Int.J.Curr.Microbiol.App.Sci (2014) 3(5): 795-809

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 5 (2014) pp. 795-809**
http://www.ijcmas.com

Original Research Article

Antagonistic activity of cellular components of *Bacillus subtilis* AN11 against bacterial pathogens

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Keywords

Textile dye effluent, Heavy metals, Microbial bioremediation

A B S T R A C T

Antagonistic effects of *Bacillus subtilis* AN11 was studied against Gram negative strains of *Aeromonas hydrophila* KJ459001 (CAHHI), *Pseudomonas aeruginosa* (ATCC 35072), *Edwardsiella tarda* JX280148 (CETMTI), *Vibrio parahaemolyticus* JF966211 (CPVP7) *Flavobacterium columnare* KF051085 (CFCCO41) and Gram positive bacterium *Staphylococcus aureus* (ATCC6538). Four different fractions of cellular component (i.e. whole cell product, heat killed whole cell product, extracellular product, outer membrane protein) of *B.subtilis* were tested for their effective and except ECP all three were effective in reducing the growth of bacterial pathogens except *Aeromonas hydrophila*. OMP showed more inhibitory effect to all the pathogens. Over a period of 96 h, the growth of the all the pathogens were reduced significantly in OMP enriched bacterial culture. The outer membrane protein of *B.subtilis* could be useful for controlling the bacterial pathogens.

Introduction

A persistent goal of global aquaculture is to maximize the efficiency of production to optimize profitability. With the increasing intensification and commercialization of aquaculture production, disease has become a major problem in the fish farming industry (Reantaso *et al*., 2005). Although vaccines are being developed and marketed, they cannot be used as a universal disease control measure in aquaculture. Indiscriminate use of antibiotics has led to the development and spread of antimicrobial resistant pathogens which were well documented (SCAN 2003; Kim *et al*., 2004; Cabello 2006, Sørum, 2006). There is a risk associated with the transmission of resistant bacteria from aquaculture environments to humans, and risk associated with the introduction in the human environment of nonpathogenic bacteria, containing antimicrobial resistance genes, and the subsequent transfer of such genes to human pathogens (FAO, 2005). On the other hand antibiotics not only inhibit or kill

beneficial micro biota in the gastrointestinal (GI) ecosystem and antibiotic residue accumulated fish products are shown to be harmful for human consumption (WHO 2006). By the above reasons since January 2006 European Union ratified a ban for the use of all sub-therapeutic antibiotics as growth-promoting agents in animal production.

The microbial ecology of the gastrointestinal tract of variety of freshwater and marine fish has been investigated intensively by many researchers during the last decade (Spanggaard *et al*., 2000; Ahmed *et al*., 2004, 2005; Ringø *et al*., 2006, 2006a; Skrodenyte- Arbaciauskiene *et al*., 2006; Hovda *et al*., 2007; Kim *et al*., 2007; Yang *et al*., 2007; Zhou *et al*., 2009).

In connection with the ban of antibiotic growth promoters (AGP) new strategies in feeding and health management in fish aquaculture practice have received much attention (Balcázar *et al*., 2006). In addition, the global demand for safe food has prompted the search for natural alternative growth promoters to be used in aquatic feeds. There has been heightened research in developing new dietary supplementation strategies in which various health and growth-promoting compounds as probiotics, prebiotics, synbiotics, phytobiotics and other functional dietary supplements have been evaluated (Denev,2008).

Control of microbial community has been regarded as difficult as it has high species diversity (Maeda, 1994, Das *et al.*, 2005) . Beneficial micro-organism could control the certain extrinsic pathogenic microorganisms can be sources of variety of bioactive natural products of basic research and commercial point of interest that have inhibitory effect on microbial growth (Das *et al*., 2005). The Gram-positive, aerobic, rod-shaped endospore-forming bacteria of the genus *Bacillus* are most impressively produced antibiotics as secondary metabolites (Kümmerer, 2009). In recent years, many studies have been emerged the antimicrobial properties of strains of *Bacillus*. Shelar *et al*. (2012) determined that bacteriocins produced by *B. atrophaeus* JS-2 showed antimicrobial properties against some Gram-positive and Gram-negative bacteria. The Gram-positive bacterium *Bacillus subtilis* is an important producer of high quality industrial enzymes and a few eukaryotic proteins. Therefore, one may anticipate that the high protein production potential of *B.subtilis* can be exploited for protein complexes and membrane proteins to facilitate their functional and structural analysis. The present study was undertaken to find out the antagonistic activity of heat resistant *B.subtilis* isolated from Bhitarkanika mangrove forest, Odisha, India against major fish bacterial pathogens either as whole or in macromolecular fractions.

Materials and Methods

The present study was carried out at Fish Health Management Division , Central Institute of FreshWater Aquaculture (CIFA), ICAR , Kausalyaganga, Bhubaneswar, Odisha during February to June 2013.

Sample collection

Bacillus sp. were collected from sediment samples the Mangrove forest of Bhitarkanika.

Antagonistic bacteria *Bacillus subtilis* ANII

Bacillus subtilis ANII (Gene bank

accession no. JX860845) previously isolated from sediment samples of Bhitarkanika mangrove forest and maintained in the Fish Health Management Division, Central institute of Fresh Water Aquaculture (CIFA), was used for the antagonistic study against fish pathogenic bacteria.

Test Pathogens

Antagonistic activity was tested against the pathogenic Gram negative strains of *Aeromonas hydrophila* KJ459001 (CAHHI), *Pseudomonas aeruginosa* (ATCC 35072), *Edwardsiella tarda* JX280148 (CETMTI), *Vibrio parahaemolyticus* JF966211(CPVP7) *Flavobacterium columnare* KF051085 (CFCCO41) and Gram positive bacteria *Staphylococcus aureus* (ATCC6538). These pathogens were maintained in the Fish Health Management Division (FHMD) CIFA Bhubaneswar were taken for the antagonistic study. Pure cultures of different bacterial strains inoculated in brain heart infusion (BHI) broth (HI media, Mumbai, India) except *V. parahaemolyticus* which was maintained in BHI broth supplemented with 2.5% NaCl and incubated at 37^0C for 18h and subsequently used for antagonistic study as whole.

Preparation of different cellular components

Four types of antigenic components e.g. heat killed whole cell products, extracellular products, outer membrane protein and whole cell culture of *B.subtilis* was used for the study. Briefly pure culture of *B.subtilis* was grown in 250 ml of BHI broth at 30^0C for 24 h. Pure culture after 24h was divided into 50ml each and taken for the preparation of heat killed and separation of extracellular products and whole cell products. The optical density (OD) of 24h old culture was taken at 546 nm and simultaneously plating was carried out in triplicate and the colony forming unit/ml was calculated. The optical density at 546 nm of *B.subtilis* was 0.5OD which corresponds to 10^6 cfu/ml.

Heat killed whole cell product (HKWCP)

B.subtilis culture was inoculated in 2.5l broth and were harvested by centrifugation at 8500 rpm for 15 minutes at 4^0C. The bacterial pellet was washed twice and resuspended in phosphate buffered saline (pH 7.2).They were heat killed at 60°C for 1h in a water bath and finally stored at -20°C.

Whole cell product (WCP)

B.subtilis culture in BHI broth were harvested after 24h incubation and centrifuged at 8500 rpm for 15min at 4^0C. The bacterial pellet was washed twice and resuspended in PBS (pH 7.2 and used for the antagonistic study.

Extracellular product (ECP)

The supernatant obtained after centrifugation 24h old culture and of *B.subtilis* in BHI broth were filtered (0.22µl). They were further concentrated with 20% PEG 6000, dialyzed against PBS (pH7.2) and used as ECP.

Outer membrane protein (OMP)

OMPs were extracted using the protocol of Austin & Rodgers (1981) with little modification. Briefly, *B.subtilis* ANII was grown in BHI broth at 37^0C for 24h and harvested by centrifugation at 8500 rpm for 10 minutes. After centrifuge supernatant was discarded and pellet

was washed with 20mM Tris buffer at pH 7.2 and finally pellet was resuspended in 10mM EDTA buffer. The bacterial cell suspensions were then subjected to sonication at 50hz for 10 mins in a sonicator to disrupt the cell wall. The unbroken cells were sedimented by centrifugation at 8500rpm and supernatant was collected. Ultracentrifugation of collected supernatant was carried out at a speed of 27400 rpm for 45 mins. The supernatant was discarded and the sediment was resuspended in Tris buffer containing 0.5% sarcosyl. Further centrifuged at 27400 rpm for 45 mins. The sediment collected after ultracentrifugation was OMP and supernatant obtained was the CMP. OMP was kept in -20 ^{0}C for further use. Protein estimation was done by Lowery method (1951).

Antagonistic study

Well diffusion assay

Overnight growth cultures of different bacterial strain of *(Staphylococcus aureus, Pseudomonas aeruginosa, Flavobacterium columnare ,Vibrio parahaemolyticus, Edwardsiella tarda* and *Aeromonas hydrophila*) were incubated on BHI agar Himedia, plates separately by the lawn culture method. Then 6mm diameter wells were made in each plate with the help of a well puncture (Sen *et al.*,1995) and 10, 20, and 30µl of whole cell product (WCP) both live and heat killed corresponding to 1.8x10^{8}cfu/ml, extracellular product (ECP) and 10, 20, 30µl of OMP corresponding to the protein concentration of 8.47mg/ml were charged in the respective wells of different bacteria of *(Staphylococcus aureus, Pseudomonas aeruginosa, Flavobacterium columnare, Vibrio parahaemolyticus, Edwardsiella tarda* and *Aeromonas hydrophila*) plates incubated at 37^{0}C for 24 h. The zone of inhibition around the charged wells was recorded after incubation. Simultaneously control samples were maintained with sterile phosphate buffer saline (pH7.2) placed into the respective wells prepared as mentioned above. In all the cases, the triplicate plates were inoculated along with the PBS control for antagonistic study.

Antibiotic Sensitivity

Selected antibiotics, furazolidone (50mcg), norfloxacin (30mcg), oxytetracycline (30 mcg) were tested in a diagnostic sensitive medium (Hi media, India) against different bacterial strain to compare the effectiveness of different cellular components of *Bacillus subtilis* AN II. A required amount of diagnostic sensitivity medium (DST) (Hi Media, India) was prepared and 20 ml DST medium were poured on to sterile petriplate (98 mm diameter) and allowed to solidify at room temperature. Cultures of pathogenic bacteria *(Staphylococcus aureus, Pseudomonas aeruginosa, Flavobacterium columnare ,Vibrio parahaemolyticus, Edwardsiella tarda* and *Aeromonas hydrophila*) were inoculated by the lawn culture method. Then the above antibiotics were placed in different culture plates and incubated at 37^{0}C for 24h. After incubation, the zone of inhibition of each product were compared with the recommended zone of inhibition of the antibiotics against each bacterial strains were treated as positive control.

Antagonistic growth kinetics against bacterial pathogens

The experiment was conducted by taking four sets of identical eppendorff tube (1.5

ml) in triplicate for each organism. A total no of five bacterial pathogens namely *Staphylococcus aureus, Pseudomonas aeruginosa, Flavobacterium columnare, Vibrio parahaemolyticus* and *Edwardsiella tarda* were taken for this study. In each eppendroff tube 24 h culture of bacterial pathogens were taken separately and 10 µl of *B.subtilis* OMP was incorporated. For each bacterium, 12 eppendroff tube was taken. Then tubes were incubated at 37 ^{0}C for 96 h. At 24 h intervals, three tubes from each bacterium were taken for measurement of optical density at 546 nm. The bacterial growth was monitored for a period of 96 h.

Statistical analysis

All the data were analyzed by running the general linear model programme available in SAS software. The means were compared using Duncan's multiple range test (Duncan, 1955) to find the difference at 5% ($P \leq 0.05$) level.

Results and Discussion

Protein estimation

The protein content of HKWCP, WCP , ECP and OMP of *B.subtilis* ANII were 5.1, 5.08, 5.02 and 8.47 mg/ml respectively.

Antagonistic study against *Edwardsiella tarda*

Results obtained from the HKWCP, WCP, ECP and OMP of *B.subtilis* ANII against *E. tarda* are shown in Fig. 1. It was found that OMP of *B.subtilis* at 30 µl concentration was found to be highly effective and produced average zone size of 36 mm. among all the different concentration of OMP starting from 10µl to 30µl per well, the zone size was increased from 9 mm to 36 mm which was fourfold increase. The HK WCP and WCP of *B.subtilis* ANII produced more or less similar zone size and they were not significantly different ($P \geq 0.05$) to each other in all the concentration tested. The antagonistic activity was increased from 10µl concentration to 30µl concentration. The antagonistic activity of OMP of *B.subtilis* was significantly different ($P \leq 0.05$) in all the concentration tested (Fig.A & Fig.1). However; the ECP of *B.subtilis* did not show any inhibitory affect to *E. tarda* in all the concentration tested.

Antagonistic activity against *Vibrio parahaemolyticus*

The zones of inhibition produced by *B.subtilis* ANII against are shown in Fig. 2. The zone sizes were more against *Vibrio parahaemolyticus* as compared to the zone sizes against *E. tarda*. The inhibitory activity of WCP of *B.subtilis* was more than twice against *V. parahaemolyticus* as compared to the *E. tarda* at 10 µl concentration. The zone of inhibition was three times more in 30 µl OMP concentration as compared to 10 µl of OMP. The antagonistic activity of *B.subtilis* ANII OMP against *V. parahaemolyticus* was significantly higher ($P \leq 0.05$).WCP of *B.subtilis* showed insignificant ($P > 0.05$) higher zone of inhibition as compared to the HKWCP in all the concentrations tested.[i] The mean zone of inhibition of *B.subtilis* WCP against *V. parahaemolyticus* ranged from 11.67mm to 39mm in 10 µl to 30 µl concentration (Fig.B & Fig. 2). The ECP did not show antagonistic activity. HKWCP, WCP and OMP showed significant ($P \leq 0.05$) inhibitory activity against *Vibrio parahaemolyticus* when compared to positive control.

Antagonistic activity against *Pseudomonas aeruginosa*

The four cellular components of *B.subtilis* ANII were tested as antagonistic / components against *P. aeruginosa* (Fig 3). Three fraction i.e. WCP, HKWCP and OMP produced zone of inhibition even at 10µl concentration and zones of inhibition were above 30 mm at 30µl concentration. The zones of inhibition of HKWCP, WCP and ECP of *B.subtilis* ANII were significantly different to each other ($P \leq 0.05$) within the treatments. The antagonistic activity of *B.subtilis* ANII OMP was significantly highest ($P \leq 0.05$) in all the three concentrations as compared to the HKWCP and WCP (Fig 3).

Antagonistic activity against *Flavobacterium columnare*

Result obtained from the HKWCP, WCP, ECP and OMP of *Bacillus subtilis* ANII against *F. columnare* as shown in Figs. D & 4. It was found that HKWCP of *Bacillus subtilis* showed an average zone of inhibition ranging from 10.33 mm to 35.33 mm from 10µl inoculation to 30 µl inoculation. Similarly WCP showed more or less similar zone of inhibition from 10 µl to 30µl concentration. However OMP showed highest zone of inhibition as compared to HKWCP and WCP against *Flavobacterium columnare* at the same concentration. The antagonistic effect was significantly higher ($P \leq 0.05$) in all the concentration as compared to both HKWCP and WCP. Highest zone of inhibition was found in 30µl of OMP (45.33mm). The zone of inhibition was significantly higher as compared to the positive control. However the ECP of *Bacillus subtilis* did not produce any inhibitory effect against *F. columnare*.

Antagonistic activity against *Staphylococcus aureus*

The inhibitory zone produced by *Bacillus subtilis* against *S. aureus* are shown in Fig 5. The zone sizes were ranged from 8.67 mm to 53.33mm in various concentrations of WCP, HKWCP and OMP (Fig.D & Fig. 5). The highest zone was recorded at 30µl concentration of OMP which was significantly higher as compared to HKWCP and WCP. Similarly the zone size was also significantly higher ($P \leq 0.05$) at 10µl and 20µl *Bacillus subtilis* OMP against *S. aureus*. HK WCP and WCP produced lesser zone size in all the concentration. However, the inhibitory effect HKWCP was not significant to each other.

Antibiotic sensitivity

Selected antibodies, furazolidone (50mcg), norfloxacin (30mcg), oxytetracycline (30 mcg) were tested in the present experiment to compare the zone of inhibition with that of four products of *B.subtilis* AN11; produed zones ranging from 8-24 mm in all the pathofenic vacteria tested (Table 1).

Growth kinetic Study

The growth kinetic of different bacterial strains to *B.subtilis* ANII OMP are depicted in Fig.6 and Fig.7. It was noticed that all the five bacteria showed significant reductions ($P \leq 0.05$) in their number over a period of 96h of inoculation. More than 90% reductions were observed in *V. parahaemolyticus* and *F. columnare* whereas 75% to 85% reductions were observed in *S. aureus* and *P. aeruginosa*. About 60% reduction was observed in *E. tarda* over a period of 72h (Fig.6).

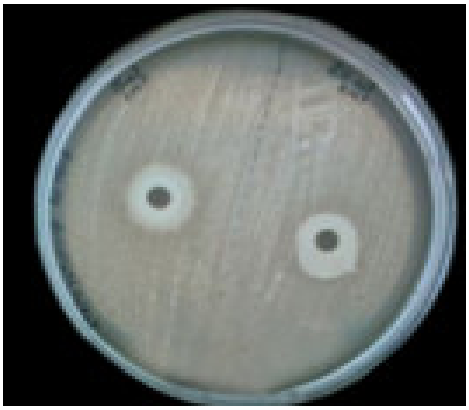

Fig A: Zone of inhibition shown by *B.subtilis* OMP(30µl) against *Edwardsiella tarda*

Fig B: Zone of inhibition shown by *B.subtilis* OMP (30µl) against *Vibrio parahaemolyticus*

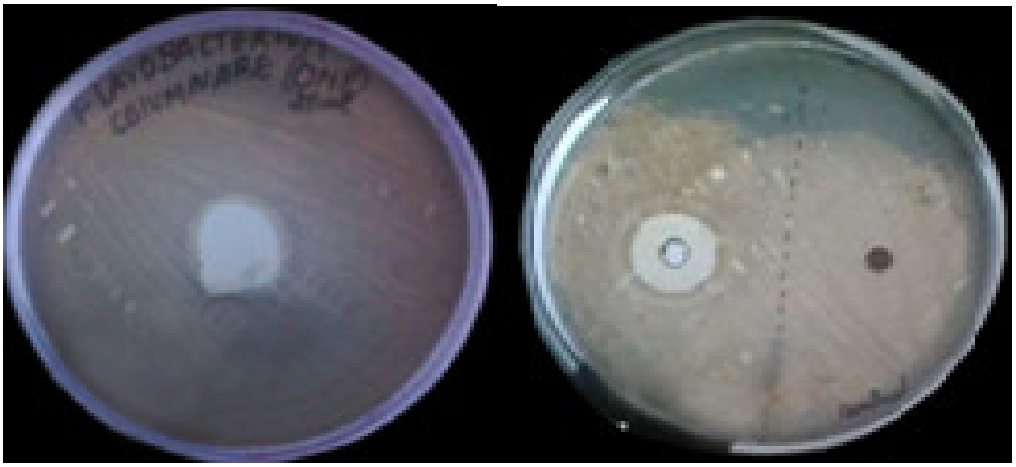

Fig C : Zone of inhibition shown by of *B.subtilis* OMP (30µl) against *Flavobacterium columnare*

Fig D : Zone of inhibition shown by of *B.subtilis* OMP(30µl) against *Staphylococcus aureus*

Table 1: Zone of inhibition (mm) of different antibiotics against fish pathogens

Antibiotics	**Disk conc.**	***A.hydrophila***	***E.tarda***	***P.aeruginosa***	***F.columnare***	***S.aureus***	***V.parahaemo lyticus***
Furazolidone	50mcg	23	24	10	22	14	18
Norfloxacin	30mcg	23	20	8	11	20	24
Oxytetracycline	30mcg	24	22	12	22	23	22

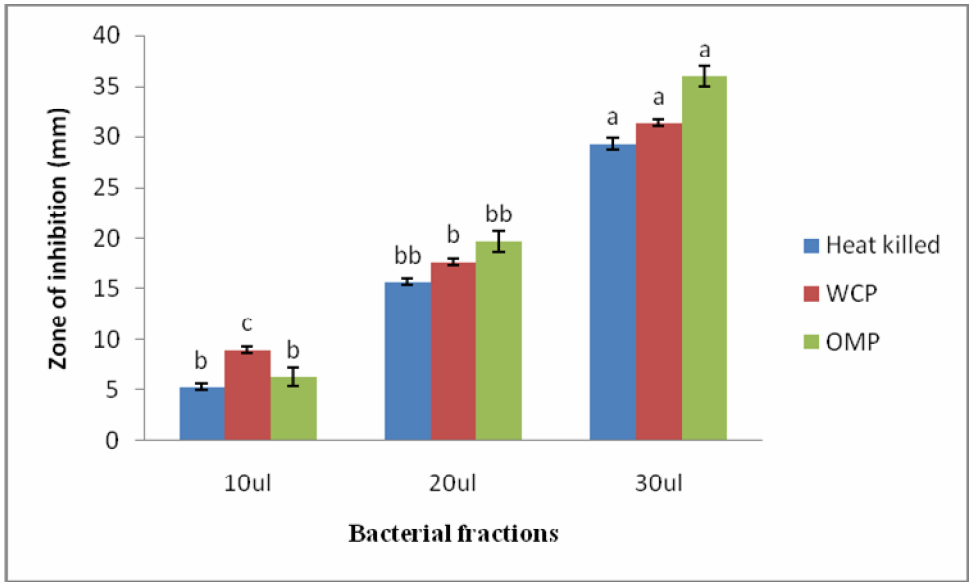

Fig 1 : Zone of inhibition (mm) of *Bacillus subtilis* AN11 bacterial fractions against *Edwardsiella tarda(*values are represented as mean ± S.E., mean bearing common superscript are not significant to each other).

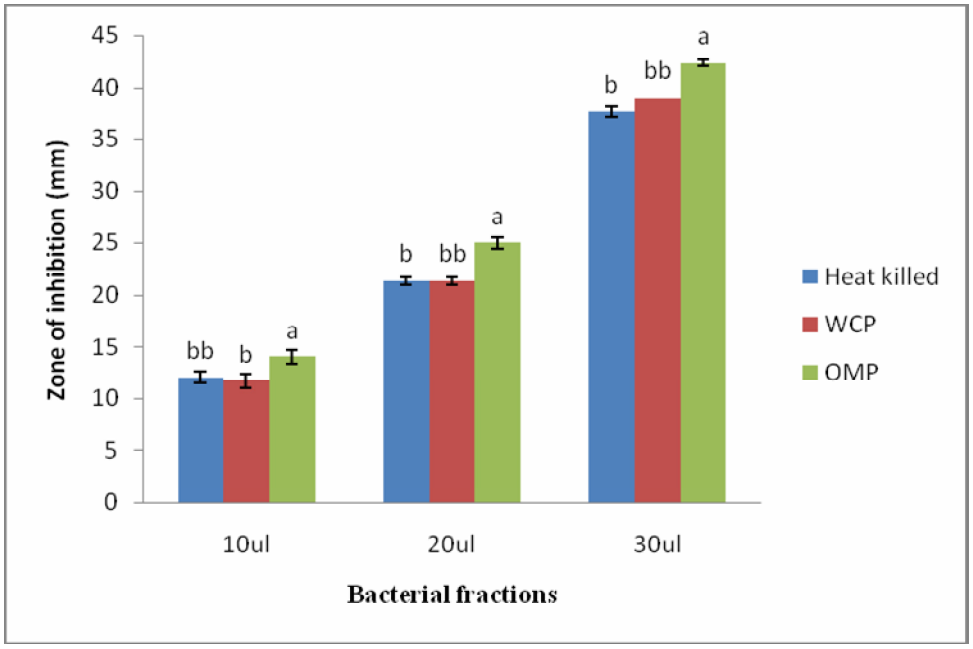

Fig2: Zone of inhibition (mm) of *Bacillus subtilis* AN11bacterial fractions against *Vibrio parahaemolyticus* **(**values are represented as mean ± S.E., mean bearing common superscript are not significant to each other).

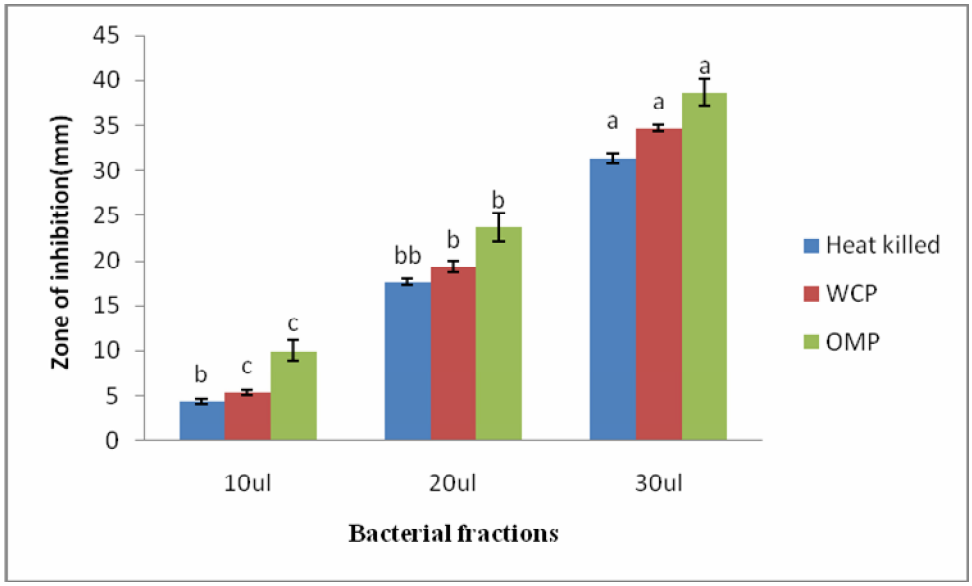

Fig 3: Zone of inhibition (mm) of *Bacillus subtilis* AN11 bacterial fractions against *Pseudomonas aeruginosa* **(**values are represented as mean ± S.E., mean bearing common superscript are not significant to each other).

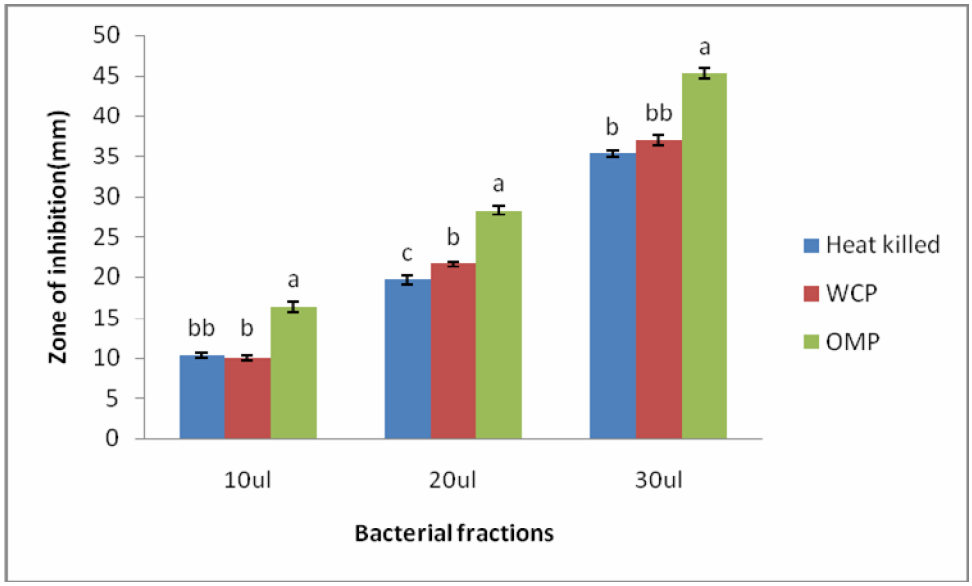

Fig 4: Zone of inhibition (mm) of *Bacillus subtilis* AN11 bacterial fractions fractions against *Flavobacterium columnare* **(**values are represented as mean ± S.E., mean bearing common superscript are not significant to each other).

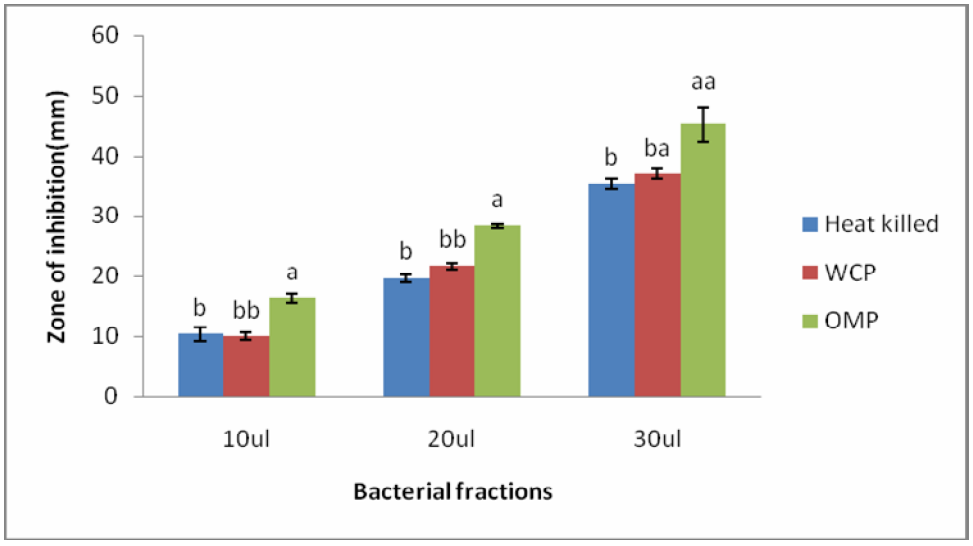

Fig 5: Zone of inhibition (mm) of *Bacillus subtilis* AN11 bacterial fractions against *Staphylococcus aureus (*values are represented as mean ± S.E., mean bearing common superscript are not significant to each other).

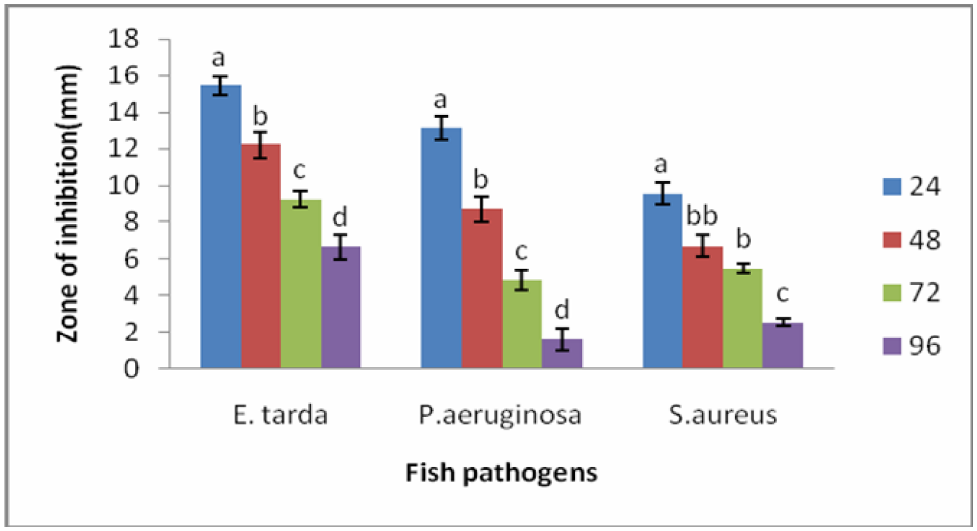

Fig 6 Antimicrobial effect of *B.subtilis* against *E.tarda, P.aeruginosa and S.aureus* in terms of zone of inhibition at various time intervals (values are represented as mean ± S.E., mean bearing common superscript are not significant to each other).

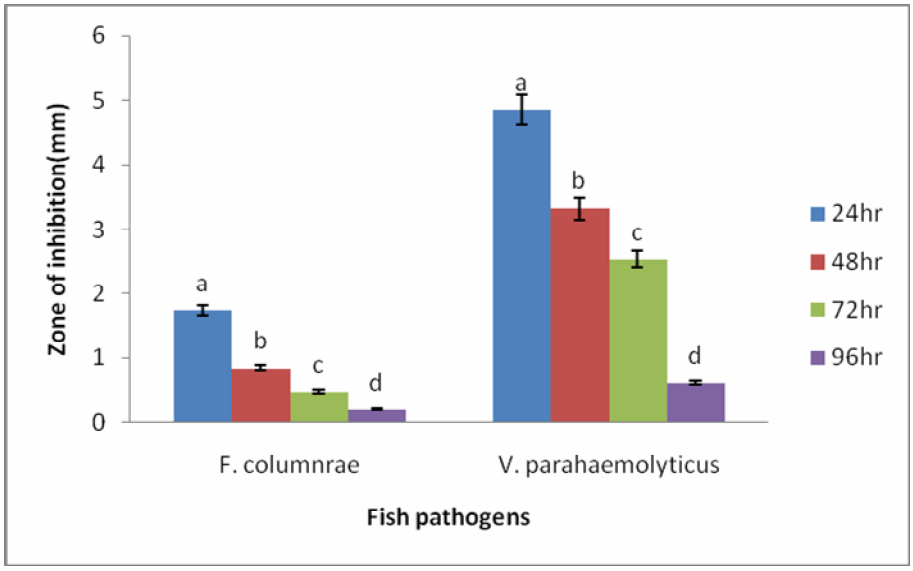

Fig.7 : Antimicrobial effect of *B.subtilis* against *F.columnarae and V.parahaemolyticus*, terms of zone of inhibition at various time intervals(values are represented as mean ± S.E., mean bearing common superscript are not significant to each other).

The use of antagonistic bacteria is widely expected to become an alternative method for the prevention and control of bacterial disease in fish. Numerous studies have shown that bacteria produce inhibitory substances that can inhibit bacterial pathogens in aquaculture system. The antagonistic effect of *Bacillus* OMP could be determined by the appearance of clear inhibitory zones around the paper discs and well diffusion assay .In the aquaculture industry, *A. hydrophila*, *V. parahaemolyticus*, *E. tarda*, *F. columnare*, *S. aureus*, cause huge economic losses. The use of antibiotics to prevent these diseases has normally been practiced .In many cases their indiscriminate use has led to increases antibiotic resistance and residual level in the products which would ultimately affect the quality of fish protein. There are many strains of the genus *Bacillus* which can produce a wide variety of antibiotics including polymyxin, colistin etc. Several bacitracins have been characterized. *Bacillus* antibiotics are generally produced at the early stages of sporulation (Eppelmann *et al*., 2001).

Sugita *et al.* (1998) isolated a strain of *Bacillus* sp. that was antagonistic to 83 percent of the isolates from fish mostly intestine targeting *Vibrio* and *Aeromonas*. Laloo *et al.* (2007) isolated several strains of *Bacillus* from *Cyprinus carpio* and carried out tests to improve the water quality in ornamental fish culture and to inhibit the growth of *A. hydrophila.* Other workers have also attempted to use other pathogens e.g. *E. tarda, E. seriolicida, Pasteurella piscicida, Yersinia ruckeri* (Austin *et al.*, 1995, Ruiz *et al.*, 1996, Sugita *et al.*, 1996, Gibson *et al*., 1998). The inhibitory effect of different cellular components i.e. ICP, ECP, WCP, or live bacteria of *P. aeruginosa, P.putida, P. fluorescens* were tested against *A. hydrophila* and found to antagonize and act as a biocontrol agent for it (Das *et*

al., 2006). Biocontrol of aquatic microbes to fish and shellfish pathogens has been reviewed by Gatesoupe (1999) and further *P.* fluorescens to *V. anguillarum* in *Lates niloticus* (Gram *et al.,* 1999) as well as *P. fluoresceans* to *A. Salmonicida.*

However the present study was targeted to find out the antagonistic activity of cellular fraction of *B.subtilis* AN II isolated from an extreme environment i.e. mangrove forest which was proved to be antagonistic to rice fungus (Muduli *et al*., 2013). Interesting ECP did not show any antagonistic properties to the pathogenic bacteria. We also found that *A. hydrophila* could not be inhibited by *B.subtilis* AN II strains.

The antibiotics produced by *Bacillus subtilis* were analyzed by well diffusion technique and migrated antibiotics were detected bioautographically by seeding cellular components *B.subtilis* on agar plates. The R.f values were compared with those given by Snell *et al.,* (1955). It was found that the antibiotics were produced by *B.subtilis* against S. aureus, *E. tarda, V. parahaemolyticus F. columnare, S. aureus* and *P. aeruginosa.* In all the fractions tested, the present study revealed that *B.subtilis* OMP showed maximum inhibitory effect to *S. aureus* followed by *F. columnare*. It also showed higher zone of inhibition to other pathogenic bacteria tested except *A. hyrdorphila.* The highest zone of inhibition produced by the OMP might be attributed by the high protein content in the OMP fractions as compared to the HKWCP, WCP and ECP. Interestingly, HKWCP and WCP showed more or less equal zone of inhibition at the same concentrations to be five fish pathogenic bacteria. The variation might be attributed due to variation of macromolecular composition that is responsible for the antagonistic study. As, there are no reports available about the role of OMP as an antagonistic for fish pathogenic bacteria, the present study is the first report regarding *B.subtilis* ANII OMP as an potential antagonistic agent for aquatic fish pathogens except *A. hydrophila.*

Outer membrane proteins possess a wide spectrum of inhibitory microbial protein that are responsible for controlling diseases at different incubation periods i.e. 24 h, 48 h, 72 h and 96 h. The antibacterial activity of OMP of *B.subtilis* was best exhibited against Gram positive *S. aureus* followed by other Gram negative pathogens. Whole cell product at the higher concentration produced lesser zone of inhibition than the OMP. The pathogenic bacteria inhibition was drastically reduced within 96 hours of incubation up to 80 percent. It is inferred that OMP of *B.subtilis* could be a best candidate for controlling the major bacterial infection in aquaculture sector.

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