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Probiotic activity and antibiotic resistance pattern of microbes isolated from shrimp ponds

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Abstract

Bacterial strains from shrimp pond sediment were isolated and identified. The microbes which showed the enzymatic hydrolysis were tested for their antagonistic activity against the fish and human bacterial pathogens. The antibiotic resistance pattern of four fish pathogens and six human pathogens were tested against twelve commercially available antibiotics. *Vibrio* 7 and *Bacillus* 1 and 2 spp. showed good antagonistic activity against the disease causing fish pathogens than the human pathogens. The Multiple Antibiotic Resistance Pattern of the isolated microbes was evaluated. *Vibrio* sp.1 and *Pseudomona*s sp. showed multiple drug resistance to three-seven of the ten antibiotics tested. The resistance pattern of the microbes may be due to the repeated use of the same drug to control bacterial diseases. The *Pseudomonas* sp. which showed multiple drug resistance was analyzed for the plasmid and the resistant gene was found in the 3000kb region.

Key words: Enzymatic hydrolysis, antagonistic activity, antibiotic resistance, MAR index, plasmid isolation.

Introduction

Antibiotics which have been used in large quantities to overcome diseases in shrimps caused by luminous *Vibrio* and/or viruses are ineffective or result in increase of virulence in pathogens. They also cause concern by promoting transfer of antibiotic resistance to human pathogens. Probiotic technology provides a solution to these problems. The microbial species composition in hatchery tanks or large aquaculture ponds can be changed by adding selected microbial species to displace deleterious normal flora. Virulence of luminous *Vibrio* species can be controlled in this manner. Microbes can play both beneficial and detrimental roles in aquaculture ponds (Rheinheimer, 1992; Baudin Laurencin and Vigneulle, 1994; Valiela, 1995; Moriarty, 1997). On the beneficial side, they are important and essential components for the nutrient and elemental cycling and the required water quality suitable for cultivation (Valiela, 1995; Moriarty, 1997). Probiotics play an important role in maintaining the health of fish. This is particularly so in water rich in organic matter as in sewage water. Further, supplementary feeding plays a major role in producing good and healthy fish. But if proper care is not taken, this food may produce harmful gases as a result of decomposition (Sharma and Bhukhar, 2000).

When pathogenic bacteria or viruses are detected, farmers apply antimicrobial compounds to feed and water. This has led to an increase in *Vibrios* and presumably other bacteria having multiple antibiotic resistances and increase in more virulent pathogens. The most commonly used microbes as probiotics in animal nutrition include *Lactobacillus* sp., *Bacillus* sp. and *Saccharomyces* sp. (Fuller, 1986).

Vibrio sp. especially the luminous *V. harveyi* has been implicated as the main bacterial pathogen of shrimps (Baticados *et al*., 1990). *V. harveyi* are

resistant to commercial antibiotics including chloramphenicol, furazolidone, oxytetracycline and streptomycin and found to be more virulent than the previous years. Antibiotics, antibacterial and antifungal drugs like penicillin, tetracycline, potentiated sulphonamides, quinolenes and nitrofurans are also being used in shrimp culture in India, though in limited quantities. This can lead to indiscriminate use of broad spectrum antibiotics which might create more problems than it can eliminate (Vijayakumaran, 1997).

It is reported that only 20-30% antibiotics are ingested by fish and the remaining 80-70% reach the environment (Dehadrai, 1977). The use of beneficial bacteria (probiotics) to displace pathogens by competitive processes is being used in aquaculture as a remedy than administering antibiotics and is now gaining acceptance for the control of pathogens (Rabinowitz and Roberts, 1986).

Materials and methods

The sediment samples were collected aseptically from different sites of the shrimp culture pond near Rajakkamangalam, Kanyakumari District, Tamil Nadu and kept in sterile plastic bags and brought to the laboratory in icebox. The sediment samples were serially diluted and plated on Zobell Marine agar plates. The plates were incubated at 27^0 C for 24 hours. The isolates were purified and stored at 4^0 C in Zobell Marine agar slants (ZMA). The isolated colonies were plated on TCBS and other selective media for the confirmation and characterization of bacteria. The confirmed bacterial cultures were sub cultured and stored in Zobell Marine agar (ZMA) slants for further use.

The isolated bacterial cultures were tested for their proteolytic activity using skim milk agar. For cellulolytic activity, a loopful of bacterial isolates was single- streaked on the centre of the basal medium and incubated at 37^0 C for 24 - 48 hours. After incubation, the plates were flooded with Congo red solution and the results were observed. The amylolytic activity was observed using starch agar plates. The cross streak method (Lemos *et al*., 1985) was used to detect the inhibitory strains among the bacterial isolates. The human and fish bacterial pathogens were streaked across the ZMA plates and incubated at room temperature. After good ribbon-like growth, the test organisms were streaked at right angles to the original streak of bacterial isolates and incubated at 27^0 C. The inhibition zone was observed after 24–48 hours for bacterial isolates and after 48–72 hours for fungi. The inhibitory activity of the bacterial isolates was indicated by the absence of growth near the control strip. A control plate was maintained only with the test organisms. Antibiotic susceptibility testing was performed by the disc diffusion method (Bauer *et al*., 1966; NCCLS, 1998).The test was performed on Muller Hinton agar using antibiotic impregnated discs. Sterile cotton swabs were dipped in the test inoculums and the entire surface of the Muller Hinton Agar (1.5% Nacl) was streaked with the swabs turning the plate at 60^0 angles between each streaking (Mosffer *et al.,* 2001).

The following ten antibiotic discs (HiMedia, India) were used in the test: ampicillin (10 µg), chloramphenicol (30 µg), bacitracin (10 µg), erythromycin (15 µg), gentamycin (10 µg), streptomycin (10 µg), oxytetracycline (30 µg), vancomycin (10 µg), penicillin (10 µg), and neomycin (30 µg). The discs were impregnated on the seeded plate aseptically with centers at least 25 mm apart. After 18 hours of incubation at 37^0 C, the strains were characterized as susceptible or resistant based on the zone of inhibition created around the discs. The bacterial strains, which showed sensitive, intermediate or resistance activity against the commercial antibiotic discs, were recorded based on the previously available guidelines (HiMedia, 1998). The plasmid responsible for the Multiple Antibiotic Resistance of the *Pseudomonas* sp. was also isolated using alkali lysis method.

Results

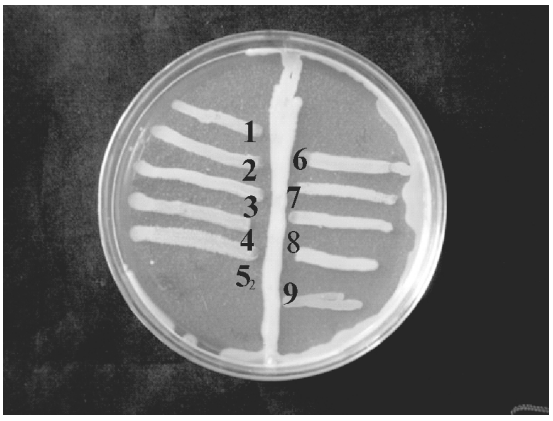
Of the 50 bacterial strains isolated from the shrimp pond bio-wastes, 21 strains were selected based on their colony morphology and biochemical characteristics. The isolated bacterial strains were identified as *Bacillus* sp., *Vibrio* sp., *Pseudomonas*

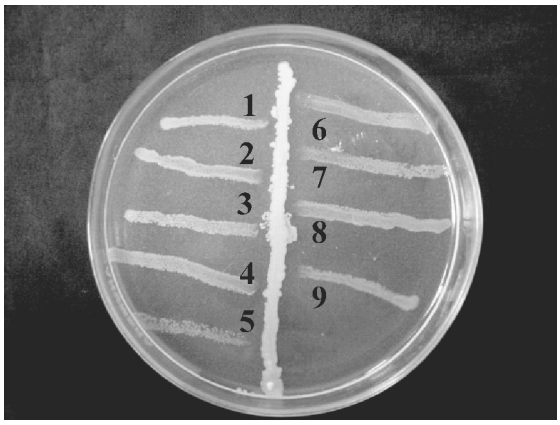
sp. and *Aeromonas* sp. The isolated *Vibrio* sp. was numbered as V 1–9; *Bacillus* sp. as B 1, 2, 3; *Pseudomonas* sp. as P1–4 and other bacterial isolates as A1–5. The enzymatic hydrolysis like amylolytic, proteolytic and cellulolytic activity of the isolated organisms like *Bacillus* sp., *Vibrio* sp., *Pseudomonas* sp. and *Aeromonas* sp. were tested. The organisms which showed good enzyme hydrolysis were further tested for their antagonistic activity against fish disease causing bacterial pathogens such as *Aeromonas hydrophila, Vibrio fischeri* and *Vibrio parahaemolyticus* and human pathogens like *Escherichia coli, Streptococcus pyogenes, Klebsiella pneumoniae, Pseudomonas aerogenosa, Salmonella* sp. and *Vibrio* sp. The antibiotic resistance patterns were tested against twelve commercially available antibiotics.

Among the 21 identified microbes, only *Vibrio* sp. V 6 and V 7 have amylolytic activity, V 5 to V 8 have cellulolytic activity and V 3 to V 5 and V 7 to V 9 have proteolytic activity. Among the other bacterial isolates, *Bacillus* 1 and 2 have amylolytic and cellulolytic activity. A 2, A 4 and A 5 have proteolytic activity. These enzymes play a major role in the degradation of protein, cellulose and starch, which are the main constituents in the shrimp farm. *Vibrio* sp. and the other bacterial isolates, which showed all the three enzymatic hydrolysis, were screened for the antagonistic activity against the pathogens *A. hydrophila, V. fischeri,* and *V. parahaemolyticus,* and human pathogens like *E. coli, S. pyogenes, K. pneumoniae, P. aerogenosa, Salmonella* sp. and *Vibrio* sp. The Vibrio sp. 7 and Bacillus 1 and 2 showed good antagonistic activity against *V. parahaemolyticus, V. fischeri* and *A.hydrophyla* when compared with the human pathogens (Figs. 1 and 2).

The results of the present study suggest that the isolated strains have the ability to degrade the bio-waste produced by the marine animals and that occurred as sewage from other sources, and thereby keep the quality of the water safe for the production of good and healthy fishes. The *Vibrio* sp. 7 and *Bacillus* 1 and 2 which showed antagonistic activity can be purified and antimicrobial compound characterized to test their possible use as the alternative chemotherapeutic probiotics on commercial scale or it can be applied directly as bio-control agents in the aquaculture ponds.

The antibiotic resistance pattern showed that *Vibrio* sp. 1, 2 (Figs. 3 and 4) were resistant towards most of the antibiotics like ampicillin, streptomycin, vancomycin, trimethoprim, gentamycin, bacitracin and penicillin.





Antagonistic activity of *Bacillus* and *Vibrio* sp. against fish and human pathogens

Fig. 1. Antagonistic activity of *Bacillus* sp.

Fig. 2. Antagonistic activity of *Vibrio* sp.

1. E. coli; 2. *Streptococcus pyogenes;* 3. *Klebsiella pneumoniae;* 4. *Pseudomonas aerogenosa;* 5. *Salmonella* sp.; 6. *Vibrio* sp.; 7. *Vibrio fischeri;* 8. *Vibrio parahemolyticus;* 9. *Aeromonas hydrophila.*

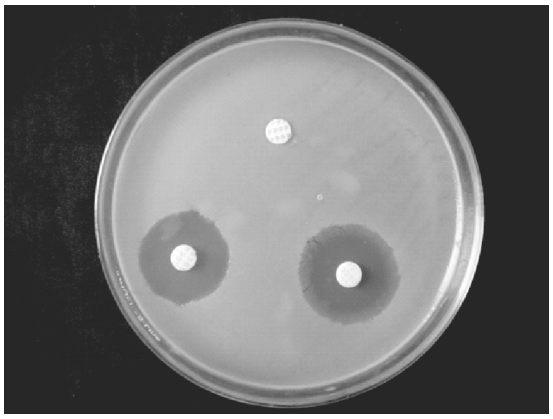


Fig. 3. Antibiotic resistant pattern of *Pseudomonas* strain against ampicillin.

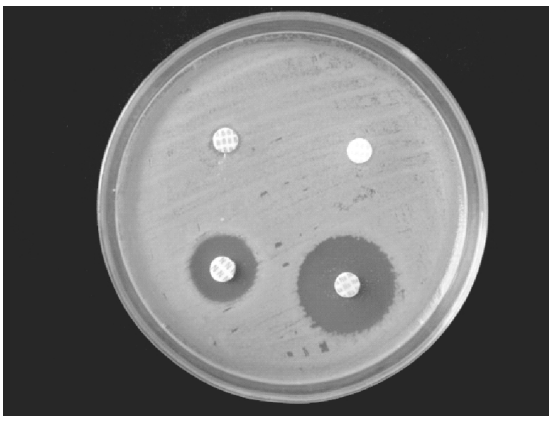


Fig. 4. Antibiotic resistant pattern of *Vibrio* against vacomycin.

Pseudomonas sp. showed resistance against ampicillin, streptomycin, vancomycin, chloramphenicol, neomycin, trimethoprim, gentamycin and *Bacillus* sp. against streptomycin, bacitracin and ampicillin (Fig 5).

Vibrio sp. 1 and *Pseudomonas* sp. showed multiple drug resistance (MAR) against five to seven of the ten tested antibiotics (Table 1), while the *Bacillus* strain showed MAR against only two antibiotics, namely, bacitracin and ampicillin. *Pseudomonas* sp., which showed resistance, was analyzed for the plasmid DNA and the resistant gene was present in the 3000 kb region.

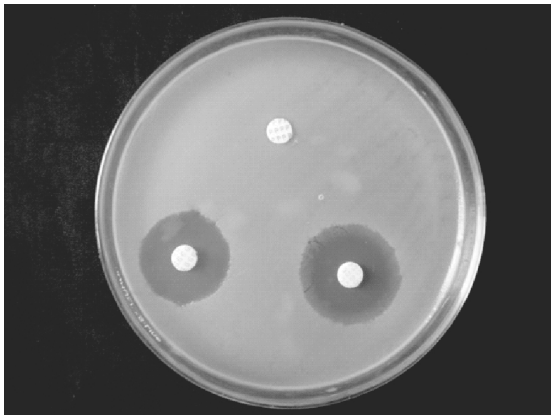


Fig 5. Antibiotic resistant pattern of *Bacillus* sp. against streptomycin and bacitracin.

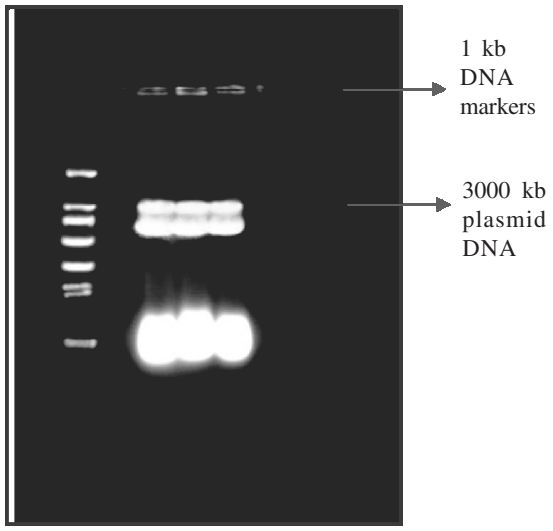


Fig. 6. Agarose gel electrophoresis photo showing the presence of plasmid DNA bands in the 3000kb region

Discussion

In aquaculture ponds, the mechanism of the action of the probiotic bacteria has the following aspects: Probiotic bacteria may (i) competitively exclude the pathogenic bacteria or produce substance that reduces the growth of pathogenic bacteria; (ii) provide essential nutrients to enhance the nutrition of the cultured animals; and (iii) directly take or decompose the organic matter or toxic materials in the water and thereby improve the quality of the water. Microbes such as *Bacillus*

sp., *Vibrio* sp., *Streptococcu*s sp., and *Aeromonas* sp. were used as probiotics by many researchers to regulate the microflora in pond water to control pathogenic micro-organisms and to enhance decomposition of the undesirable organic substances as they have bacteriostatic or bactericidal effects on pathogenic bacteria.

In the present study, *Vibrio* sp. 1 and *Bacillus* sp. 1 and 2 showed better antagonistic activity against the fish-borne pathogens *viz*; *V. parahaemolyticus, V. fischeri* and *A. hydrophyla* when compared with the human pathogens. The results coincide with the observations of Grien and Meyer (1958) who reported that the isolate of *Streptomycetes* from marine sediments are inhibitory to *Bacillus cereus, B. subtilis, E. coli, S. aureus, K. pneumoniae* etc. *Streptomycetes* exhibiting antibacterial activity have also been isolated from decaying material found in the littoral zone (Chandramohan *et al*., 1974).

However, aquatic *Pseudomonas* is often antagonistic against other micro-organisms (Lemos *et al*., 1985; Gram, 1993) including fish pathogenic bacteria (Smith and Davey, 1993) and fungi (Bly *et al*., 1997; Hatai, 1988). The use of probiotics can improve the immunity of cultured animals to pathogenic micro-organisms to a certain extent. In intensive culture systems, there is usually an accumulation of high load of organic material in the pond bottom due to uneaten feed, faeces and plankton die-offs. Avnimelech *et al*. (1995) suggested that the water quality in intensive aquaculture systems is controlled by the microbial biodegradation of organic residues. Microbial processes affect water quality mainly due to utilization of oxygen, regeneration of inorganic nutrients and production of toxic metabolites like ammonia, nitrite and sulphide (Moriarty, 1996; Jory, 1998).

Yasuda and Taga (1980) reported that bacteria would be useful both as food and as biological control agents of fish disease and as activators of the rate of nutrient regeneration in aquaculture. *Vibrio* sp. and *Pseudomonas* sp. showed resistance pattern against most of the tested antibiotics. The use of drugs to control diseases may cause antibiotic resistance of the bacteria found in aquaculture ponds. About 10-20% of the studied strains showed Multiple Antibiotic Resistance pattern. *Vibrio* sp. 1 and *Pseudomonas* sp. showed MAR index to 3-7 of the ten antibiotics used. Previous studies by Cattabiani *et al*. (1992); Khaitovich *et al*. (1992) and Li *et al*. (1998) showed that streptomycin, rifambicin, kanamycin, tetracycline, and polymyxin B were active against *Vibrio* sp. Li *et al*. (1998) reported multiple resistances in *Vibrio* sp. to ampicillin and trimethoprim and to chloramphenicol, tetracycline and ampicillin. The multi drug resistance against 3-7 antibiotics shown by *Pseudomonas* sp and *Vibrio* sp. means that these micro-organisms are capable of detoxicating those antibacterial substances when present in the system.

The *Pseudomonas* sp. analyzed for the antibiotic resistance have been found in the plasmids. The resistant gene was found in the 3000kb region (Fig 6). The selective process leading to the emergence and maintenance of bacterial resistance to antibiotics are mainly brought about by incorrect or abusive utilization of the drugs (Anderson, 1968). In *V. cholerae,* the antibiotic resistance genes have been found on transmissible plasmids. Durpont *et al*. (1985) reported that, in addition to plasmids, integrons has also been described as vehicle in the transport of resistant genes. According to Chandrasekaran *et al*. (1998), Tendencia and de La Penta (2001) and Mudryk (2005), antibiotic resistance in marine bacteria results from terrestrial bacteria with antibiotic resistant plasmid entering the sea water, and this may be responsible for the observed prevalence of resistant genes in the marine environment.

Resistance occurs due to increased disease problems and the limited number of drugs suitable for the control of fish pathogens (Hastein, 1995). When prophylactic drug use is for the generalized prevention of infections, it is always a failure (Weinstein, 1954; Sande and Mandell, 1990). Regardless of whether the resistance is mediated by excessive or multiple exposures to antibiotics or by other mechanisms, the antibiotic - resistant bacteria may have public health significance. Dixon

et al. (1990) reported that among the two (Tetracycline and Romet-30®) permitted antibacterial compounds in USA for food fish culture, 67% of the bacterial strains were resistant to Romet-30®.

It is concluded that disease problems in shrimp ponds can be overcome by applying probiotic biotechnology. It makes use of the natural mechanisms by which bacteria compete against each other. With the right combination of bacteria and aeration, water exchange can be minimized and water can be recycled between crops and the likelihood of introducing pathogens causing environmental impacts can be reduced. The transfer of antibiotic resistance to human pathogenic bacteria, which is exacerbated by the abuse of antibiotics in aquaculture, will decrease. The bacterial resistance problems already recognized in human and veterinary medicine need to be studied further among bacterial pathogens occurring in aquaculture.

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