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Chitin degrading bacteria from the prawn *Metapenaeus dobsoni* M. and their control

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Studies on isolation and characterisation of chitin degrading bacteria of wild prawns from Goa waters were conducted. Investigations revealed that *Vibrios* were the major group of bacteria involved.From biochemical tests it was found that the associated bacterial flora were represented by *Vibrio angillarum* and *Vibrio parahaemolyticus*. Study on the effect of antibiotics and plant extracts indicated the combination of antibiotics with plant extracts (*Ocimum sanctum, Azardirchta indica, Phyllanthus niruri*) were found to be more effective indicating the synergestic effect of the plant extract.

Bacterial diseases caused by *Vibrios* have often been reported from the cultured prawns[1,2]. Earlier reports[3-5] suggest that the *Vibrio* spp isolated from aquatic environment are pathogenic for human population and responsible for larval mortalities in prawn hatcheries.

Modern aquafarming involves manipulations in growing conditions through fertilisation and supplimentary diet contributing about 16 % of total aquatic production[6]. Much attention has been paid towards farming of economically important species without proper management of water quality leading to deterioration of the environment[7]. Marine *Vibrio* infection among humans is known to occur in high intensities in Japan, where eating raw fish is common. The seasonal association of few species of *Vibrios* with copepod reveals that copepod shells provide a source of carbon, nitrogen and energy[8-10] in addition to sugars and proteins[11]and also for mutual benefits[12]. The bacterial population isolated from such crustaceans are known to support microbial population of chitin degrading bacteria.

Aquaculture practices use variety of antibiotics to control the bacterial population[4,13]. The different types of antibiotics used for control of *Vibrios* suggest that few drugs are sensitive whereas, others are found to be resistant[14] due to indiscriminate use of such antibiotics leading to production of resistant strains. In the present paper, isolation, identification and characterisation of chitin degrading bacteria associated with carapace of the prawn *Metapenaeus dobsoni* are mentioned. An attempt has been made to study the effect of some antibiotics and plant extracts on the bacterial growth.

The prawns (*Metapenaeus dobsoni*) were collected from the creek located at Siridao, Goa. The live prawns were transported to the laboratory and transferred to the aerated water tanks maintained at room temperature. The laboratory reared prawns were fed with animal protein diet for a period of one week. Then the prawns were removed and killed by keeping in iced water. The prawn exoskeleton was removed by using sterile scissors, crushed and homogenised in phosphate buffer (0.05 *M*; *p*H=7.0) using a sterile homogeniser. Freshly collected leaves of terrestrial plant species of *O. sanctum* (Fam: Lamiaceae), *A. indica* (Fam: Meliaceae) , *P. niruri* (Fam: Euphorbiaceae) were washed with distilled water and dried at 60°C. The dried leaves were extracted with methanol. A blackish green material was obtained after drying. The crude extract was completely dried to constant weight *in vacuo*. A stock solution containing 1,000 µg/ml of crude plant extract was made in sterile distilled water. Further, dilutions were made and added to agar plates to get a concentration of 250 µg/ml.

For isolation of marine bacteria a known volume of buffer was inoculated to marine 2216 agar[15] and were kept for incubation at room temperature for 7-9 days. The different bacterial colonies were selected for isolation.

Chitinase producing marine bacteria were selected using chitin agar plates[16] containing 0.3% chitin. The isolated marine bacteria were inoculated on the plate and incubated at 25°C for 7-9 days. A chitinase producing marine bacterium formed a clear halo around the colony on the plate. The isolated strains were identified using biochemical tests[17].

To assess the effect of antibiotics on the growth of isolated bacteria, seven antibiotics namely Carbenicillin (Car), Gentamycin (Gen), Chloramphenicol (Chl), Erythromycin (Ery), Kanamycin (Kan), Streptomycin (Str) and Tetracycline (Tet) at four dosages (0.25, 0.50, 1 0 and 2.0 mg/ml) were tested. A stock solution of these antibiotics was prepared and adequate quantities were added to the chitin agar plates to monitor the zone of inhibition during the incubation period of 3-4 days. Further, in order to assess

the effect of combination of antibiotics (0.5 mg/ml) and plant extract (250 µg/ml), both these components were mixed. The plant extracts used for the study were from *Azadirchta indica* (A), *Ocimum sanctum* (O) and *Phyllanthus niruri* (P) as they are known to have medicinal value. All the experiments were done in triplicate and a mean of them is given in the results. The antibiotics alone were studied to understand and compare the effect to control chitin degrading bacteria when a combination of antibiotic and plant extract was used.

The bacterial flora isolated from the carapace of the prawn were subjected to standard biochemical and physiological tests and the results obtained are given in Table 1. The bacteria were Gram negative, motile rods varying from white to yellowish colour. The catalase and oxidase tests were positive, whereas the indole production was negative. The results obtained from various biochemical tests suggest that the isolated colonies belong to the genus *Vibrio*. The most common bacteria associated with marine crustacea are *Vibrios* and they are known to produce extracellular chitinase[18]. de la Pena, *et al.*[4] reported Gram negative motile sensitive short rods as the causative bacteria of vibriosis in the prawn, *Penaeus japonicus*. The results of various biochemical tests were compared with earlier reports[19] which agreed with most of the morphological and biochemical properties except indole production and methyl red test. Investigations reveal that *Vibrios* are the major group of bacteria responsible for degradation of exoskeleton thereby making these prawns more vulnerable to secondary infections.

The isolated bacteria were incubated on the chitin agar plates. The observations displayed a zone of clearance, indicating that the chitin has been taken up as a source of energy by the bacterial cultures isolated in the present study. Nearhose[12] reported that *Vibrios* are adapted to marine environment, often living with mutually beneficial relationship with fish, prawn and squid species. Kaneko & Cowell[8] reported adsorption of *V. parahaemolyticus* to chitinous exoskeleton which becomes beneficial to bacteria to cause necrotic lesions in crustaceans. Further, such lesions formed chitin degrading bacteria make these host organisms more prone to seconday invaders[10].

The results obtained from the application of seven antibiotics on the zone of inhibition of chitin degraders are shown in Table 2. Among these antibiotics Chloramphenicol showed marked effect at low dosages (0.25 and 0.50 mg/ml) whereas, Carbenicillin was more effective at high dosages (1.0 and 2.0 mg/ml). Published reports[13,14,20] suggest that *Vibrios* are more sensitive to Chloramphenicol and display resistance against Erythromycin, Kanamycin and Streptomycin. *Vibrio* spp isolated from different stages of prawn larvae varied in their resistance pattern as well as frequency of multiple resistance[13]. In semi-intensive prawn culture, Tetracycline is being used widely to reduce the rate of infection among

Table 1—Morphological and biochemical tests for taxonomic studies

Tests	Species 1	Species 2
1. Size	Big mucoid	Big mucoid
2. Shape	Rods	Rods
3. Colour	Pale Yellow	Pale Yellow
4. Elevation	Low convex	High convex
5. Gram stain	–	–
6. Motility	+	+
7. Aerobic growth	+	+
8. Oxidase test	+	+
9. Catalase test	+	+
10. Utilisation of CHO		
a. Glucose	+	+
b. Lactose	–	–
c. Fructose	+	NC
d. Sucrose	–	NC
e. Maltose	+	NC
11. Indole production	–	–
12. Methyl red test	–	–
13. Voges-Proskauer test	–	+
14. Citric acid	+	+
15. Nitrate reduction	+	–
16. Urease test	–	–
17. Ammonia production to arginine	+	–
18. Hydrogen sulfide production	–	+
19. Lypolytic activity	+	+
20. Salt tolerance test (NaCl)		
a. 0%	–	–
b. 0.5%	–	–
c. 3%	–	–
d. 6%	–	–
e. 8%	–	–
21. Arginine decarboxylase	–	–
22. Lysin decarboxylase	–	–
23. Ornithine decarboxylase	–	–
24. Sensitivity to 0/129	+	+

+ = positive reaction; -- = Negative reaction; NC - Not clear; 0/129 = *Vibrio* static coumpound

Table 2—Effect of antibiotics on the zone of inhibition of *Vibrio* spp

Antibiotics concentration (mg/ml)	Zone of inhibition (cm)						
	Car	Chl	Ery	Gen	Kan	Sttr	Tet
0.25	1.0	2.2	–	1.1	0.9	1.2	0.8
0.50	1.8	2.3	0.5	1.5	1.0	1.4	0.8
1.00	3.0	2.5	0.7	2.2	1.6	1.6	1.6
2.00	3.2	3.0	1.6	2.3	1.6	1.6	1.6

Car = carbenicillin; Chl = Chloramphenicol; Ery = Erythromycin; Gen = Gentamycin Kan = Kanamycin; Str = Streptomycin; Tet = Tetracycline

Table 3—Comparative effect of antibiotics and antibiotics incorporated in plant extracts along with Minimum Inhibition Concentration (MIC)

Antibiotics	Zone of inhibition (cm)					
	Inhibition diameter (cm)	MIC (mcg)	Antibiotic concentration (0.5 mg/ml)	(Plant extract + antibiotics) (O)	(A)	(P)
Carbenicillin	1.7	100	1.8±0.6	2.0±0.4	2.4±0.3	2.2±0.5
Chloramphenicol	1.2	30	2.3±0.2	3.0±0.5	2.9±0.6	2.5±0.5
Erythromycin	1.3	15	0.5±0.1	2.0±0.2	1.7±0.3	1.5±0.2
Gentamycin	1.2	10	1.5±0.3	1.5±0.2	2.0±0.2	1.5±0.4
Kanamycin	1.3	30	1.0±0.3	1.4±0.2	1.3±0.1	1.1±0.1
Streptomycin	1.1	10	1.4±0.1	1.8±0.2	2.0±0.3	1.6±0.4
Tetracycline	1.4	30	0.8±0.1	1.8±0.3	1.8±0.4	1.9±0.5

O = *Ocimum sanctum*; A = *Azadirchta indica*; P = *Phyllanthus niruri*

Table 4—ANOVA table for antibiotics and antibiotics incorporated in different plant extracts

Sourcee of variation	Sum of squares	d.f.	F ratio	Significance level (p)
Antibiotics	1.1842	4	0.483	0.742
Antibiotics + O	1.2492	4	0.609	0.6983
Antibiotics + A	2.2692	4	90.771	0.0784
Antibiotics + P	1.6942	4	1.461	0.445
Residual	1.6100	2	–	–

O = *Ocimum sanctum*; A = *Azadirchta indica*; P = *Phyllanthus niruri*

cultured species. Based on the results obtained in the present study it is imperative that Carbenicillin in the dosages of 1.0 and 2.0 mg/ml could be a better alternative drug in the control of *Vibrio* spp. However, Gentamycin at higher dosages appeared to be more effective as compared to Erythromycin, Kanamycin, Streptomycin and Tetracycline. Rahim & Aziz[20] isolated *Aeromonas* spp from few prawns and demonstrated that the above species could be controlled by the effective dosage of Gentamycin.

A compartaive effect of various antibiotics (0.5 mg/ml) incorporated in three plant extracts on the zone of inhibition of bacteria are given in Table 3. The results indicate that Chloramphenicol when mixed with plant extract of *Ocimum sanctum* has maximum effect followed by the same incorporated in *Azadirchta indica*. Available literature[21,22] indicate that the plant extracts are known for their antibacterial activity. A crude leaf extract of the plant *Juniperus communis*[22] showed antibacterial activity at concentration of 250 µg/ml. ANOVA analyses were carried out on the data obtained from the effect of antibiotics and antibiotics incorporated in different plant extracts (Table 4). It was found that the effect of antibiotics incorporated in *Azadirchta indica* was highly significant ($p = 0.0784$) as compared to other treatments.The present set of observations suggest that most of the antibiotics except Gentamycin and Kanamycin in low dosages (0.5 mg/ml) in combination with plant extract from natural sources are more effective than antibiotics alone. It also provides sufficient scope to use such cheap, effective and economical herbal formulations to control the bacterial infections in aquatic environment and use of such combinations would regulate the excessive use of antibiotics in aquaculture practices.

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