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Antifungal Potentiality of Marine Microbial Chitinases Isolated from Crustacean Shells

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Six Chitin degrading bacterial species were identified and isolated from shells of crustacean samples namely Prawns and Crab shells. The isolates were screened on the basis of their formation of clearance zone in Colloidal chitin agar plate. The chitinolytic strains obtained were assessed for their antifungal potential against the three fungal strains namely *Fusarium Oxysporum, Aspergillus niger* and *Aspergillus flavus*. The fungal growth inhibition by the bacterial strains that is likely to be due to the secretion of extracellular hydrolytic enzymes is observed on Potato Dextrose Agar Plate for a duration of 24-72 hours. The pure chitinase extracted from these bacterial samples were assessed for the lysis of fungi and formation of protoplast. Of the various fungal species, *Fusarium oxysporum* and *Aspergillus flavus* showed the highest sensitivity to all the chitinase producing bacterial isolates. *Aspergillus niger* showed resistivity against most of the bacterial strains with an exception of *V.aestuarianus*. Further, different strains showed different degree of antagonism towards various phyto pathogens.

Key words : Microbial Chitinase, Antifungal activity, *Fusarium oxysporum, Aspergillus niger* and *Aspergillus flavus*.

Chitin is potentially an important nutrient source for marine bacteria since it is produced by many marine organisms, including zooplankton and several phytoplankton species[1]. The human processing of shellfish generates a huge quantity of chitin that creates a major waste disposal problem. Microbial chitinase is important in the conversion of waste chitin to N-Acetyl glucosamine which, in turn, is assimilated by other micro-organisms to produce some fermentation products such as edible yeast and ethanol. Chitinases may also be used in the biological preparations and in the cell fusion of fungi. Several enzyme inhibitors with various industrial uses were isolated from bacteria and actinomycetes living in the marine environment. These inhibitors are useful in medicine and agriculture. Many bacteria, including *Bacillus circulans*, *Aeromonas* sp. and *Serratia marcescens* has been shown to produce multiple chitinases from different genes, and the efficient degradation of chitin is assumed to be achieved by the combined action of the multiple chitinases.

Fungal plant diseases are one of the major concerns to agricultural food production world wide. Soil borne pathogenic fungi such as, *Fusarium*, *Rhizoctonia* and *Phytopthora* attack most of the economically important crop plants resulting in loss of billions of dollars. The abuse of chemical pesticides or fungicides to cure or prevent plant diseases has caused soil pollution and

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detrement effects in humans. Biological control of soil-borne plant pathogens by antagonistic microorganisms is a potential non-chemical mean of plant disease control. Chitinases are pathogenesis-related proteins that catalyse the random cleavage of internal β-1,4 glycosidic linkages in chitin, a major constituent of fungal cell walls and insect exoskeletons. These enzymes have been identified in various tissues of numerous plants species, despite the fact that no known plant substrate for the enzyme has been identified[2]. Chitinases exhibit antifungal activity[3] by lysing fungal tips and inhibiting growth[4]. Chitin is particularly abundant in the marine environment being the principle component of the exoskeletons of crustaceans and many molluscs. Despite the huge turnover of chitin in the marine and soil environments and the importance of microbial chitinases in this process, an attempt was made to prove the efficacy of chitinase from marine crustacean source as antifungal agents.

MATERIAL AND METHODS

Screening of chitin degrading bacteria

Chitinase producing bacterial strains were isolated from different places along the coastal areas of Chennai. All the isolates were identified to various genera representing species of *Vibrio, Flavobacterium, Shewenella, Exiguobacterium* and *Bacillus*. Screening of potent strains capable of maximum production of chitinase was detected by spotting on colloidal chitin medium and the clear zones produced around the colonies were observed. The strains were thus identified as *Vibrio aestuarianus, Flavobacterium odoratus, Shewenella putrefaciens, Exiguobacterium strain, Bacillus subtilis* and *Bacillus atrophaeus.*

In vitro antifungal activity

The antifungal activity was assayed *in vitro* by inhibiting the growth of phytopathogenic fungus on PDA media (Himedia)[5]. The bacterial inoculum of the six chitinolytic isolates were picked aseptically and streaked in the center of petridish. Fungal inocula consisted of agar disc (1cm diameter) punched out with sterilized corkborer from the growing margin of colonies and was placed on either side of bacteria inoculated plates. The Petri plates were incubated at 28 ºC for three days.

Fungal cell production

The fungi were grown in potato dextrose agar plates for 10 days at 28°C. The spore germination was carried out in 250 mL Erlenmeyer flasks containing 50 mL of a culture medium which was composed of 10 g/L soluble starch; 0.3 g/L vitamin-free casein; 2.0 g/L KNO_3; 2.0 g/L NaCl; 0.05 g/L $MgSO_4$ 7 H_2O; 0.01 g/L $FeSO_4$ 7 H_2O; and 0.02 g/L $CaCO_3$, at 28°C and 150 rpm for 20 hrs. Then the material was centrifuged at 2,822 *x g* for 6 minutes at 5°C. The mycelium was washed 3 times with distilled water and re-suspended in 0.2M phosphate buffer with pH 5.8 [6].

Application of purified chitinase in the lysis of fungi and formation of Protoplasts

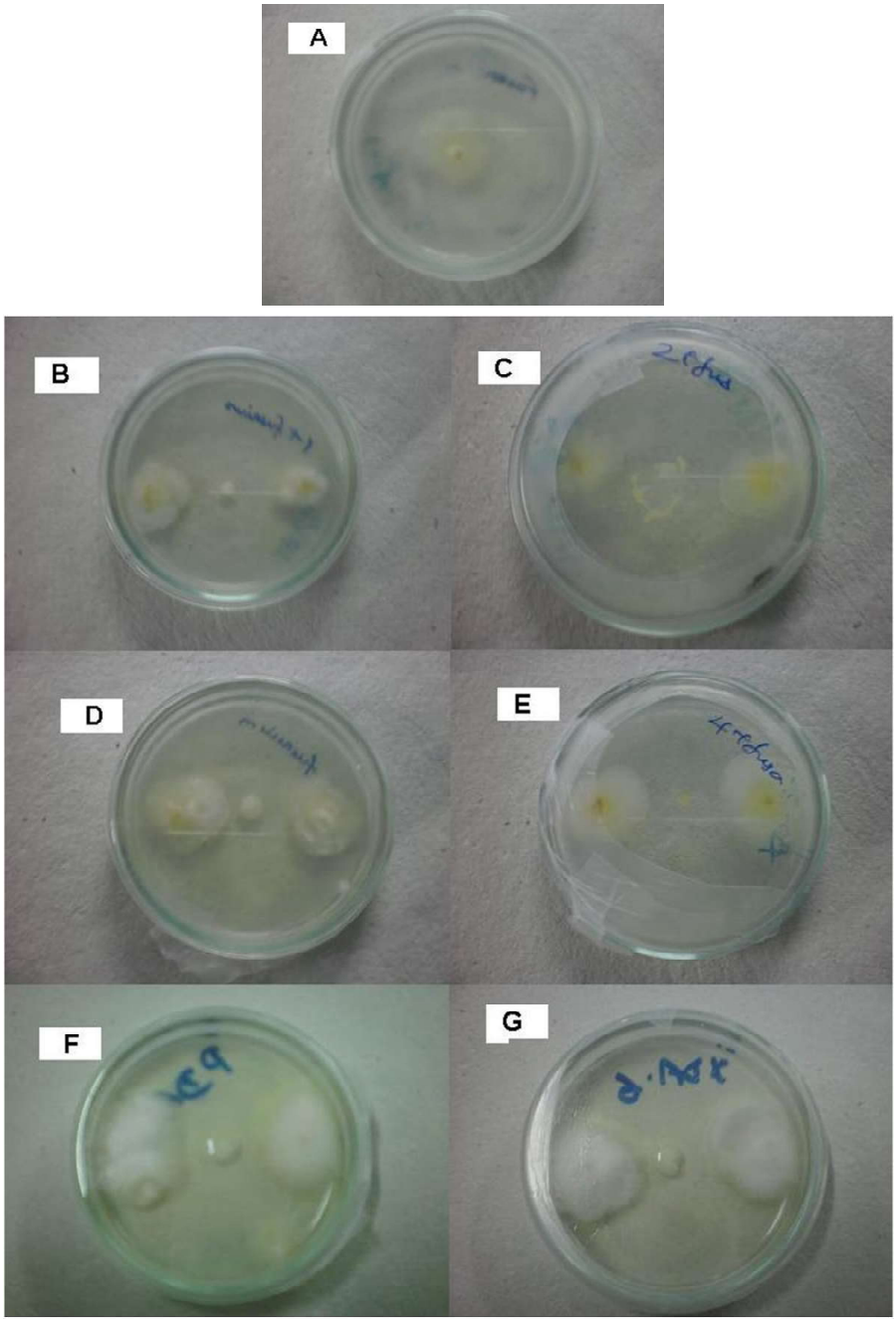
The reaction mixture containing 0.5 mL of fungal suspension in 0.2 M phosphate buffer, pH 5.8 and 0.5 U purified chitinase suspension/ml fungal suspension, was incubated at 30°C for 2 hours and was stirred at regular intervals. Cell lysis was observed under an optical microscope with immersion. The reaction mixture containing the fungal suspension, without the addition of the enzyme and incubated for the same time and same temperature, was used as control.

RESULTS

Bacteria's architecture and resistance to antimicrobial agents vary considerably with strain, age, and nutrition, this difference being most pronounced between Gram-positive and Gram-negative organisms[7]. It was incubated on Petri plates containing 15ml PDA under sterile conditions at room temperature [(28±2) °C] for 5 days to prepare a conidial suspension for later use.

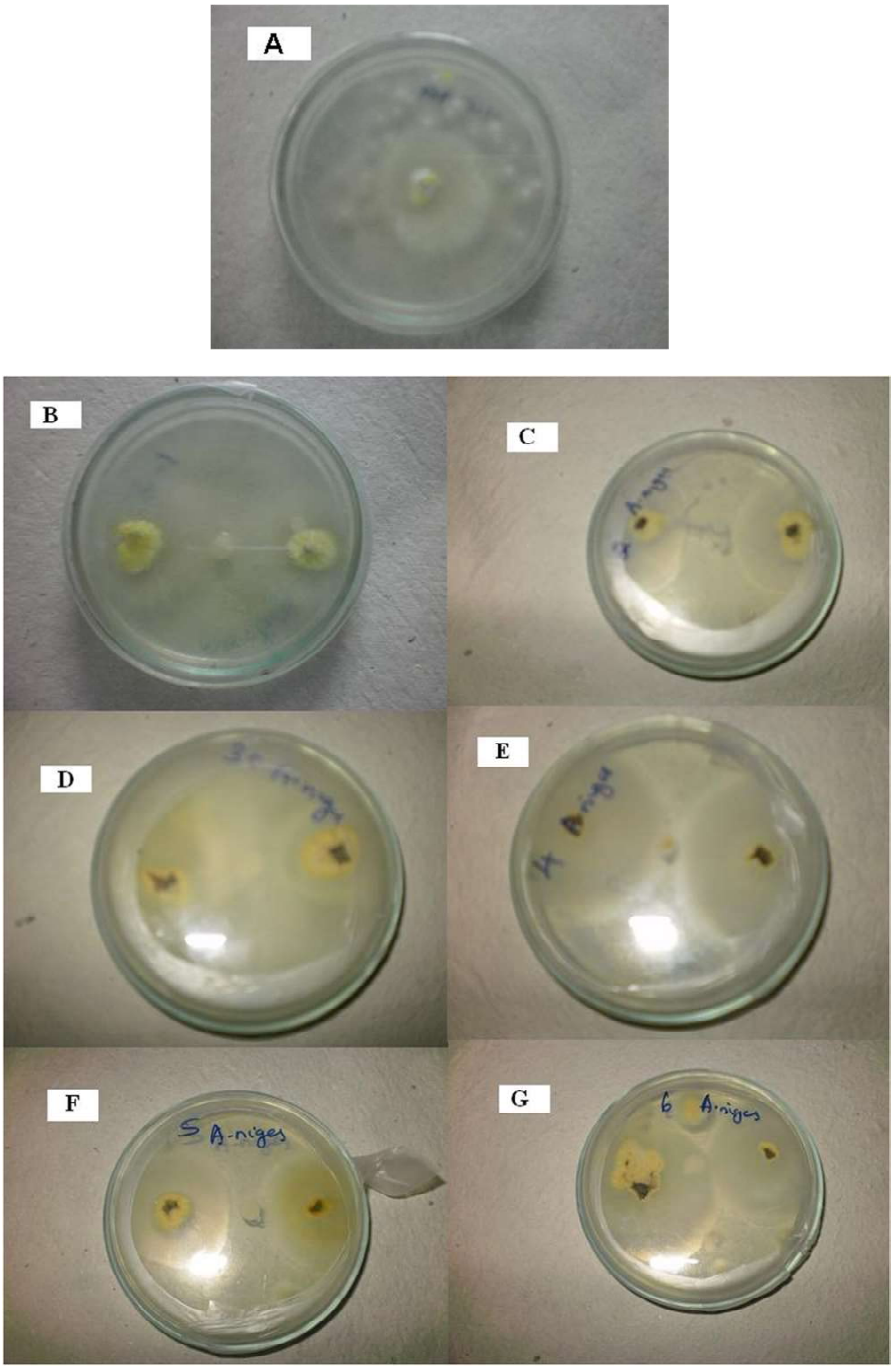
The six strains showed different inhibitory activity towards different fungal species. The inhibitory effect of chitinolytic strains on phytopathogens were determined by their level of inhibition towards the growth of fungal species on Potato Dextrose Agar plate incubated at room temperature for three days. The Potato Dextrose Agar plate inoculated with fungal species alone serves as the control.

Antifungal activity of all the six chitin degrading bacterial strains toward *Fusarium oxysporum* (Fig. 1-A to G), *Aspergillus niger* (Fig. 2-A to G) and *Aspergillus flavus* (Fig. 3-A to G) was observed. Of the various fungal species,



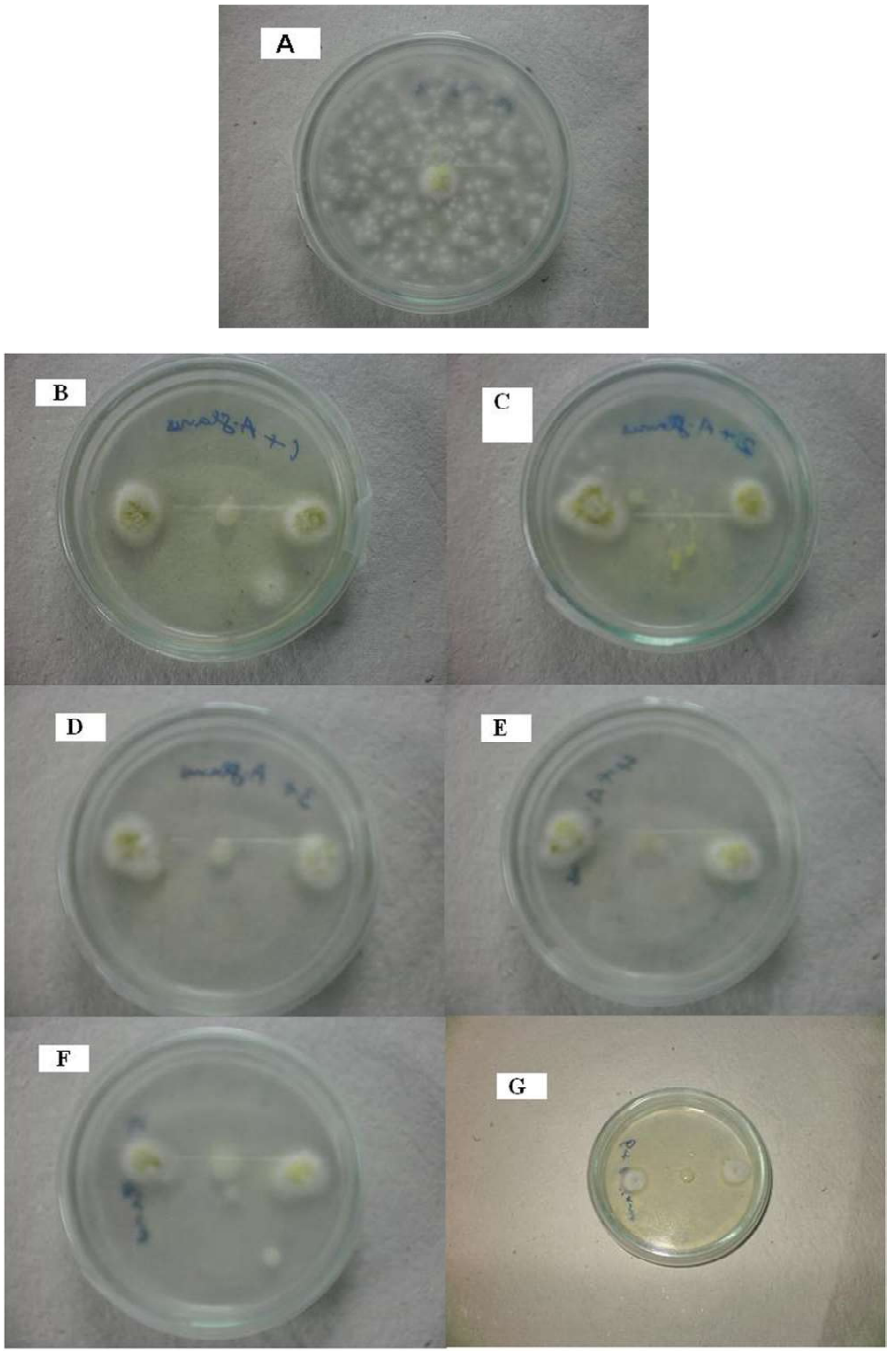
A: Control Potato Dextrose Agar Plate Showing the growth of *F. oxysporum* alone.
B: Test 1: PDA with *F. oxysporum* along the periphery and the bacteria *V.aestuarianus* at the center.
C: Test 2: PDA with *F. oxysporum* along the periphery and the bacteria *F.odoratus* at the center.
D: Test3: PDA with *F. oxysporum* along the periphery and the bacteria *S. putrefaciens* at the center.
E: Test 4: PDA with *F. oxysporum* along the periphery and the bacteria *Exiguobacterium* at the center.
F: Test 5: PDA with *F. oxysporum* along the periphery and the bacteria *B.subtilis* at the center.
G: Test 6: PDA with *F. oxysporum* along the periphery and the bacteria *B.atrophaeus* at the center.

Fig. 1. Antifungal assay of bacterial strains towards *Fusarium oxysporum*



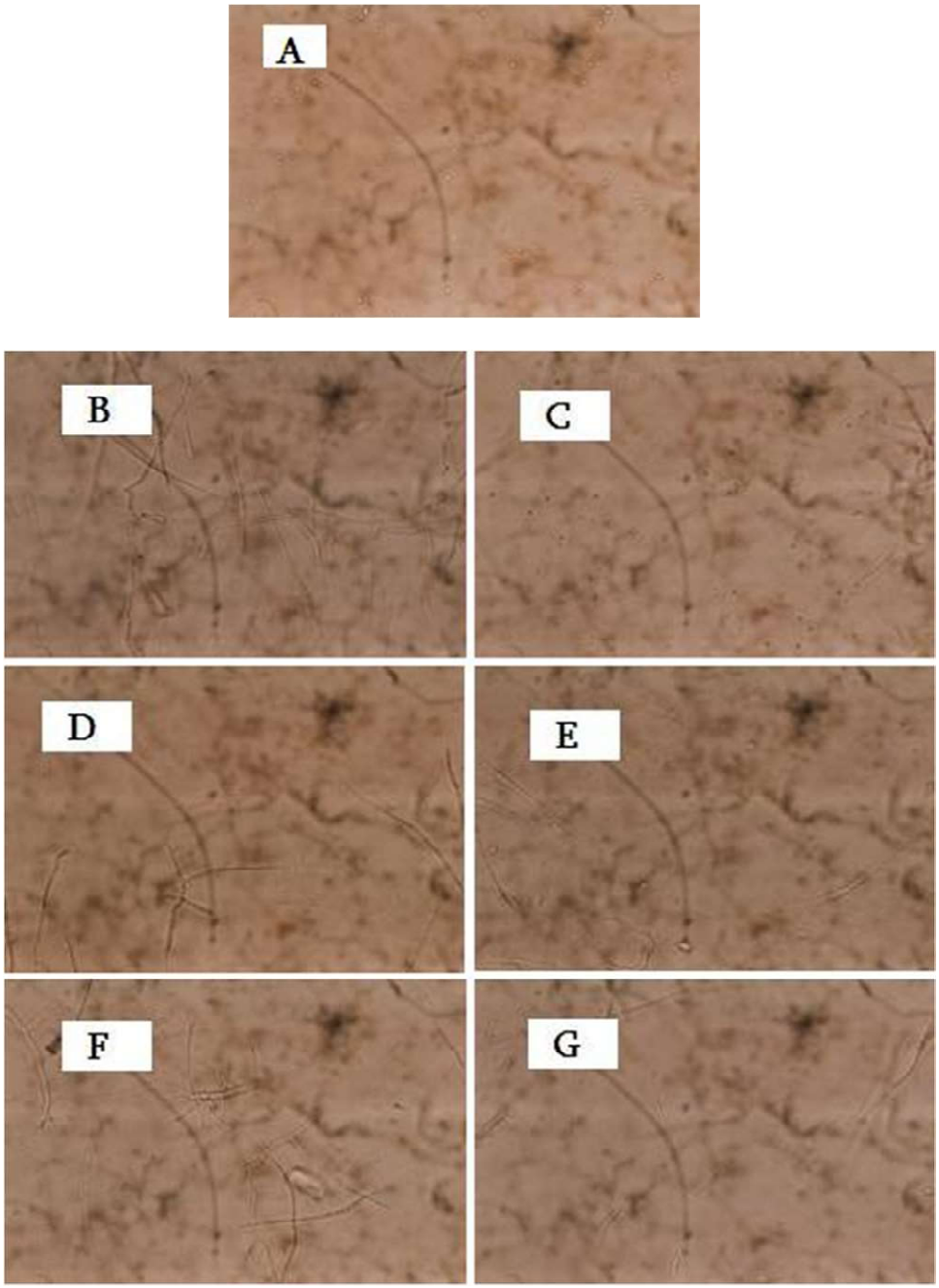
A: Control Potato Dextrose Agar Plate Showing the growth of *A. niger* alone.
B: Test 1: PDA with *A. niger* along the periphery and the bacteria *V.aestuarianus* at the center.
C: Test 2: PDA with *A. niger* along the periphery and the bacteria *F.odoratus* at the center.
D: Test3: PDA with *A. niger* along the periphery and the bacteria *S. putrefaciens* at the center.
E: Test 4: PDA with *A. niger* along the periphery and the bacteria *Exiguobacterium* at the center.
F: Test 5: PDA with *A. niger* along the periphery and the bacteria *B.subtilis* at the center.
G: Test 6: PDA with *A. niger* along the periphery and the bacteria *B.atrophaeus* at the center.

Fig. 2. Antifungal assay of bacterial strains towards *Aspergillus niger*



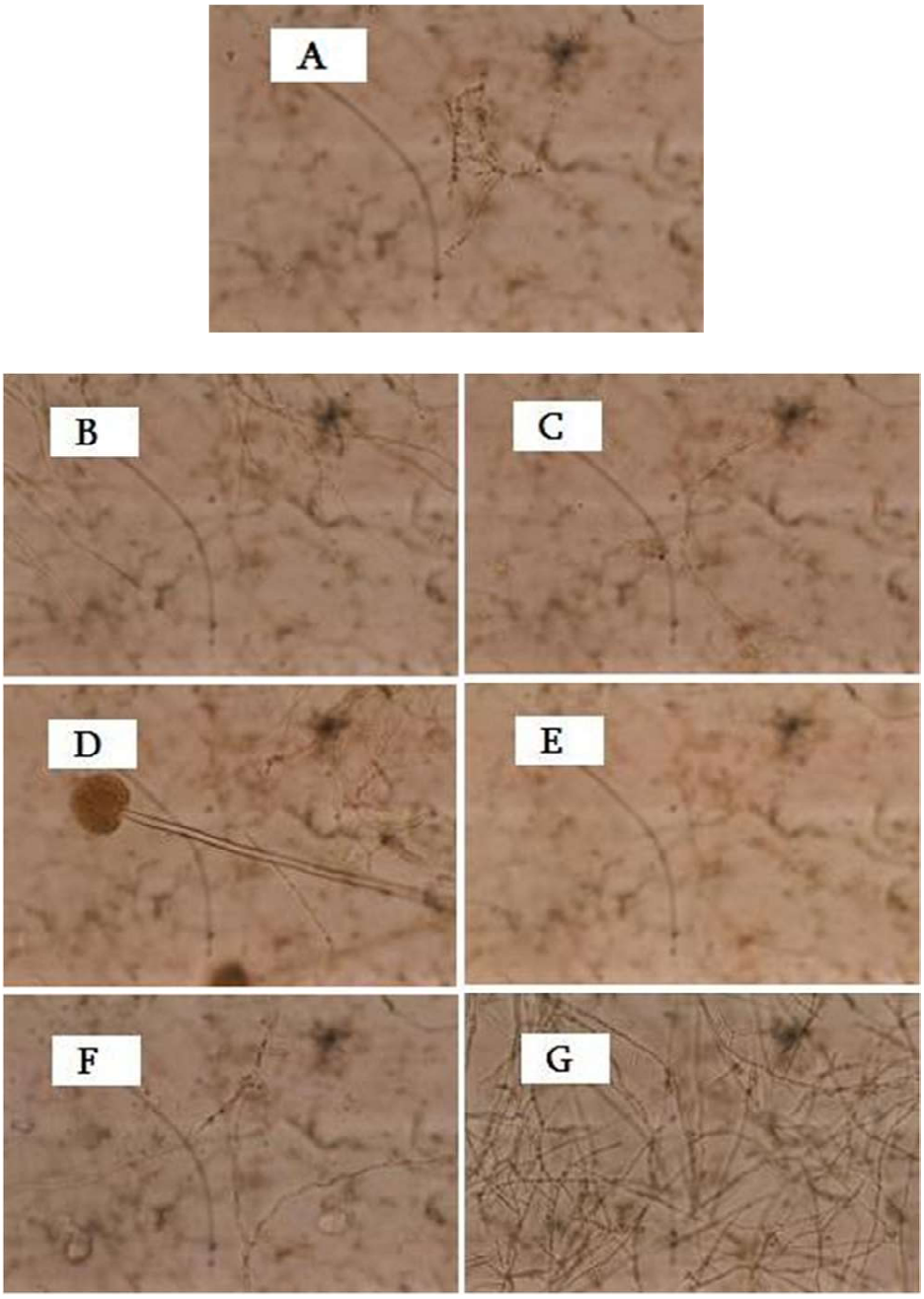
A: Control Potato Dextrose Agar Plate Showing the growth of *A. flavus* alone.
B: Test 1: PDA with *A. flavus* along the periphery and the bacteria *V.aestuarianus* at the center.
C: Test 2: PDA with *A. flavus* along the periphery and the bacteria *F.odoratus* at the center.
D: Test3: PDA with *A. flavus* along the periphery and the bacteria *S. putrefaciens* at the center.
E: Test 4: PDA with *A. flavus* along the periphery and the bacteria *Exiguobacterium* at the center.
F: Test 5: PDA with *A. flavus* along the periphery and the bacteria *B.subtilis* at the center.
G: Test 6: PDA with *A. flavus* along the periphery and the bacteria *B.atrophaeus* at the center.

Fig. 3. Antifungal assay of bacterial strains towards *Aspergillus flavus*



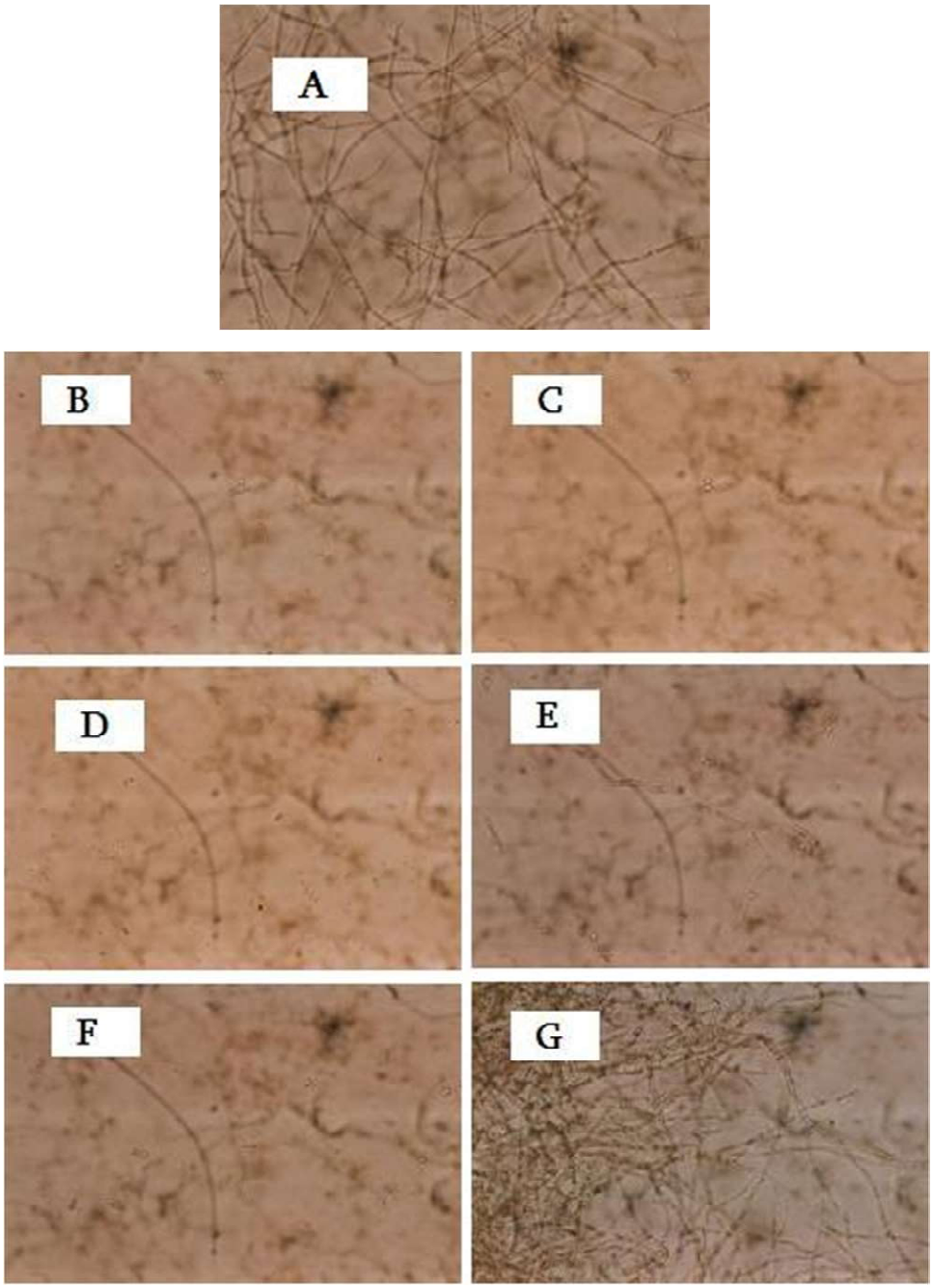
A: Control *Fusarium Oxysporum*.
B: Test 1: *Fusarium Oxysporum* treated with pure chitinase from *V.aestuarianus*.
C: Test 2: *Fusarium Oxysporum* treated with pure chitinase from *F.odoratus* .
D: Test3: *Fusarium Oxysporum* treated with pure chitinase from *S. putrefaciens* .
E: Test 4: *Fusarium Oxysporum* treated with pure chitinase from *Exiguobacterium*.
F: Test 5: *Fusarium Oxysporum* treated with pure chitinase from *B.subtilis*.
G: Test 6: *Fusarium Oxysporum* treated with pure chitinase from *B.atrophaeus*.

Fig. 4. Observation of cell wall lysis by the pure chitinase from six chitin degrading bacterial strains towards *Fusarium Oxysporum*



A: Control *Aspergillus niger*.
B: Test 1: *Aspergillus niger* treated with pure chitinase from V.*aestuarianus*.
C: Test 2: *Aspergillus niger* treated with pure chitinase from *F.odoratus* .
D: Test3: *Aspergillus niger* treated with pure chitinase from *Shewenella putrefaciens*.
E: Test 4: *Aspergillus niger* treated with pure chitinase from *Exiguobacterium*.
F: Test 5: *Aspergillus niger* treated with pure chitinase from *B.subtilis*.
G: Test 6: *Aspergillus niger* treated with pure chitinase from *B.atrophaeus*.

Fig. 5. Observation of cell wall lysis by the pure chitinase from six chitin degrading bacterial strains towards *Aspergillus niger*



A: Control *Aspergillus flavus*.
B: Test 1: *Aspergillus flavus* treated with pure chitinase from V.*aestuarianus*.
C: Test 2: *Aspergillus flavus* treated with pure chitinase from *F.odoratus* .
D: Test3: *Aspergillus flavus* treated with pure chitinase from *Shewenella putrefaciens*.
E: Test 4: *Aspergillus flavus* treated with pure chitinase from *Exiguobacterium*.
F: Test 5: *Aspergillus flavus* treated with pure chitinase from *B.subtilis*.
G: Test 6: *Aspergillus flavus* treated with pure chitinase from *B.atrophaeus*.

Fig. 6. Observation of cell wall lysis by the pure chitinase from six chitin degrading bacterial strains towards *Aspergillus flavus*

Fusarium oxysporum and *Aspergillus flavus* showed the highest sensitivity to all the chitinase producing bacterial isolates. *Aspergillus niger* showed resistivity against most of the bacterial strains with an exception of *V.aestuarianus.*

The results towards *Fusarium oxysporum* showed that *V.aestuarianus* inhibited the mycelial growth of the fungus very prominently when compared to all the other isolates. The fungal phyto pathogen *Aspergillus flavus* showed almost same degree of sensitivity towards all the chitinolytic strains tested. The plates tested against *Aspergillus niger* shows that it's growth is well affected by *V.aestuarianus* whereas considerable growth of mycelia was observed in the presence of all other chitinolytic strains incubated for same period at same temperature.

The figure illustrates the lysis of the fungi *Fusarium oxysporum* (Fig. 4: A to G), *Aspergillus niger* (Fig. 5: A to G) *and Aspergillus flavus* (Fig. 6: A to G) by the chitinase purified from the chitin degrading bacterial strains as compared to their respective controls. With the exception of *Aspergillus niger*, the fungi *Fusarium oxysporum and Aspergillus flavus* were highly hydrolysed, since only fragments were found on the slides when examined under the optical microscope with immersion.

The fungi *A. niger* presented no alteration in cell structure after treatment with 0.5 U of purified chitinase/mL of suspension, as compared to their respective controls. There are many differences in the composition and organization of fungal cell walls and this difference probably hindered the action of chitinase.

DISCUSSION

Conclusive structure/function analyses of chitinase enzymatic and antifungal activities have been limited because the proteins are typically isolated from plants or fungi and are not easily purified to homogeneity in large quantities, i.e. often several chitinase isoforms are co-purified[3,4,8,9]. There is a wealth of data supporting the important role of chitinolytic enzymes in antifungal activities of various antagonistic microorganisms[10-14]. Several recent studies reported the purification and characterization of chitinases and β-1, 3-glucanases produced by *Trichoderma* spp. and *Talaromyces flavus* and highlighted their role in the mycoparasitism of soilborne pathogens such as *Sclerotium rolfsii*, *Rhizoctonia solani*, and *Fusarium* spp. [15,16]. Although the majority of the available evidence has been interpreted in terms of a model in which the *in vivo* role of chitinases is to protect the host from fungal invasion directly by hydrolyzing chitin in the fungal cell wall, it has been suggested that the role of chitinases in the plant disease resistance mechanism is to release elicitors of defense-related lignification from fungal cell walls.

Although various bacterial strains such as *Aeromonas*[12], *Enterobacter*[13], *Chromobacterium*[17], and *Serratia* [10] have been reported to be effective biological control agents of *S. rolfsii* and *R. solani*, the data related to suppression of *Fusarium* wilts by chitinolytic bacteria is limited[18]. The complexity of the soil ecosystem is a constraint that makes biological control of these root pathogens by introduced antagonists a challenge[19].

Though there are several reports regarding the antifungal activity of chitinase, the plant chitinase stands at the level of self defense against fungal pathogens and the bacterial chitinase provide an insight into the biopotentiality of microbial community. However, there are no datas pertaining to the determination of antifungal activity from the marine microbial species, that too from the marine animals. The antifungal activity of the chitinolytic strains belonging to the genera *Vibrio, Flavobacterium, Shewenella* and *Exiguobacterium* strains has not been studied yet. Hence, the result obtained from this study clearly suggests the utilization of bacterial chitinase from a pure marine environment as fungicides towards phyto pathogens. The chitinolytic strains described here were assessed for their ability to suppress the growth of several phytopathogenic fungi in vitro, and no enrichment techniques were used to specifically select chitinolytic bacteria. Hence, Chitinolytic activity against insects, nematodes, and fungi raises the use of chitinases as alternative pesticides which are environmentally-friendly compared to chemical pesticides. This finding enhances the potential of protein modification research that could substantially improve the function of chitinases in host plant defense.

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