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Research Article

Identification of Protease Synthesizing *Micrococcus Yunnanensis* from the Gut of Mud Crab *Scylla Serrata*

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ABSTRACT

In the present study screening of bacteria from the gut of mud crab *Scylla serrata* collected from Karwar coast (N-13°, 05.722'; E- 079°, 48.658'), Karnataka has been resulted in the isolation of a yellow pigmented, protease producing gram positive bacterial strain AN-06. It was able to utilize casein as substrate for growth and showed zones of clearance around the colony in Skim milk agar medium, and hence was selected for molecular characterization by 16S rRNA sequencing. The strain AN-06 was identified as *Micrococcus yunnanensis* based on the phenotypic characteristics and 16S rRNA sequence analysis (99% similarity) (Genbank accession number KU937307). *Micrococcus yunnanensis* is gram-positive, aerobic, non-endospore-forming, non- motile cocci. Optimal growth was observed at temperature, 28°C; pH, 8.0 and NaCl, 15%. There is only very little information on gut microbes from marine crab and their functional diversity. It is the first time report on the occurrence of protease producing *Micrococcus yunnanensis* from the gut of a healthy marine crab. The role of these bacteria may be for protein digestion in marine crabs. However, detailed studies are required to know the source and exact role of this bacterium *in Scylla serrata gut*

Keywords: Scylla serrata; *Micrococcus yunnanensis*; Protease; Gut bacteria

INTRODUCTION

The digestive tract of both marine as well as freshwater fishes host regular microflora, which are autochthonous and allochthonous [1]. Information on the interaction between the gut microbiota and intestinal immunity in fish and crustaceans is available is remarkably less. The connections between gut flora and host are one of the most important factors that influence aquatic animal health [2]. The intestinal flora has been reported as vital to the development [3].Immunity and disease resistance of gut [4,5]. Gastrointestinal bacteria also have significant role in nutrients digestion and provide the host with physiologically active materials, like enzymes, amino acids and vitamins Symbiotic bacteria in an animal's digestive tract often produce enzymes that would complement for digestion of foods as well as synthesize compounds that are assimilated by the host [6]. Harris suggested that gut bacteria may contribute significantly to nutrient gain by aquatic hosts [7,8]. Giant prawn *Macro rachium rosen ergi* (Colorni), the *Polychaete hiepussetosus* [9] prawns upogenbia africana and Callinessa kraussi halothuroid *Psychropoles sp* Deep sea amphipod *yssianssidae hirondella* and sea urchin *chinus e ilentus* (Unkls) have been shown to maintain a permanent and consistent microbiota in the gut, which is significantly different from that of the surroundings. In many studies there is no indication to suggest the bacteria isolated from the gut are anything other than ingested, transient bacteria. Nagasawa and Nemoto reported that bacteria may be an important food source for some marine invertebrates . The most commonly isolated bacterial genera are *Pseudomonas, lavo acterium, eromonas , Micrococcus and Staphly coccus* [10]. Gut microflora plays an important role in the in the digestive process, growth and disease resistance of the host although few reports concerning microbial enzyme production in the gastrointestinal tract of fish are available [11] information on the distribution of these enzyme-producing endosymbionts in different regions of the gut are scarce [12].

Enzymes are considered as a vital resource utilized by the food, chemical and allied industries to produce a wide range of biotechnology products and have already been recognized as valuable catalysts for various organic transformations and

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production of fine chemicals and pharmaceuticals [13-15]. The important protease producing bacteria reported from fishes are species of *acillus, Pseudomonas, alomonas, rthro acter* and *Serratia* . Proteolytic enzymes are found in all living organisms, playing an essential role in cell growth and differentiation. The extracellular proteases have commercial value and have been applied in multiple processes in various industrial sectors. Microorganisms are the most desired sources for enzyme production because of their rapid growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications [16].

Mud crabs are widely used in aquaculture practices owing to its winsome qualities such as faster growth, larger size, high reproductive capacity, disease resistance, marketability, adaptability to farming systems etc. The mud crab, Scylla serrata is an ecologically important crab and it is widely distributed in the Pacific and Indian oceans. In addition, they have a high tolerance to abiotic factors and the gut content analysis provided important insights into feeding patterns. Studied the food and feeding habits of *Scylla serrata* from Karwar waters reported that fish accounted as 23.57%, crustaceans as 18.37%, and detritus as 35.7% in its gut [17].

Taxonomically, Micrococcus belongs to the kingdom bacteria, Phylum:Actinobacteria, Class: Actinobacteria, order: Actinomycetales, family:Micrococcaceae, and genus:Micrococcus. Micrococcus species are widely distributed in nature and their potential to grow and secrete active protease at high salt and alkaline pH makes them an ideal agent for treatment of high alkaline and saline effluents from leather and other industries using the concept of bacterial biofilm. *Micrococcus yunnanensis* is one of six species of genus *Micrococcus* . It can be isolated from plant roots of polyspora axillaris. These bacteria that colonizes the internal tissue of the plant showing no external sign of infection or negative effect on their host. Reported that proteolytic activity of protease from *Micrococcus aloeverae* and *Micrococcus yunnanensis* isolated from aloe vera leaf were active at high temperature, pH and salt conditions indicating that these factors are not inhibitory for production of protease from this species, pH required for induction of protease production varied from genus to genus. Also reported protease production in alkaline medium at pH 10 and 10.5 respectively by the bacillus species and showed optimum production of protease at pH8 with continuous decrease in alkalinity [18].

Being a crab species with high demand, it is essential to know about the microbes associated with gut. Such information's will be of highly useful to understand the immunity aspects of the animal and also to ensure the safety for human consumption. keeping this idea, a preliminary analysis was initiated to find out the microbial population as well as to find out any useful bacteria living in the gut of the mud crab [19].

MATERIALS and METHODS

Collection of crab

Mud crab *Scylla serrata* were collected live from shore seines operated along Karwar coast (N- 13°, 05.722'; E- 079°, 48.658'), Karnataka with the help of the local fishermen Four live Scylla serrata specimens were transferred aseptically to an ice box and transported to the laboratory at Karwar Research Centre of Central Marine Fisheries Research Institute (CMFRI) for further processing. In the laboratory, the digestive tracts were removed, cleaned and cut into pieces. The pieces were transferred to sterile petri-plates and thoroughly flushed with chilled sterile saline (pH 7.4; 0.89% NaCl), and homogenized using sterile saline (10:1; volume: weight). The homogenate thus obtained was used as inoculum for bacterial culture [20].

Isolation of bacteria from GI tract contents

Serial dilution (10-1-10-6) of the gut homogenate was performed by using sterile normal saline. The dilutions were plated on nutrient agar medium by spread plate method and incubated at 37°C for 24 h.

Characterization of bacterial isolate

All bacteria isolated from crab gut contents were characterized by following Bergey's Manual of Systematic Bacteriology. Individual isolate which had shown casein hydrolysis was maintained in nutrient agar slants at 4°C in refrigerator with periodic sub culture.

Screening of protease producing bacteria

After 24 h growth the different colonies were picked and transferred to fresh plates of nutrient agar medium. To get pure cultures the isolates were streaked several times on nutrient agar slants. The morphology of the bacteria and purity of the cultures were examined with a stereoscope microscope. Single colony of the purified isolates were tested for protease activity by streaking on skim milk agar medium (peptone: 5 g L-1, beef extract: 3 g L-1, skim milk: 5 g L-1 and agar: 15 g L-1) and incubated at 37°C for 24 h. Occurrence of zone of clearance was recorded for proof of casein hydrolysis by the strain.

Optimization of growth and protease production

The pure bacterial isolate showing casein hydrolysis was further screened to study their tolerance to temperature, pH and salt (NaCl) at different levels. Salt tolerance was tested in nutrient broth medium at different concentrations of NaCl (0, 2, 5, 10, 15, and 20% w/v). The temperature resistance of the isolates was studied by growing them in temperatures ranging from 5 to 500C in nutrient agar. The pH tolerance was examined by growing the isolates in nutrient broth medium at different levels of pH (from 4 to12; adjusted using 0.1 N HCl and 0.1 N NaOH).

Molecular characterization of selected bacterial strain

For PCR amplification of the 16S rRNA, sequencing and phylogenetic analysis, DNA was extracted from selected culture using phenol chloroform extraction method [21]. The extracted DNA was checked through 1.2% agarose gel (10 × 4 cm) electrophoresis with ethidium bromide incorporated in 1X TAE buffer for 30 minutes at constant voltage (90 V). After electrophoresis, the gel was observed in ultraviolet light and documented using the BioDoc-ItTM imaging system (UVP). 16S rRNA genes were amplified with the universal primers 27F and 1492R by using the Polymerase Chain Reaction (PCR). The nearest taxa of the 16S rRNA gene sequence (1418-1542 bases) were identified by BLAST sequence similarity. The CLUSTAL W software was used to align 16S rRNA gene sequences and Maximum Likelihood (ML) and neighbor joining methods with MEGA version 5 were used to construct the phylogenetic tree [22].

RESULTS

Isolation and enumeration of bacteria

The numbers of cultivable bacterial cells present in crab gut were estimated after isolation and growth on nutrient agar medium supplemented with 2% (w/v) sodium chloride and incubated at 35±2°C for 48 h. The total heterotrophic bacterial load was observed to be 3.20×105 cfu g-1

Characterisation of isolated bacteria

A total of 3 bacterial strains were isolated from gut of mud crab *Scylla serrata* and classified into 3 taxonomic groups by Pseudomonas, Arthrobacter and Micrococcus (Table 1).

SI. No.	Name of the Test	Scylla serrata G-1 Pseudomonas sp.	Scylla serrata G-2 Arthrobacter sp.	Scylla serrata G-3 (AN-06) Micrococcus sp.
1	Gram's stain	G(-)	G(+)	G(+)
2	Cell shape	Rods	Rods	Cocci
3	Density	Trans.	Opaque	Opaque
4	Elevation	Convex	Convex	convex
5	Margin	Entire	Entire	Entire
6	Shape	Round	Round	Round
7	Pigments	Off white	Off white	Yellow
8	H2S production	-	-	-
9	MacCon agar test	NLF	NG	NG
10	Fluorescence	-	-	-
11	Motility	M	NM	NM
12	Catalase	+	+	+
13	Oxidase	+	-	-
14	Indole	-	-	-
15	MR	-	+	-
16	VP	-	-	-
17	Citrate utilization	+	+	-
18	O/F test	-	-	-
19	Nitrate reduction	+	+	-
20	Penicillin sensitivity	R	R	R
21	Gelatin liquefaction	-	-	-
22	Starch Utilization	-	-	-
23	Cellulose hydrolysis	-	-	-
24	Protease activity	-	-	+
Sugar utilization Test				
1	Adonitol	-	-	-
2	Arabinose	-	-	-
3	Cellobiose	-	-	-
4	Dulcitol	-	-	-
5	Fructose	+	-	-
6	Glucose	+	-	-
7	Inositol	-	-	-
8	Lactose	+	+	-
9	Mannitol	-	+	-
10	Mellibiose	-	-	+

11	Raffnose	-	-	-
12	Rhamnose	-	-	-
13	Salicin	-	-	-
14	Sorbitol	-	-	-
15	Sucrose	+	+	-
16	Trehalose	-	-	+
17	Xylose	-	-	-

Table 1: Biochemical characteristic of bacteria isolated from *Scylla serrata*.

All the isolated bacteria were tested for their biochemical characters which showed the isolates were gram positive cocci, gram negative and positive rods. Among the isolates one of the strains showed positive results on protease active and yellow pigmented, two were utilized citrate and nitrate reduction by two. All strains were positive in catalase. The protease active and yellow pigmented bacterial strain obtained in the study was selected for further molecular level characterization (Figures 1 and 2).

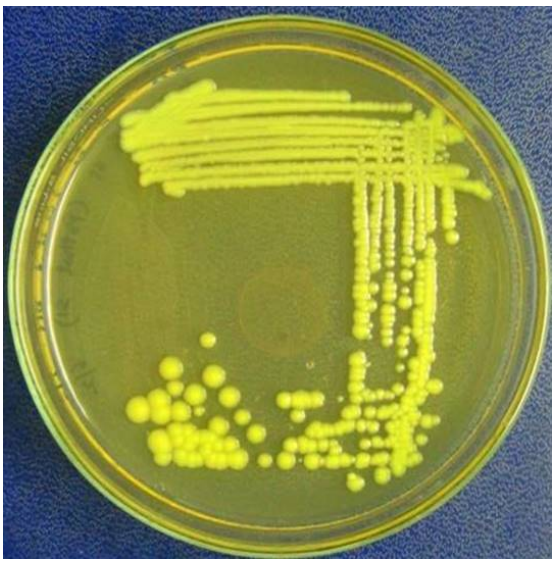

Figure 1: Yellow Colour bacteria on agar plates from gut of mud crab Scylla serrate.

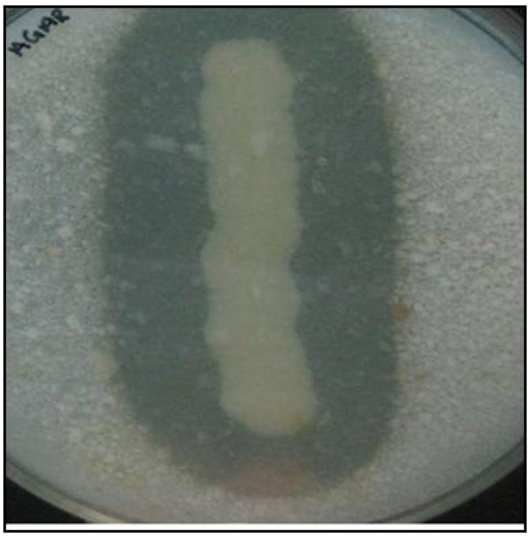

Figure 2: The plate showing the protease activity by Micrococcus yunnanensis using Skim Milk Agar Plate.

Molecular characterization of selected bacterial strain

The DNA of bacterial strain was extracted and used as template for further studies. DNA extraction resulted in bright bands in the very high base pair range of a gel electrophoresis. An OD260:280 ratios between 1.8 to 2.0 was obtained for the extracted DNA samples, thereby indicating that DNA preparation of the isolates was proper, and the samples were pure and free from protein or phenol contamination.

Identification and phylogeny

Sequence analysis of PCR products revealed that, for the isolate there was 1% or no differences with the most closely matched sequences in the databank. The isolate was identified as: AN-06 as Micrococcus yunnanensis and the sequences were deposited at GenBank with the Accession number KU937307. The phylogenetic tree (Figure 3.) was inferred from Kimura 2-parameter by the neighbour-joining method. The analysis of 16S rRNA gene sequence indicates the position of the native identified isolates in the same cluster with respect to their reference group.

Figure 3: Mud crab Scylla serrate.

Description of AN-06 micrococcus yunnanensis

The strain was gram positive, non-motile non-endospore-forming cocci. It was found to be catalase positive and oxidase negative; gelatin and starch were not hydrolysed by it. The strain was negative with tests for methyl red, Voges Proskauer reaction and reduction of nitrate. The isolate was identified as Micrococcus sp. based on the results of biochemical and physiological characteristics. Thus isolated colony of Micrococcus sp. was used to record production of protease enzyme using Skim Milk Agar medium for 24 h. Clear zone around the colony in plates were the indication of protease production. Protease was synthesized by the strain for utilization of protein in the skim milk agar medium [23].

Optimization of growth and protease production

The characterization of the isolate was optimized by using physiological tests. The effect of temperature on protease production was recorded at different temperature regimes ranging from 5-50°C. Maximum zone of clearance 22 mm was recorded at 25°C in skimmed Milk Agar medium. The effect of pH on protease production was studied by growing the bacteria in the medium at different pH (4, 5, 6, 7, 8, 9, 10, 11 and 12). At a pH of 8 maximum zone of clearance 22 mm was recorded. The effect of NaCl on protease production was also tested (NaCl 0-20%) and at 15% NaCl maximum zone of clearance 23 mm was recorded [24,25].

DISCUSSION

The aim of this study was to isolate and characterize bacteria from the mud crab gut. It was earlier reported that the crustacean gut as sterile. However, in the present investigation the mean Total Viable Count (TVC) of bacteria in the gut of Scylla serrata was 3.20×105 cfu g-1. Using a variety of phenotypic tests we tried to characterize the isolates. Using 16S rRNA sequencing, presumptive identities and relationships between bacteria isolated could be made more easily. In this study *Micrococcus Yunnanensis* a gram positive protease producing aerobic gut bacteria from mud crab Scylla serrata was isolated and characterized by following conventional phenotypic tests and by molecular method. The mud crab is a detritivore and scavenger so the diet would include protein, polysaccharide and other complex carbohydrates. We have observed that bacteria isolated from Scylla serrata gut can hydrolyse protein and that they grow in complex media. This could suggest that they play a role in the digestion of the crab. Before any conclusions can be drawn about whether any of this bacterial strain is indeed a symbiont of Scylla serrata or are merely ingested with the food of the crab further investigations need to be carried out. It has been reported that proteolytic enzyme producers are helpful for the health of the ecosystems of this earth as these microbes decompose the dead and decaying animal or plant tissues in water or land and they can create pollution free environment and are responsible for the recycling of nutrients.

Micrococcus Yunnanensis isolated from the crab digestive tracts in the present study can be beneficially used as a probiotic while formulating the diet for cultivable crab species, especially in the larval stages. The main strategy to use probiotic is to isolate intestinal bacteria with favorable properties from mature animals and include large numbers of these bacteria in the feed of immature animals of the same species. The use of probiotics in commercial aquaculture is necessary for formulating diets at larval stages to minimize the cost of feed preparation. Few reports on proteolytic enzymes isolated from marine origin have been reported. Have been reported protease producing actinobacteria from the gut content of the shrimp.

It was observed in the present study that *Micrococcus Yunnanensis* can grow at high temperature, pH and salt conditions indicating that these factors are not inhibitory for production of protease for the species. Protease production was active and stable in extreme physiological conditions for the isolate. Rahman et al. reported that enzyme synthesis and energy metabolism of bacteria was controlled by temperature. Moreover, temperature significantly regulated the synthesis and secretion of bacterial extracellular protease enzyme. Effect of pH on protease production has also been reported by other researchers in different species of bacteria including Micrococcus, Bacillus, Pseudomonas, Burkholderia, and Geobacillus. The pH of the culture strongly affects many enzymatic processes and transport of compounds across the cell membrane. The protease activities of the isolate were also affected by the pH. Increase in pH resulted in corresponding increase in protease production. The supplementation of sodium chloride was used as inducers for protease secretion. Increased the concentration of NaCl increased the protease production. It was earlier established that bacteria utilizes the sodium-driven solute transport systems for their survival and adaptation in high pH environments. Hence, our study was focused to optimize temperature, pH and NaCl for the efficient production of protease enzyme. It was found that optimum temperature of 28oC, pH 8 and NaCl 15% was effective condition to produce high amount of protease enzyme when compared with others.

CONCLUSION

In conclusion, we report that *Micrococcus Yunnanensis* as an efficient protease producers from the gut of mud crab Scylla serrata. Further studies have to be carried out in order to apply in different commercial fields.

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