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Screening and isolation of bacteriocin producing *Bacillus pumilus* from *Penaeus monodon*

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Abstract
The present study was carried out to isolate and screen bacteriocin producing bacteria from the gut of *Penaeus monodon*. A total of 68 bacterial isolates were isolated and the strains, M1, M2, M3, M4, M5, M6, M7, M8 and M9 showed inherent capacity of controlling the shrimp pathogens which was screened by acid production test and well diffusion method. Based on morphological and biochemical characters, the potent bacteriocin producing isolate was identified as *Bacillus pumilus*.

Keywords: probiotic, bacteriocin, *Bacillus pumilus*, antibiotic resistance, shrimp

1. Introduction
Probiotic organisms possess antimicrobial potentialities and their ability to modify the intestinal microbial flora, secrete antibacterial agents such as, bacteriocins and organic acids, compete against pathogens to prevent their adhesion to the intestine, compete for nutrients necessary for pathogen survival, and stimulate antitoxins thus reducing the requirement of antibiotics in aquaculture sector. Probiotic was used as feed additives to improve stress tolerance and reproductive performances in edible fish species [1]. The application of probiotics in aquaculture was intended as a replacement of the massively used antibiotics. Probiotic microbes have the ability to synthesize extracellular enzymes such as lipases, amylases and proteases well as provide the host with growth factors such as fatty acids, vitamins and amino acids [2]. The physio-chemical parameter of water such as, dissolve oxygen, pH, dissolved carbon dioxide, minerals and organic load were very important in bacterial colonization. Variation of these physio-chemical parameters, increasing favour on the growth of several facultative or obligate or various pathogenic bacterial strains such as *Edwardsiella*, *Staphlycoccous*, *Aeromonas*, *Pseudomonas*, *Citrobacter*, *Streptococcus*, *Proteus* and various species of *Vibrio*, which cause much mortality in both freshwater and brackish water fish [3].
Phototropic bacteria play a critical role in marine ecosystem, which enhance the water quality in term of nitrate reduction, phosphorous availability, and salt enhancement. Addition of probiotics in shrimp culture pond reduces the toxic material concentration and enhances the water quality index [4]. Various industries directly release effluent into water bodies without any prior processing, which cause pollution and unbalance the ecosystem. Addition of probiotic bacteria reduce the heavy metal like Cd, Pb, Ni, Hg, and maintain a healthy condition for aquatic animals. In aquaculture, the probiotic bacteria mainly serve as a positive agent in maintaining water quality such as, decrease organic load, increase beneficial bacterial population decrease algal growth, increase dissolved oxygen concentration, increase nutrients concentration and inhibit potential pathogens [5]. The aquaculture is one of the rapidly growing food producing sectors in countries like India, China, India, Norway, Brazil, Sri Lanka, Malaysia, Sri Lanka, USA and Japan which mainly produce shrimps, molluscs, fish and crabs. At present, various types of probiotics or probiotics mixtures are used, but their applications in aquaculture sectors are still limited. In the present study, probiotic bacteria were isolated from the gut of *Penaeus monodon* and screened for its antibacterial activity against bacterial pathogens.

2. Materials and methods
2.1. Isolation of bacteria from the gut of *P. monodon*
P. monodon was collected from the culture pond from Rajakkamangalam, Kanyakumari District, Tamilnadu, India under free-living conditions. The physical parameters showed a water temperature between 28- 30 °C, 20 ppt and pH of 7.8. *P. monodan* was caught by cast net. Shrimp of medium size (30 ± 2 g) was taken for analysis assuming that they might have a well-established pattern of intestinal microbial flora. Shrimps were transferred to water collected from the pond and brought into the laboratory in live condition. Upon reaching the laboratory, analysis of the intestinal microflora was done on samples consisting of excised washed intestines pooled from ten batches each. Serial dilutions were prepared from the homogenates. The samples were spreaded on Zobell Marine Agar plates and individual colony was further isolated by repeat streaking.

2.2 Acid production test for the initial screening of probiotics
Isolated cultures were allowed for fermentation for 2 days and at 37 °C and the medium were collected. It was centrifuged at 10000 rpm for 20 min at room temperature. The supernatant obtained were analyzed for acid production using bromothymol blue solution. Acid producing colonies changed their color of the solution to yellow while bacteriocin producing colonies did not show any colour change.

2.3 Agar well diffusion method

The isolates showed negative results in acid production test were inferred to be bacteriocin producing. Further quantitative analysis of the colonies was done using agar well diffusion. Five indicator strains were used and the activity of the bacteriocin produced by selected isolates was determined. Isolates were inoculated in 100 ml nutrient broth medium and incubated for 48 h at 37 °C. These samples were than centrifuged at 10,000 rpm for 10 min to obtain the cell-free supernatant and 50 µl of which was then was aliquoted in the wells of Meuller Hinton Agar (MHA) plates.

2.4 Identification of the bacterial isolates

The isolate was initially characterized on the basis of morphological and biochemical characteristic features.

3. Results

In the present study, nine probiotic bacteria were isolated from the fish gut based on morphological and biochemical characters of bacteria. The isolated probiotics showed activity against *Micrococcus* sp. *Bacillus* sp., *Pseudomonas* sp., *Vibrio* sp. and *Staphylococcus* sp.. It was interesting to observe that these selected probiotics was able to control the growth of fish pathogen. The isolate M1 exhibited a zone of inhibition of 10 mm against *Micrococcus* sp. 10 mm in *Pseudomonas* sp. But it was not able to control the growth of *Vibrio* sp. The probiotic isolate M3 was able to control the growth of *Bacillus* sp. with 11 mm zone of inhibition. But it was not able to control *Pseudomonas* sp. and *Vibrio* sp. The strain M4 was able to control *Micrococcus* sp. with 10 mm, *Pseudomonas* sp. with 12 mm and *Vibrio* sp. with 10 mm zone of inhibition. The isolate M5 exhibit zone of inhibition against *Micrococcus* sp. with 14 mm, *Pseudomonas* sp. with 12 mm and *Vibrio* sp. with 10 mm, and proved good controlling effect against the tested pathogens. The isolate M6 has no activity against the tested pathogens. The probiotic M7 possess activity against *Micrococcus* sp. with 10 mm zone of inhibition, and *Pseudomonas* sp. with 11 mm zone of inhibition, but did not show any activity against *Vibrio* sp. The probiotic M8 did not show any controlling effect on the tested pathogens. The probiotic isolate M9 showed potent zone of inhibition of *Micrococcus* sp. with 15 mm, *Pseudomonas* sp. with 12 mm and *Vibrio* sp. with 14 mm and thus proved as a good probiotic candidate among the isolated gut probiotics The gut bacterial isolates were further screened for acid production test and only nine bacterial isolates (M1, M2, M3, M4, M5, M6, M7, M8 and M9) were bacteriocin positive. The results showed that 9 isolates gave positive inhibition zones (7 – 21 mm) (Fig. 1 and 2). The antimicrobial activity of *Bacillus pumilus* was relatively higher than the other screened probiotic bacterial isolates. The identified strain was Gram positive, rod shaped, motile, hydrolyzed starch, catalase- and casein positive. Whereas, this strain showed negative results towards urea hydrolysis, nitrate reduction, indole test and citrate test. Based on morphological and biochemical tests, this organism was identified as *Bacillus pumilis.*

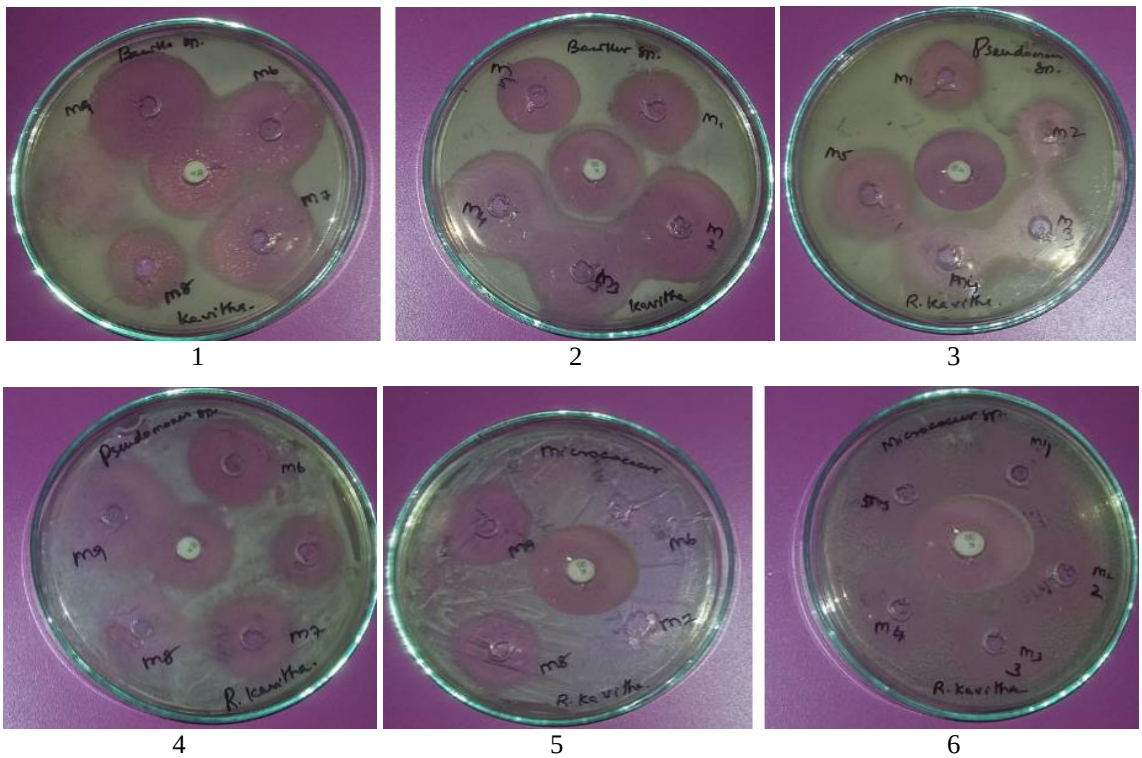

Fig 1: Antibacterial activity of bacteriocin from bacteria isolated from the fish gut, *P. monodon* against (1 and 2) *Bacillus* sp., (3 and 4) *Pseudomonas aeruginosa* and (5 and 6) *Micrococcus* sp.

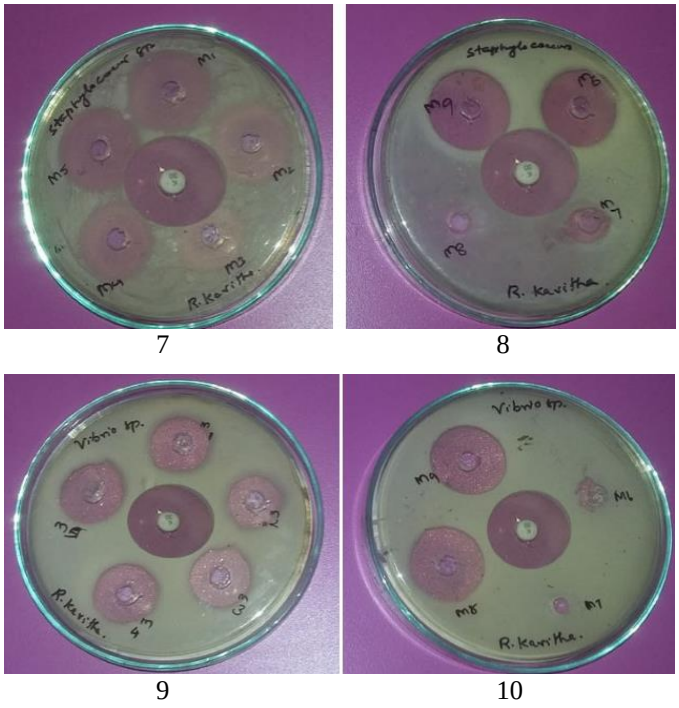

Fig 2: Antibacterial activity of bacteriocin from bacteria isolated from the fish gut, *Penaeus monodon* against *Staphylococcus aureus* (7 and 8) *Vibrio* sp. (9 and 10).

4. Discussion

In the present study, various bacterial strains were isolated from the gut of *P. monodon*. A total of 68 bacterial isolates were isolated from the fish gut based on morphological characters of bacteria. Among the bacterial isolates, strain KA1 showed inherent capacity of controlling the marine pathogens and the isolate was found to be *B. pumilis* based on biochemical properties. There are many bacterial strains from the genus *Bacillus* which can produce a wide variety of antibiotics including bacitracin, polymyxin, and colistin. Members of the *Bacillus* sp. are endospore forming, Gram positive and aerobic bacteria that are catalase production, rod shaped and distributed widely. They found in many environments such as aquatic environments, rocks and gastrointestinal tracts of various organisms [6].

The antibacterial activity of the selected bacterial strain against indicator strains such as *Micrococcus* sp. *Bacillus* sp. and *Pseudomonas* sp., *Vibrio* sp. and *Staphylococcus* sp. was analyzed. The isolated bacterial isolates showed potent activity against these indicator bacteria. Among the 68 gut bacterial isolates, only nine showed positive result for bacteriocin production after acid hydrolysis test. The negative strains were discarded and these nine isolates were further subjected to antibacterial assay by well diffusion assay. The isolated strain M9 showed potent activity against the tested pathogenic bacteria. It was previously reported inhibitory activity of bacteriocin from various bacterial species against fish pathogens. Marahiel *et al*. [7] isolated *Bacillus subtilis* C126 from sugar cane fermentation, which produced a polypeptide antibiotic, bacitracin, which inhibited the growth of *Micrococcus flavus*. Mendo *et al*. [8] isolated a *Bacillus licheniformis* strain, 189, from a hot spring environment in the Azores, Portugal, was found to strongly inhibit growth of Gram-positive bacteria by producing peptide antibiotic. In the search for active molecules produced by *Bacillus* species, especially *Bacillus cereus*, *Bacillus subtilis* and *Bacillus licheniformis*, produced several antifungal compounds, mainly peptide molecules [9].

The probiotics bacterial strains are very much responsible for beneficial measures such as, growth promotion, maintaining water quality and immune modulation and extracellular enzyme production. In recent year, various microorganisms such as yeast and bacteria have used randomly used as probiotic candidates in fish industries [10]. Outbreaks of bacterial, viral and fungal infections have caused more economic losses throughout the world in aquaculture sector. In addition, substantial broodstock mortality was reported in farms mainly due to poor environmental conditions, generation of toxins, genetic factors and unbalanced nutrition [11]. In past few decades, control and prevention of animal diseases were mainly achieved by using veterinary drugs, chemical additives especially antibiotics, even though such methods were later found to be responsible for generating significant risks to public health by promoting the selection, propagation and persistence of bacterial-resistant strains [12].

5. Reference

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