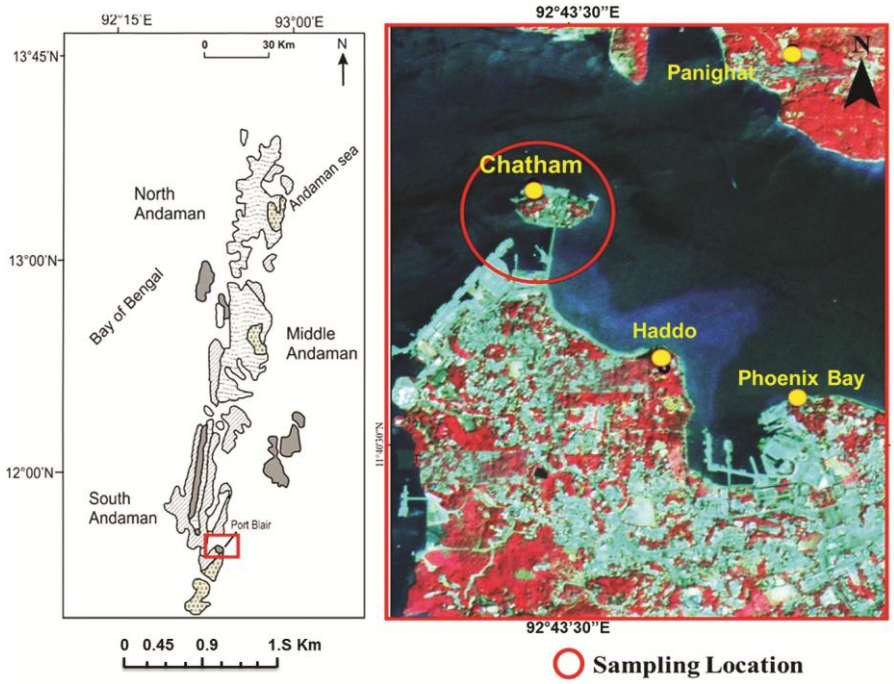

Fig. 1 — Map showing sampling location Chatam

help of local fishermen using squid jigs. Specimens were collected during evening in dim light and transported to the laboratory. Specimens were identified using standard taxonomic keys (Food and Agriculture Organization, 1984)[15], and were identified as *Sepioteuthis lessoniana*.

Isolation of luminescent bacteria

Swabs samples taken from the surface, caecal fluid, gut and ink gland were plated on Luminescent Agar[16] and incubated over night at 37 °C for 24 hours. Subsequently after the incubation period, petri plates were observed for luminous colonies in a dark room after eyes were dark-adapted for 5-10 minutes. Luminous colonies were picked with sterile toothpicks, transferred onto the same agar and purified by streaking. Pure isolates obtained were maintained on Nutrient agar containing 3 % NaCl for further studies.

Biochemical tests

Biochemical tests were performed according to Benson[17], and bacterial identification was carried out with standard biochemical identification keys[18]. For reliable identification, IDENTAX- Bacterial Identifier[19] and ABIS Online software's were also used.

Screening of extracellular enzymes

Skim Milk agar (HIMEDIA) with 2 % NaCl was used for screening of protease activity[17]. For lipase activity, spirit blue agar (HIMEDIA) was prepared by adding 3 % lipase substrate prepared as an emulsion by mixing 1 ml Tween-80, 100 ml olive oil and 400 ml of warm distilled water[17,20]. Yeast Extract agar (HIMEDIA) with 2 % NaCl and 1 % colloidal chitin as substrate was used for testing chitinase activity[21]. Petri-plates for the enzyme tests were incubated at 37 °C for 2 to 5 days. After incubation, plates were checked for clear zones around the colonies which indicated positive result for particular enzyme.

Antibiotic resistance profile

Different antibiotic discs such as Ampicillin, Penicillin, Streptomycin, Kanamycin, Tetracyclin, Gentamycin, Polymixin B, Nalidixic Acid and Chloramphenicol (HIMEDIA) with effective concentrations were tested against luminous isolates using disc diffusion method[22]. Antibiotic discs were placed on Muller Hinton Agar (2 % NaCl) plates seeded with overnight grown pure luminous bacterial broth cultures and incubated at 37 °C for 24 hours and subsequently zone of inhibitions were measured.

Results

A total of 50 luminous isolates were isolated, and among these, 14 strains showing intense luminescence were selected and subjected to biochemical identification. All the 14 strains were Gram negative, motile small rods. All 14 strains were positive to oxidase, catalase and negative to urease and citrate test. Strains CS4IG, LGo1, LGo2, and LS1 showed yellow colour on TCBS agar, while remaining strains displayed green colour. Based on these biochemical tests, five luminous bacterial species- *Vibrio campbellii*, *Photobacterium damselae*, *Vibrio vulnificus B2*, *Vibrio alginolyticus* and *Vibrio harveyi* were identified (Table 1). Most of the isolated luminous strains produced industrially important extracellular enzymes like protease, chitinase and lipase. While strain CS1S did not produce protease and chitinase but showed positive to lipase (Table 2). All 14 luminous strains were sensitive to most of the antibiotics tested. Highest sensitivity was observed against Chlopramphenicol and showed resistance against Ampicillin and Penicillin. The diameters of inhibition zones were measured in millimetre (mm) (Table 3).

Discussion

Studies on symbiotic luminous bacteria from squid of the genera *Uroteuthis*, *Loliolus* and *Euprymna* have been carried out extensively[23], and considerably with special emphasis on bobtail squid, *Euprymna scolopes*[24]. It was reported that sepiolid squids are known to contain two luminous *Vibrio* species, *V. fischeri* and *V. logei*[25,26], however, it was evident that light organs of the sepiolid genera *Rondeletiola* and *Sepiolina* found to contain *Photobacterium leiognathi*[27,28]. It was evident that *Photobacterium* species were more commonly found in the loliginid squid genera *Uroteuthis* and *Loliolus*[26]. Though studies have detailed the association of luminous bacterial species composition from different light organ containing squids[8], yet the non-luminous squids that lack in light organs have not been investigated. The present study demonstrated the non-species specific association of five luminous bacterial species with a non-luminous big fin reef squid *Sepioteuthis lessoniana*.

Studies by Mochizuki[29] and Lee *et al*.[30] have reported the tissue and ink potential of *S. lessoniana* in antibacterial and antiseptic activities[29,30]. Mohanraju *et al*.[31] have showed antibacterial activity in methanolic extracts of body tissue of *S. lessoniana*

Table 1 — Biochemical results of fourteen luminous isolates

Tests	Isolates													
	CCSW1	CCSW2	CS1S	CS2S	CS3G	CS4CF	CS4G	CS4IG	CS5I	CS5S	LGo1	LGo2	LS1	LS2
Gram staining	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Colour on TCBS	G	G	G	G	G	G	G	Y	G	G	Y	Y	Y	G
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arginine carboxylase	-	-	+	+	-	-	+	-	-	+	-	-	-	-
Lysine carboxylase	+	+	-	+	+	+	+	+	+	-	+	-	+	+
Ornithine carboxylase	+	+	-	-	+	+	+	+	+	-	+	-	+	+
Bile Esculine	+	+	-	-	+	+	-	+	+	-	-	-	+	+
Simmon Citrate test	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole test	+	+	-	-	+	-	-	+	-	-	+	+	+	+
MR test	+	+	+	+	+	+	+	+	+	+	+	+	+	+
VP test	-	-	+	+	-	+	+	-	+	+	-	-	-	-
H_2S production	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0% Salt	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6% Salt	+	+	-	-	+	+	+	+	+	-	+	+	+	+
8%Salt	+	+	-	-	+	+	+	+	+	-	+	+	+	+
10% Salt	+	+	-	-	+	+	+	-	+	-	-	-	-	-
Sucrose	-	-	-	-	-	+	+	+	-	-	+	-	+	-
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannose	+	+	+	+	+	-	+	+	+	+	+	+	+	+
Mannitol	+	+	-	-	+	+	+	+	+	-	+	+	+	+
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Salicin	-	-	-	-	-	-	-	-	-	-	+	+	-	-
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7° C	+	+	+	+	+	+	+	+	+	+	+	+	+	+
20° C	+	+	+	+	+	+	+	+	+	+	+	+	+	+
40° C	-	+	-	-	+	+	+	+	+	+	-	+	+	+
Urease	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatinase	+	+	-	+	+	-	-	+	-	-	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+

collected from Andaman Islands[31]. While antibacterial activities of different luminous bacterial species against human pathogenic bacteria are also demonstrated[32]. However, studies have not focused on composition of luminous bacterial diversity from this squid *S. lessoniana*. In the present study, luminous bacterial species *V. campbellii*, *P. damselae*, *V. vulnificus* B2, *V. alginolyticus* and *V. harveyi* were isolated from *S. lessoniana* and found significant change in luminous bacterial species composition. Studies by Balan *et al*.[33] reported the occurrence of different luminous bacterial species, *Shewanella hanedai*, *V. splendidus*, *V. mediterranei*, *V.* orientalis, *V. fischeri* and *V. harveyi* from squids[33]. While Guerrero-Ferreira *et al*.[34] reported *Vibrio* and *Photobacterium* from luminous loliginid and sepiolid squids from Australia[34]. Very recently, genetic diversity of luminous bacteria in pristine Andaman environment showed widely distributed luminous bacterial species in non-luminous samples including several plant animal components[35]. The results from the present and earlier studies showed the occurrence

Table 2 — Enzyme activity of luminous isolates

Isolates	Enzymes			Identified bacterial species
	Protease	Chitinase	Lipase	
CCSW1	+	+	+	*Vibrio campbellii*
CCSW2	+	+	+	*V. campbellii*
CS1S	-	-	+	*Photobacterium damselae*
CS2S	-	+	+	*Vibrio vulnificus* B2
CS3G	+	+	+	*V. campbellii*
CS4CF	-	+	+	*Vibrio alginolyticus*
CS4G	-	+	+	*V. alginolyticus*
CS4IG	+	+	+	*Vibrio harveyi*
CS5I	-	+	+	*V. alginolyticus*
CS5S	-	+	+	*P. damselae*
LGo1	+	+	+	*V. haryevi*
LGo2	+	+	+	*V. harveyi*
LS1	+	+	+	*V. harveyi*
LS2	+	+	+	*V. campbellii*

Table 3 — In vitro antibiotic sensitivity and resistance profile of luminous isolates

Isolates	Zone of Inhibition (mm)								
	AMP	P	S	K	TE	HLG	PB	NA	C
CCSW1	-	-	13	14	20	17	9	19	25
CCSW2	-	-	15	12	20	21	9	20	28
CS1S	12	9	15	16	26	24	17	28	32
CS2S	-	-	15	15	26	28	19	27	30
CS3G	-	-	15	12	24	21	10	24	25
CS4CF	-	-	16	12	21	21	10	20	21
CS4G	-	11	16	15	19	25	10	20	30
CS4IG	-	-	14	11	19	21	8	18	23
CS55I	-	-	16	13	18	20	11	28	25
CS5S	12	9	13	15	25	26	17	28	32
LGo1	-	-	14	12	22	20	8	16	27
LGo2	-	-	15	15	22	20	9	16	30
LS1	-	-	12	13	20	19	7	20	25
LS2	-	-	14	13	19	20	10	10	26

of distinct variation in luminous bacterial composition; this perhaps may be due to different bio-geographical factors which influence the bacterial composition in squids.

Principally these luminous bacterial association help squids for counter illumination to evade predators. Regardless of this purpose, luminous bacteria are also involved in production of different extracellular enzymes such as proteases, chitinases[36] and lipases which are necessary for bio-degradation of organic material available in marine environment. Proteases from Vibrios have been used for the breakdown of feather waste[37]. Lipases have a broad spectrum of specificity in degrading Tweens, phospholipids and also widely used as biocatalysts in industrial applications. Chitinases are enzymes which break down chitin[38]. Extracellular enzymes produced by the luminous strains isolated in this study may further be focused for industrial applications.

Conversely, luminous Vibrios are known to cause luminous vibriosis in aquaculture industries worldwide[39]. Although antibiotics have been used to control the outbreaks of this disease in aquaculture farms, there has been a limited success as application of antibiotics leads to antibiotic resistance among luminous bacteria. However, studies are being carried out to improve, control and treat the disease[40]. Therefore, it is important to know the sensitivity and resistance of these luminous bacterial isolates to different antibiotics. All the isolated strains in this study displayed antibiotic sensitivity and mostly to Chloramphenicol, while resistance was detected

merely against Ampicillin and Penicillin. The luminous bacterial species composition in *S. lessoniana* is an interesting incidence to understand their QS communication via AHL molecules. As indicated by earlier studies[5-7], species specific luminous bacteria usually display strong communication through via AHL molecules. However, the present study highlights a rare incidence where mixed luminous bacterial species produce luminescence, and this incidence is yet to be determined whether they produce luminescence due to QS displayed by individuals or combined activity.

Conclusion

The present study evinced the luminous bacterial species composition in big fin reef squid *S. lessoniana*. It is evident that this loliginid squid found to possess distinct luminous bacterial species that have not been previously described as associates. Further studies are yet to be ascertained on non-species-specific association between these luminous bacterial species with this squid and their quorum sensing mechanism.

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Conflict of Interest

Authors do not have any conflict of interest to declare.

Author Contributions

AZ, CHR and RM did the research conceptualization. AZ performed all experimental work. AZ and CHR wrote the Manuscript. CHR and RM guided AZ. Manuscript correction, editing, and approval was done by CHR and RM.

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