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Bioprospecting for industrially important biomolecules from gut microflora of the mud crab (*Scylla serrata*) from an estuary of the Mandovi River in Goa, India

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Original Article

Abstract

Scylla serrata generally known as the mud crab is a common inhabitant of estuarine waters and mangrove sediments in the Indo-Pacific region. Being a dweller of estuarine habitats and mangrove sediments along with having diverse feeding habits, it is an appropriate candidate for a bioprospecting study. The bacteria associated with its gut were isolated and screened for industrially important enzymes such as amylase, cellulase, chitinase and ligninase. The exopolysaccharide production, calcium carbonate dissolution and bioluminescence properties were also studied. Multiple distinctive bacteria that possess these characters were isolated. In this study we report for the first time, the presence of bacterium *Macrococcus caseolyticus* in the gut of the mud crab inhabiting an estuary of the river Mandovi in North Goa, India. Confirmatory identification studies were conducted by means of biochemical and molecular analysis and supplemented with data from scanning electron microscopy. We focus here on isolating and screening the gut microflora of the mud crab, *S. serrata* to understand its true industrial potential. We hope that the presence of bacterial isolates that have massive biotechnological applications as demonstrated in this study, can attract attention leading to investigations on the scantly studied gut microbiomes of marine crabs.

Keywords: *Mud crab, bioprospecting, enzymes, gut microflora, Macrococcus caseolyticus*

Introduction

The gut of an organism acts as a natural habitat and a nutritious environment for bacteria that are either beneficial or detrimental to the organism. As the gut is involved in the uptake of nutrients and water, the symbionts are likely to produce enzymes and other bioactive molecules that aid the host's digestive processes. In the marine environment, microorganisms have the choice and not dependency to live within a host unlike their counterparts in terrestrial habitats (Harris, 1993). However, similar to terrestrial mammals, host phylogeny, diet and growth state of the host seem to influence the composition of gut microbiota (Zhang *et al.*, 2016). Today, there is a rising need to find molecules that contribute to the advancement of human life. Biomolecules such as those derived from microorganisms are industrially important alternatives to chemical molecules. In this regard, marine environment have been explored much lesser than terrestrial biomes. Organisms from marine ecosystems have to withstand a myriad of environmental variations that can give rise to biomolecules of unique features. Primary metabolites such as

enzymes are known to be highly conserved. (Kathiresan *et al.*, 2008). Many of the industrially important enzymes today belong to the groups of amylases, cellulases, chitinases and ligninases.

Amylase is responsible for the breakdown of starch molecules into glucose monomers. They are heavily used in the detergent industry in formulation of detergents, production of biofuel and de-sizing process in textile industry (Souza *et al.*, 2010). Cellulases are enzymes that convert cellulose into simpler monosaccharides like glucose. They have various applications in industries like pulp and paper, laundry, textile, food and feed industry, brewing, bioethanol production and agriculture (Kuhad *et al.*, 2011). Chitinases are enzymes capable of degrading chitin to simpler sugars. Chitin being the most abundant polymer in the oceans, is a component of insect exoskeleton, fungi and exoskeleton of crustaceans (Souza *et al.*, 2011). They find applications in waste management and in agriculture as alternative to chemical pesticides against pathogenic fungi (Rathore *et al.*, 2015). Enzymes that breakdown lignin thereby modifying lignocelluloses and making carbohydrates more accessible for bioconversion are ligninases (Yadav *et al.*, 2015). Removal of recalcitrant compounds from the environment, dye degradation, wastewater treatment and soil treatment (Bandounas *et al.*, 2011; Jhadhav *et al.*, 2016; Yadav *et al.*, 2015).

Scylla serrata or the mud crab is found commonly in estuarine waters and mangrove sediments. They are mainly carnivorous in nature and feed on smaller crabs, worms, molluscs, etc. (Hill, 1979; Thimdee *et al.*, 2001). However, examination of the gut, revealed plant materials also (Hill, 1976; Thimdee *et al.*, 2001). Crabs receive microbes in the gut through water and organic matter that is populated with bacteria (Anandkumar *et al.*, 2014). These gut microbes often produce enzymes and bioactive compounds for their survival that may be industrially important (Anandkumar *et al.*, 2014; Haygood *et al.*, 1999; Harris, 1993; Hoyoux *et al.*, 2009; Priya *et al.*, 2019).

Given the spatial occurrence of *S. serrata* and its varied forms of nourishment, the gut microflora is presumed to produce a wide variety of enzymes and other bioactive compounds that aid in its digestive processes essential for its survival. However, marine crabs and their microbiomes have been the focus of only a handful of bioprospecting studies (Rameshkumar *et al.*, 2009; Anandkumar *et al.*, 2014). Hence, this study was undertaken to explore the industrial potential of gut bacteria of the mud crab.

Material and methods

Procurement of crabs

A male mud crab was purchased from Malim Jetty in Panjim, Goa, India. It was collected from nearby estuarine waters and identified as *S. serrata* (Fig. 1) due to the presence of H-shaped groove on the carapace, spines on the outer portion of carpus and presence of polygonal markings on all legs (Padate *et al.*, 2013).

Fig.1. *Scylla serrata*

Sample preparation

The crab was sacrificed according to the guidelines given by the Royal Society for the Prevention of Cruelty to Animals (RSPCA) (Najiah *et al.*, 2010). Prior to dissection, the crab was washed 2-3 times with sterile distilled water, followed by sterile saline washing twice. The surface was wiped using 70% ethanol to prevent surface contamination (Talpur *et al.*, 2011). Carapace was opened using blunt-end scissors and the gut was dissected into fore-gut and hind-gut (procedure adapted from Barker and Gibson, 1978.) The gut tissue was separately washed in sterile phosphate-buffered saline to remove the food bolus. Then the tissue was weighed and homogenized separately in a saline solution using a sterile mortar and pestle.

Enumeration of viable count and selection of isolates

100-fold dilutions (1:100 and 1:10000) were prepared from the individual tissue homogenates, and 0.1ml was plated onto Zobell marine agar (HiMedia, Mumbai) and nutrient agar (HiMedia, Mumbai). After incubation at room temperature for 24 hours the number of bacterial colonies were counted and the viable count was calculated as CFU/gram of sample. 8 isolates were selected based on colony morphologies such as pigmented colonies, gliding colonies, etc. (Al-Amoudi *et al.*, 2016).

Testing for the production of hydrolytic enzymes

The 8 isolates were screened for four different enzymes viz. amylase, cellulase, chitinase and ligninase by streaking on starch agar (HiMedia, Mumbai), carboxymethyl cellulose agar (Babavalian *et al*., 2012), chitin agar (Souza *et al*., 2009) and methylene blue –supplemented Luria agar (Bandounas *et al*., 2011; Jadhav *et al*., 2016) respectively. Positive isolates were selected based on zones around colonies after incubation for 24-72 hours at room temperature.

Exopolysaccharide (E.P.S) Production

The production of E. P. S was checked by streaking the 8 isolates on Congo red agar (Freeman *et al*., 1989). The plates were incubated at room temperature for 24 hours and checked for black colonies.

Test for bioluminescence

The 8 isolates were streaked on luminescent agar (Badar *et al*., 2012). After 24 hours of incubation at room temperature, the plates were checked in the dark for glowing colonies.

Calcium carbonate ($CaCO_3$) dissolution

The isolates were tested for their ability to dissolve calcium carbonate by streaking them on calcium carbonate agar (HiMedia M1900). The plates were checked for clearance around the colonies after 24-48 hours of incubation at room temperature.

Morphological, biochemical and molecular characterization

The isolate that showed positive results for most tests (*2*.4-2.7) was further analyzed via morphological, biochemical and molecular characterization.

Scanning electron microscopy: A glass slide (1cm x 1cm) was placed in a flask containing nutrient broth and inoculated with the isolate. The flask containing the sample was incubated for 18 hours at room temperature. Simultaneously, a loopful of an 18-hour old bacterial culture on nutrient agar was smeared on another glass slide (1cm x 1cm). Both the slides were fixed with 3% glutaraldehyde solution for 3 hours and then washed with an increasing gradient of ethanol dilutions in water (v/v) (10, 30, 50, 70, 90 and 100%). The samples were sputter-coated with gold and visualized using the scanning electron microscope (ZEISS- EVO18 Special Edition).

Biochemical characterization: For characterization of the MGN4 isolate, we studied the gram character of the cell wall, colony pigmentation, catalase, oxidase, sugar fermentations of D-Mannitol, D-Glucose, D-Lactose and D-Sucrose, methyl red test and Hugh Liefsons test.

Molecular characterization: DNA was isolated from MGN4 and the molecular characterization was carried out by 16s rRNA gene sequencing. The FASTA sequence was compared to the closest related species according to the EzBioCloud database.

Results

The isolation and estimation of bacteria associated with the gut of mud crab was performed via viable count. Viable count was expressed as x 10^4 colony forming units (CFU) per gram of gut tissue plated on Zobell marine medium and nutrient medium respectively: fore-gut: 23.3, 21.9; hind-gut: 680, 273. Viable count is seen to be higher in comparison to the average CFU present in the waters of the Mandovi Estuary (Rodrigues *et al*., 2011). This relatively high viable count in the gut tissues is possibly due to the colonization of the gut wall (Harris, 1993) by the rich bacterial flora present in the mangrove and estuarine sediments (Venkateswaran *et al*., 1981; Rodrigues *et al*., 2011).

8 distinct isolates from the total observed colonies were selected for primary screening based on distinguishing characteristics such as pigmentation of colonies, gliding colonies, production of EPS layer, etc. These isolates were named FGN1, MGN4, HGN1, HGN2, MGZ2, HGZ1, HGZ2 and FGN2.

S. serrata has a complex diet of plankton, plants, smaller crabs etc. The presumption that the gut microbiota could produce many different enzymes corroborated with our results as 6 of the 8 selected isolates showed positive results for at least one enzyme tested (Figs. 2-5). One isolate in particular, MGN4 exhibited positive results for the production of four enzymes i.e., amylase, cellulase, chitinase and ligninase. 5 of the 8 isolates i.e., FGN2, HGZ1, MGN4, HGN1, HGN2 appeared as black colonies on Congo red agar indicating exo-polysaccharide production (Fig 6). None of the isolates were found to be positive

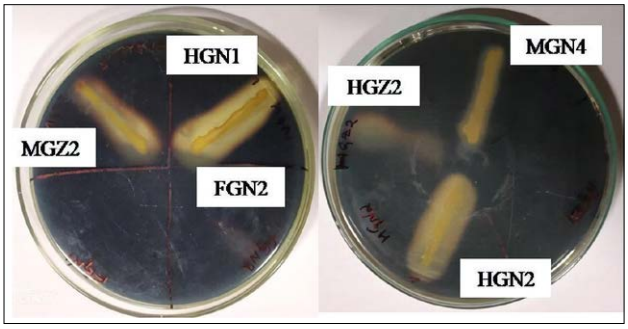

Fig. 2. Amylase producing isolates- as indicated by zones around the colonies

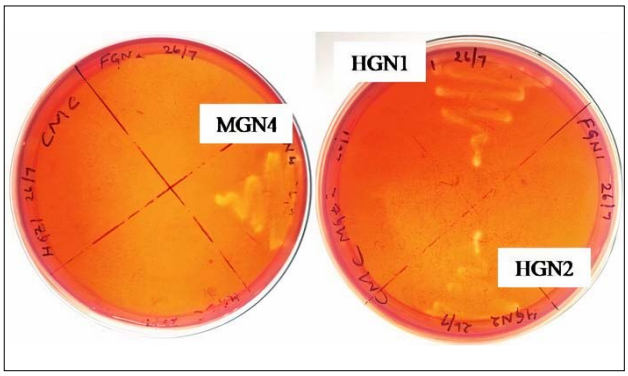

Fig. 3. Cellulase producing isolates-plates with cellulase positive isolates having clear zones around the colonies

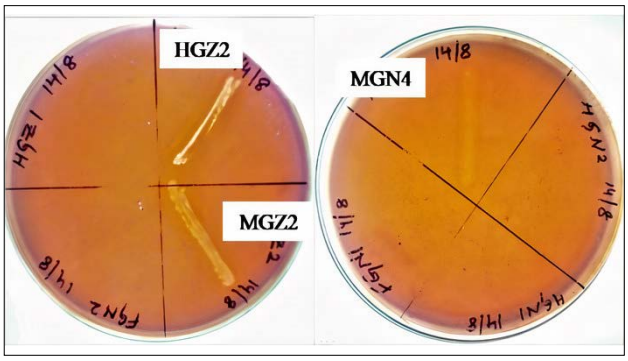

Fig. 4. Chitinase producing isolates- as indicated by the clear zones around the colonies

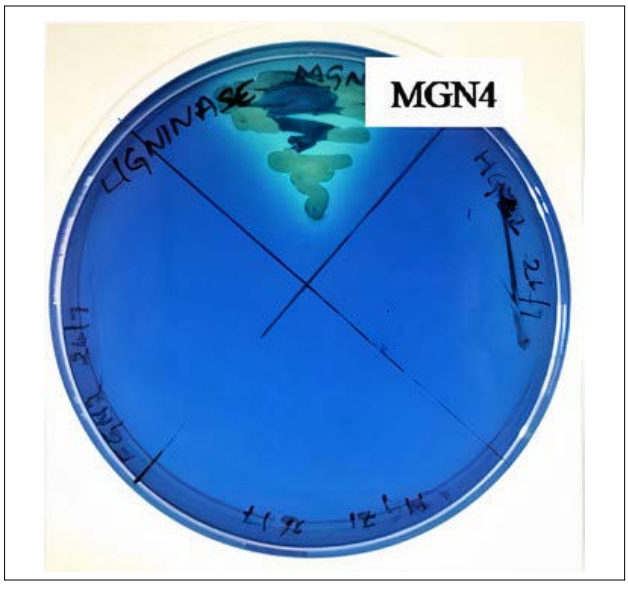

Fig. 5. Ligninase producing isolates-the isolate MGN4, which is positive for Ligninase, indicated by a clear zone around the colonies.

for bioluminescence and solely MGN4 showed positive result for Calcium carbonate dissolution (Fig. 7).

The isolate MGN4 was studied using SEM, molecular identification and biochemical characterization. In the SEM

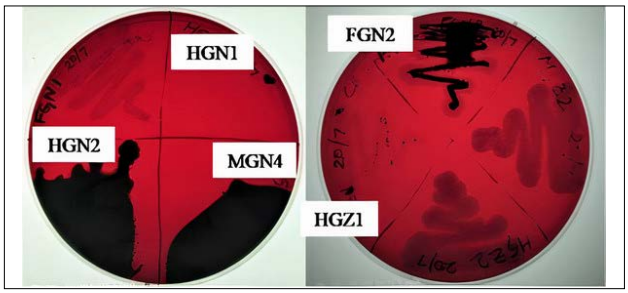

Fig. 6. Exopolysaccharide producing isolates-indicated by black coloured colonies

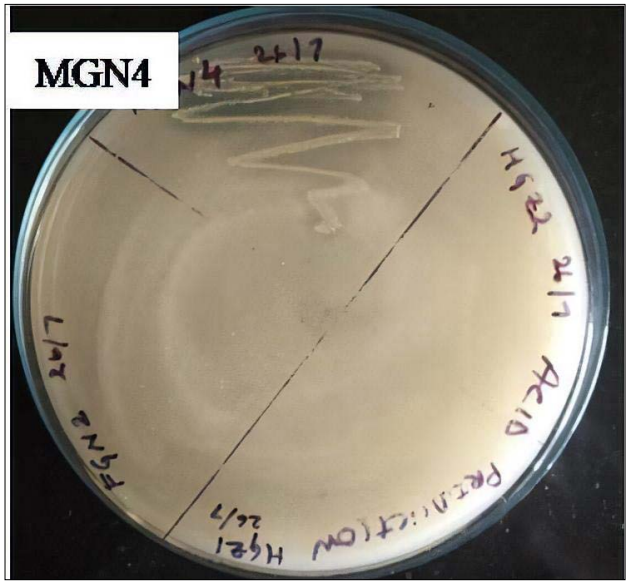

Fig. 7.Calcium carbonate dissolution-the isolate MGN4 shows dissolution of Calcium carbonate as indicated by the clear zone around the colonies.

image globular, cocci-shaped cells were observed packed in cluster type arrangement. The cell size was measured to be in between 1.0-1.3 μm. in diameter (Fig. 8).

The 16s rRNA gene sequence showed 100% sequence homology

Table 1. Biochemical and morphological characterization

TEST	RESULT
Gram Character	+ve
Shape	Cocci, Arranged in clusters
Cell Size	1–1.3 μm
Colony Pigmentation	Yellow
Catalase	+
Oxidase	+
Urease	-
Methyl Red	+
D-Mannitol (fermentation)	-
D-Glucose	+
Lactose	-
Sucrose	-
Hugh Leifson's*	Fermentative*Hugh Leifson's test result (+, +) is interpreted as presence of fermentative type of metabolism of glucose.

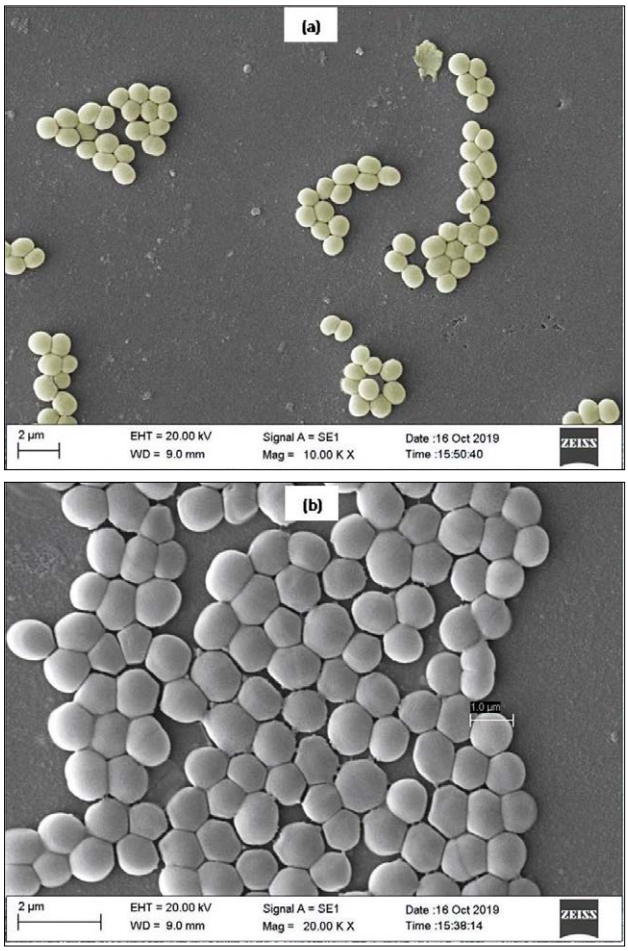

Fig.8. (a) and (b) show Scanning Electron Microscope Image under 10,000X and 20,000X magnifications respectively. Fig. 8(a) is artificially coloured for illustration purposes

to *Macrococcus caseolyticus* ATCC 13548(T) when matched to the EzBioCloud database. The biochemical characterization of this isolate was found to corroborate this finding (Table 1). (Garrity and Holt, 2001).

Discussion

M. caseolyticus was previously included among the *Staphylococcus* genus, but later assigned its own genus due to the fact that it has a significantly smaller genome and is deficient in several sugar and amino acid metabolism pathways and genes that confer virulence that are present in *Staphylococci* (Baba *et al*., 2009; Kloos *et al*., 1998*). M. caseolyticus* has predominantly been isolated from animal skin, meat and dairy goods although marine sources have also been reported. In Ganesan *et al.* (2010) isolated the bacterium in the marine environment from the green algae *Ulva lactuca.* An older study reported isolation from skin of pilot whales (Kloos *et al*., 1998). However, to the best of our knowledge, this is the first report of *M. caseolyticus* to be isolated from the gut of *S. serrata* or any other crabs. The strain was deposited at NCMR, Pune, India with the accession number MCC 4302.

This isolate has demonstrated immense potential for application in various industries. Its amylase, cellulase and ligninase activity could find use in the paper and pulp and textile industry as well as applications in dye degradation and bioremediation of recalcitrant compounds in the environment (Souza *et al*., 2010; Kuhad *et al*., 2011; Bandounas *et al*., 2011; Jhadhav *et al*., 2016; Yadav *et al*., 2015). The chitinase and calcium carbonate dissolution ability may find an application in the management of crab and shrimp shell waste and in the reclamation of sodic soils (Kumar *et al*., 2018; Tamilselvi *et al*., 2016; Rana *et al*., 2015). According to Harris (1993), resident bacteria in the gut of marine invertebrates can either form permanent and stable populations by inhabiting the crevices and pouches in the gut or may colonize the gut contents or be attached to the epithelial lining. In crustaceans, microbiota have been reported in the mouth, stomach, midgut, hindgut and the digestive glands (Harris, 1993; Hoyoux *et al*., 2009; Li *et al*., 2012; Chen *et al*., 2015; Zhang *et al*., 2016). In a study conducted on Chinese mitten crab *Eriocheir sinensis*, it was reported that the microbes associated with the gut of the crab, differed from the microbes associated with the gills, suggesting site specific microbiota like mammals (Zhang *et al*., 2016). In yet another study, habitat-specific gut microbiota was observed in the Crustacean *Idotea blathica* (Mattila *et al*., 2014). Research on mud crabs (*S. paramamosain)* suggested that intestinal microbial diversity greatly differed on the basis of whether the crab was a wild crab, a healthy cultured crab or a diseased crab, implying the role of the gut microbiota on the physiological health of the host (Li *et al*., 2012). The gut microbiome in Galatheid crabs (*Munidopsis andamanica*) were presumed to be involved in the digestion of wood falls in the Pacific Ocean (Hoyoux *et al*., 2009). Microbes associated with crustaceans have also been known to produce proteases, lipases and chitinases which aid in digestive processes (Harris, 1993). A few recent studies have also reiterated the same. An example of the same is the bacterium *Bacillus subtilis* isolated from the gut of crab *Portunus pelagicus*, that was found to be a protease enzyme producer (Priya *et al*., 2019).

Our results indicate that the mud crab gut is an attractive source for the study of industrially important microorganisms and biomolecules useful in various biotechnology sectors. Through this paper we highlight the necessity of focusing future bioprospecting studies on the gut microbiota of marine invertebrates especially crabs from various environments.

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