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ORIGINAL ARTICLE **OPEN ACCESS**

Antagonistic activity of potential enzyme producing *Bacillus subtilis* from saltpan against the shrimp pathogens

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ABSTRACT
A common way to screen the candidate probiotics is to perform in-vitroantagonism test, since bacterial antagonism is a common phenomenon in nature.Therefore, several investigators have used this phenomenon to select candidates by in-vitro tests, The potential probiotics microorganisms were isolated from extreme environments such as acidic, thermophilic, hypersaline and arid regions are important'hot spots' of microbial 'mega diversity'. In the present investigation, potential Bacillus subtilis was isolated and identified from saltpan environment. Cells free extract of B. subtilis species showed antibacterial activity against Vibrio species and the diameters of the inhibition zones were about 1 to 13mm. The maximum antibacterial activity (13mm) was recorded in V. paraheamolyticus and lowest was recorded in V. mimicus (1 mm).
Key Words: *Antagonism; Enzyme producing; Vibriosis; Penaeus monodon*;

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INTRODUCTION

Marine microorganisms are of considerable interest as a new and promising search of biologically active compound [1]. They produce avariety of metabolites some of which can be used for drug development [6]. Microorganisms have important role in industrialapplications where they are involved in production of antibiotics, herbicides, pesticides, insecticides and even solvents as precursors for the manufacture of plastics. The increased practice of aquaculture has led to a high number of disease outbreaks with an increasing range of pathogens. As a result of the extensive usage of antibiotics leads to drug resistance [3]. An important part of the natural products from the group of small molecular secondary metabolites are their incredible array of unique chemical structures andtheir very frequent occurrence and versatile bioactivities [4]. The prevention and control of diseases many remedies has been proposed use of antibiotics, vaccines and immunostimulant *etc*. The use antibiotics will lead theformation of drug resistant forms, it is more difficult to control and eradication of these kind of bacterial forms, Salt pan organisms are defined as halophilic microbial forms, which usually inhabit salt rich environments.

In the biological control in aquaculture emerge and since then the research effort has continually increased. *Bacillus* sp. is often antagonistic against other fresh water fish pathogenic bacteria [5, 7]. Generally bacteria play two major roles as beneficial bacteria and pathogenic forms, beneficial bacteria are helpful in nutrient recycling and organic matter degradationand thus clear the environment [8]. Pathogenic bacteria are the causativeagents of bad water quality, stress and diseases as they act as primary and secondary pathogens [9]. Lactobacilli of human intestinal origin have been shown to exhibit antagonistic activity against both gram positive and gram negative bacteria. Many strains belonging to the, *L. acidophilus* have been reported to produce antimicrobial compounds, which show a great variety as to their inhibition spectrum. Antimicrobial substance produced are smaller peptides and do not contribute to the formation of resistance to pathogens.

Feed conservation rate is decreased due to the fact that many of the probiotic microorganisms produce enzymes like amylase, protease and cellulose whether in gut or in environment.

A common way to screen the candidate probiotics bacterial antagonism to select candidates by *in-vitro* tests, in which pathogens are exposed to the candidate probiotics or their extracellular products in liquid and/or solid medium enzymes [10]. The present study, potential probiotics microorganisms were isolated from extreme environments such as acidic, thermophilic, hypersaline andarid regions are important 'hot spots' of microbial 'mega diversity'. These are habitatsof microorganisms which have the genetic and

physiological capacity to survive and grow under these harsh or extreme conditions through which they have evolved while shaping the environment as well as known it today. To live and survive under extreme conditions require structural and physiological adaptations of the organism [11, 12]. In recent years many reports are available for the application of halophilic bacterial forms as a probiotic in shrimp aquaculture [13, 14].

MATERIAL AND METHODS

Collection of samples

The sediment samples were collected from reservoir ponds of the Marakkanam saltpan environment by using pre-sterilized plastic container. Sample collections from corner of square ponds were avoided, as it contains the wind accumulated organic matter and debris. The collected sediment samples were transferred to the laboratory in a ice box and bacteriological analyses were made immediately.

Preparation of samples for bacteriological analysis

The halophilic bacterial populations were estimated by adapting the spread plate technique. 9 ml of water sample blank was prepared using 50% aged sea water and sterilized in autoclave at 121^{o}C (15lbs) for 15 minutes. One ml of sample was pipette out using a sterilized piped and transferred into 9 ml water blank and appropriate dilution made on further transfer to sterile blank.

Isolation of halophilic bacteria

Isolation of halophilic bacterial forms are achieved by using the halophilicagar (Hi-Media Mumbai). Suspend 32.5 grams of halophilic agar; Casein acid hydrolysate 10 g; Yeast extract 5 g; Trisodium citrate 3 g; Potassium chloride 2 g; Magnesium sulphate 25 g; Sodium chloride 250 g; Agar 20 g and Distilled water 1000 ml in 1000ml distilled water. The media was boiled and then sterilized by autoclavingat 15Ibs pressure (121ºC) for 15 minutes. The serially diluted samples were plated by spread plate technique and the agar plate were incubated up to 5 week at 37 ºC for the isolation of halophilic bacterial forms and the result were expressed as CFU^{-1}g by Berge's Manual of systematic bacteriology [26].

Phenotypic characteristics and identification of isolates

The cultures were purified on the same salt concentration and medium from which they were isolated. The selected cultures were tested for other biochemical characteristics such as indole, Methyl red, Voges proscauer, and Citrate, Urease, TSI and carbohydrate utilization. All isolates studied were determined and compared to phenotypic date of known organisms described in the Berge's Manual of systematic bacteriology [23, 24, 27,

Morphological characteristic

Colony morphology of bacterial isolates were evaluated from first selected colonies from the original plate of soil dilution plate count. Gram stain reaction, cell morphology and motility of bacterial isolates were examined as described by Cappuccino and Sherman [28].

Isolation of shrimp pathogen

The infected shrimp hepatopancreas, gill and gut were dissected and removedfor pathogenic bacteria isolation. The samples are serially diluted from 10^{-1} to 10^{-9} and 0.1 ml of samples from 10^{-5}, 10^{-6} and 10^{-7} were plated on TCBS (Thiosulfate Citrate Bile Salt sucrose Agar- Peptone-10.00g, Yeast extract- 5.00g, sodium citrate- 10.0g, sodium thiosulphate- 10.0g, sodium cholate- 3.0g, Oxgall- 5.00g, sucrose- 20.0g, sodium chloride- 10.0g, Ferric citrate- 1.00g, Bromo thymolblue-0.04g, Thymol blue-0.04g, Agar- 14.00g, pH of 8.6 ± 0.1). The incubation period was maintained for 24 to 48 hours at 37^{o}C for the plates, for pathogenic bacteria population density analysis [21].

Preparation of pathogenic bacteria culture

Nutrient broth medium was prepared and sterilized in an autoclave at 15 Ibs pressure, and inoculated with the pathogenic bacterial strains, namely *Vibrio paraheamolyticus, V. vulnificus, V. mimicus, V. damsel, V. anguillarum, V. cholera, V. fluvialis, V. harveyi, V. splendidus* and *V. alginolyticus* and incubated at28 ± 2 ° C for 18-24 hours. 24hours old bacterial broth culture was inoculated in Mullar Hinton Agar medium by using a sterile cotton swab.

Antibacterial activity by well diffusion methods

In the agar well diffusion method, the pathogenic strains were prepared in Miller Hinton broth and were plated into Miller –Hinton agar in using sterile swabs wells (6mm diameter) were punched in the plates using a sterile stainless steel borer. An overnight culture of isolated bacteria strains were grown in nutrient agar broth at 28° C for 24h. After incubation, cells were removed by centrifugation at 10,000rpm for 15 min., the supernatant was collected and filtered through 0.22 µm membranes. Each well was filled with 50 µm of filter- sterilized supernatant (Test sample - T). All the assays were carried out in triplicate, after incubation at 28 ºC for 24h.The diameter (mm) of the inhibition zone around the well was measured.

RESULTS

In the present investigation, *B. subtilis* was isolated and identified from saltpanenvironment (Table 1). Among these, cells free extract of *B. subtilis* species showed antibacterial activity against *Vibrio* species and the diameters of the inhibition zones were about 1 to 13mm (Fig.1). The maximum antibacterial activity (13mm) was recorded in *V. paraheamolyticus* and lowest was recorded in *V. mimicus* (1 mm) (Fig.2).

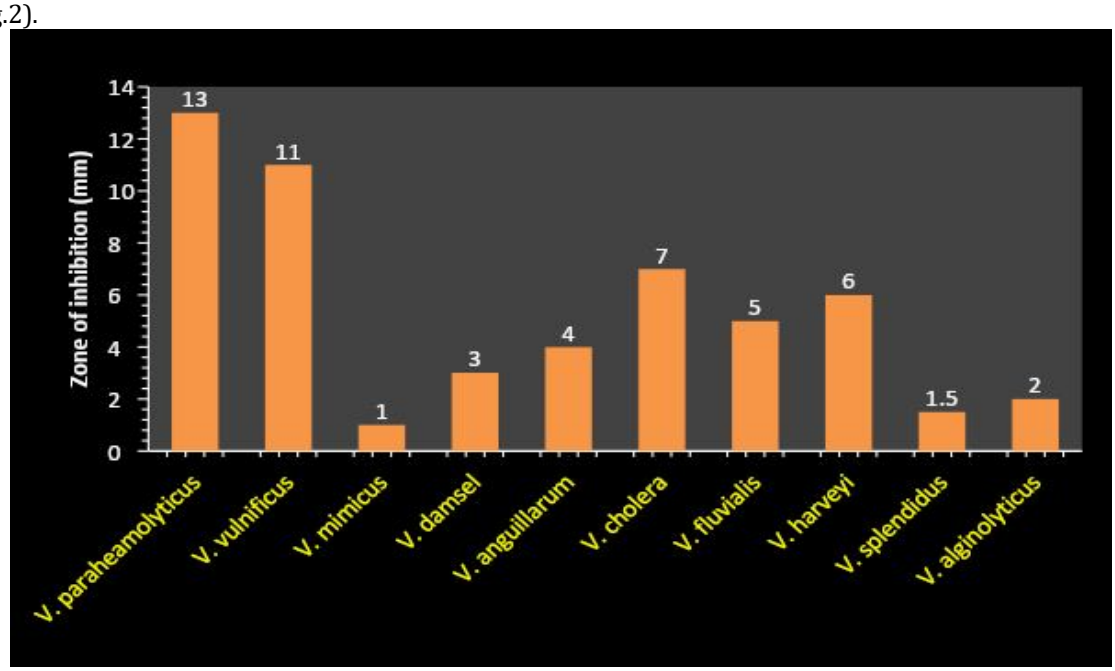

Fig.1. Anti bacterial activity against shrimp pathogens

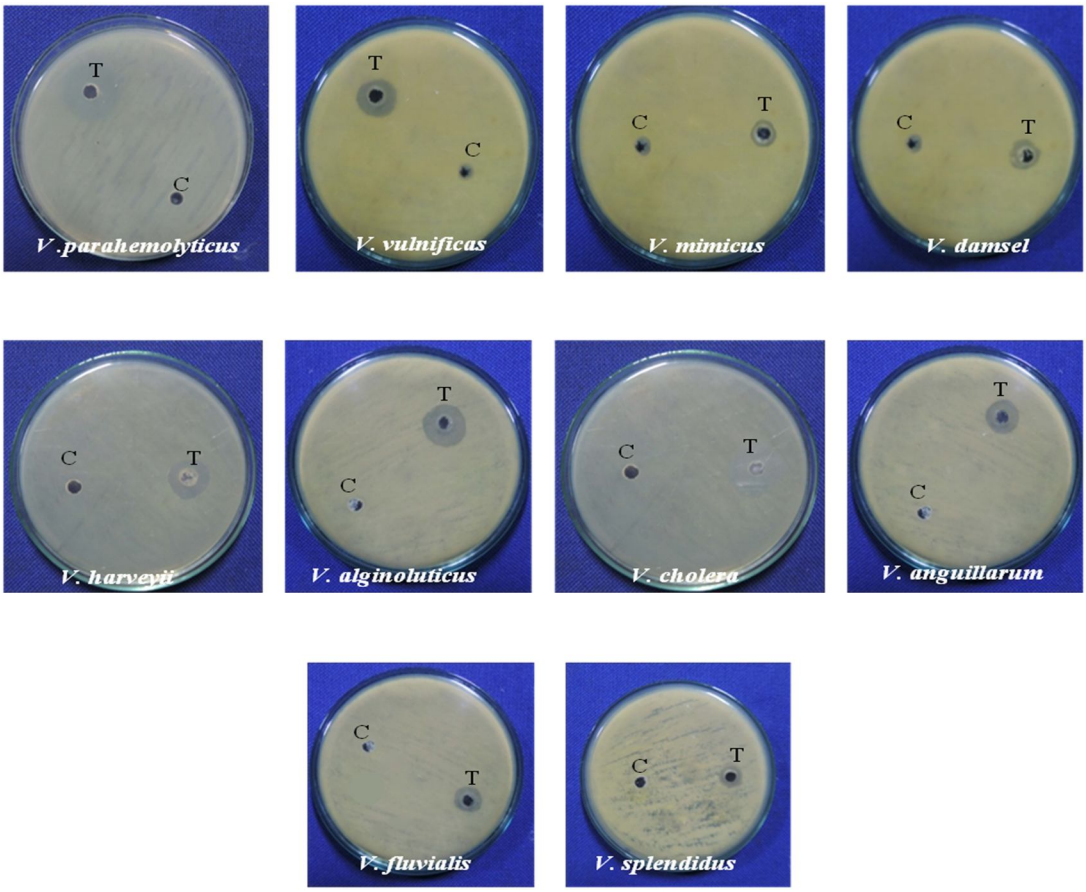

Fig.2. Anti bacterial activity against shrimp pathogens

DISCUSSION

In recent years, the culture of shellfish has made tremendous strides, resulting in the development of major industries around the propagation of crustaceans (shrimp,lobster, crabs). Crustaceans also constitute a major component of both the species and biomass in aquatic ecosystems, making them of pivotal importance to the ecosystem integrity. Diseases affect both cultured and wild crustacean populations and the types of diseases affecting shellfish are in most cases similar to those affecting aquatic vertebrates and terrestrial mammals. Parasites, fungi, bacteria, viruses and non-infectious diseases cause problems. Various toxins and other water quality stressors can also affect crustacean health. With the increasing importance of crustacean aquaculture as well as mounting pressures on fisheries, there is a need for reliable accurate means for developing the health of crustaceans [22]. Vibriosis is one of the major diseases caused in the aquatic organisms.

This is usually caused by the species belonging to the genes *Vibrio*. Several speciesare known to be pathogenic to aquatic animals as well as humans. They are highly abundant in aquatic environments. Several cultivation-dependent and independentstudies have showed that Vibrios appear at particularly high densities in or on marine organisms such as corals, fish, molluscs, seagrass, sponges, shrimp and zooplankton [1]. Even though, there are several remedies in controlling *Vibrio* there exist several constraints such as the prevalence of the antibiotic resistancebacteria. In this present study designed to find the potential of application of halophilic bacteria was used to control the crisis of Vibriosis in the culture of *L. vannamei.* A growing number of studies has demonstrated the use of probitics in aquaculture and their ability to control potential pathogens [2, 10]. In the present study, halophilic bacteria of *B. subtilis* isolated from saltpan environment in Marakkanam, showed acceptable activities towards the shrimp pathogens.

Halophilies are the salt-loving organisms that inhabit hyper saline environments and it offers a multitude of actual or various potential applications in various fields of biotechnology [5, 20] notably for producing enzymes able to function at low water activities (Amylases, proteases, lipases, nucleases) of industrial interest and accumulating a variety oforganic compounds, called compatible solutes, useful as enzymes or cell-stabilizing agents, salt antagonists or stress-protective agents. Similar aspect present study has been under taken to screen biologically potent halophilic microorganism from saltpan environment and it application in aquaculture as a probiotics. Halophilic bacterial forms produce several secondary metabolites such as bacteriocins, bacteriocins like substances and antibacterial lipopeptides and these metabolites have received considerable attention as biological control agents in pharmaceutical industry because they are generally recognized as safe (GRAS), have low toxicity, high biodegradability, and are environmentally friendly [16]. In present study, the halophilic bacterial strain *B. subtilis* showed antagonistic against shrimp pathogens such as *V. paraheamolyticus (13mm), V. vulnificus (11mm), V. mimicus (1mm), V. damsel (3mm), V. anguillarum (4mm), V. cholera (7mm), V. fluvialis (5mm), V. harveyi (6mm), V. splendidus (1.5mm)* and *V. alginolyticus (2mm.)* Similarly, Lee *etal,* [12] have reported a novel analyze from *B. subtilis* SC-8 antagonistic to *B. cereus.*

Prem Anand [17] has observed the presence of *V. parahaemolyticus* from the shrimp culture pond. As described by several authors, *Bacillus* bacteria have been widely used as probiotics and also especially *B. subtilis* a gram- positive non- pathogenic, spore- forming bacterium it has been widely using for oral bacterial therapy prophylaxis of gastrointestinal disorders, improving culture environmental quality and the promote the survival of cultured organisms [14, 23].

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