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Protective algal consortium in the sporocarp of woodrotting fungus from bacterial chitinase activity

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The wood rotting fungi grow on the bark of any tree; usually they are pigmented because of the lignin decomposing ability of these fungi. They are degraded by the chitinase activity of different bacterial pathogens. In this investigation, fruiting bodies of *Ganoderma* sp. of different ages were collected from the base of a coconut trunk in Tamil Nadu off the coast of Bay of Bengal near the Golden Beach, about 25 Km from Chennai. The bacteria isolated from the surface of the fruiting body were identified on the basis of enzyme characteristics and proved to be a Gram negative coccobacillus *Enterobacter* sp. They were shown to be producing chitinase enzyme for degrading the fruit body. But in one such fruiting body, an algal mesh formed of *Pandorina*, identified on the basis of microscopic character in BG 11 medium effectively protected the basidiocarp from the enzymatic action of the bacterial chitinase. This was further proved by the growth of bacteria and algae in collateral medium showing inhibitory action of the algae on the bacteria.

Thus it can be concluded that the phenomenon of hyper-parasitism on saprophytic organisms is controlled by photosynthetic algal consortium and thereby, in future, this algal consortium can be used to free the waterbody from coliform infestation.

Key words: Chitinase, *Enterobacter* sp. *Ganoderma* sp. *Pandorina*, algae, BG11 media, collateral media

INTRODUCTION

Coconut plants growing along the coastline are subjected to serious stress because of high wind speed, pollution, tidal waves and wood rotting fungi. (Billot and Broschat, 2001).

One such fungus was isolated from an uprooted coconut plant and found to be *Ganoderma* sp. characterized by basidiocarps that are large perennial woody brackets called conks (Billott *et al.*, 2001), it is a white rot fungus and has enzymes which allow it to breakdown wood components such as lignin and cellulose. These wood degrading enzymes have industrial applications such as biopulping and bioremediation (Yuen and Gohel, 2005).
Chitin present in the *Ganoderma* sp. is degraded by the enzyme chitinase. Chitinase (E.C. 3.2.1.14) are a group of antifungal proteins that catalyses the hydrolytic cleavage of β - 1,4-glycoside bond present in the biopolymers of N-acetyl-D-glucosamine mainly in chitin.

In our work the *Ganoderma* sp. has been isolated from coconut plant in southern coastal part of India. The enzyme chitinase is also produced by the bacteria which are found to be associated with the isolated sample of *Ganoderma* sp.

So, it might as well be expected that the presence of the enzyme chitinase degrades the structural integrity of *Ganoderma* sp. But the *Ganoderma* sp. that was isolated is still intact. Surprisingly the isolated *Ganoderma* sp. is covered by unique algal consortium containing green algae. We have tried to establish that the algae may have antagonistic relationship with chitinase producing bacteria,

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which inhibits or decelerates its chitinase production, which explains the existence of fungal colony.

MATERIALS AND METHODS

Sample material

The fungal sample was collected from an uprooted coconut tree, 58 km away from Pondicherry on the Chennai-Pondicherry Highway

Identification of green algae

The alga was isolated and observed under microscope.

Isolation of bacteria

Bacterial colonies were isolated from the surface of the fungus, which was covered by algal consortium. Bacteria were isolated into pure culture by dilution plate method. The bacteria were grown on nutrient agar medium and incubated at 37°C for 24-48 hrs. Four different colonies were found- the colonies were preferentially transferred to fresh media to obtain pure cultures.

Characterization of bacteria and measurement of chitinase assay

The gram reaction of each of the pure cultures was determined by Gram staining followed by measurement of their chitinase activity. The bacterial colony showing highest chitinase activity was selected for further characterization.

Maintenance of pure culture

The bacterial colony with highest chitinase activity was selected for maintenance of pure culture. The cultures were maintained by repeated subculturing. Subcultures were maintained by streak plating technique. These bacteria were used for further biochemical tests.

Gas production by bacteria

The bacteria were inoculated in lactose broth and Durham's tube was inserted to observe bubble formation. Five test tubes containing Durham's tube inserted were inoculated.

Isolation of gas producing bacteria on EMB agar

The EMB (Eosin Methylene Blue) agar plates were inoculated with positive 24-48 hrs. lactose broth culture with sterilised inoculation loop.

- The plates were incubated for 24-48 hrs. at 37°C in an inverted position.
- The incubated plates were observed carefully.

Identification of bacteria by biochemical tests

The following biochemical tests were performed during identification of bacterial colonies:-

Catalase test

This test detects the presence of enzyme catalase, which converts H_2O_2 to H_2O and O_2.

Generally a colony is taken from a culture and added to a slide containing H_2O_2. The formation of bubbles, i.e., production of O_2, suggests a positive catalase test. Absence of bubbles gives a negative catalase test.

Oxidase test

This test detects the presence of cytochrome C oxidase that is able to reduce O_2 and artificial electron acceptors.

Filter paper was moistened with a few drops of 1% tetramethyl-p-phenylenediamine dihydrochloride. Now a single colony was taken from the culture and smeared on the paper. A positive test was given by the formation of purple color within 10 seconds.

Nitrate reduction test

This test detects the capability of a bacterium to reduce nitrates to nitrites and this is detected by the addition of alpha-naphthylamine and sulfanilic acid when it gives a reddish colouration.

No colour development can be interpreted in 2 ways- true negative result where nitrate has not been reduced and a false negative where nitrates have been reduced to products other than nitrites- since test reagents detect only nitrites, nitrate reduction forming other products cannot be understood.

Gelatin liquefaction test

This test detects whether or not a bacterium has the ability to produce proteases that hydrolyze gelatin and liquefy solid gelatin medium. The tubes needed to be put in a refrigerator for checking en-

zyme liquefaction since gelatin liquefies at 37 °C. If gelatin was hydrolyzed by gelatinase (protease), it remained a fluid and hence flow when the test tube was tilted giving a positive test. A negative test was when gelatin did not flow even when the test tube was tilted.

Indole test

This test detects the production of indole from the amino acid tryptophan.

The test organism was inoculated in tryptone broth, which was a rich source of tryptophan. Indole positive bacteria produced tryptophanase enzyme cleaving tryptophan, producing indole and other products. When Kovac's reagent (p-dimethylaminobenzaldehyde) was added to a broth containing indole, a dark pink or reddish pink color developed. Orange yellow color was negative test.

Methyl red test and Voges Proskauer test

The Methyl Red (MR) and the Voges Proskauer (VP) tests are determined from a single incubated tube of MR-VP broth. After 24-48 hrs. of incubation, the MR-VP culture was splitted into two tubes; one tube was used for the MR test and the other for the VP test.

MR-VP medium contains glucose and peptone. All enterics oxidized glucose for energy; however, the end products are different depending on bacterial enzymes. Both MR and VP tests are used to determine the end products formed after glucose degradation.

Citrate test

This test determines whether or not the bacterium can use sodium citrate as a sole source of carbon. The citrate test utilizes Simmon's citrate medium which contains bromothymol blue, a pH indicator with a range of 6 to 7.6. The medium also contains inorganic ammonium salts which are utilized as the sources of nitrogen. Use of citrate involves the enzyme citritase, which breaks down citrate to oxaloacetate and acetate. Oxaloacetate is further broken down into pyruvate and carbon dioxide. Production of sodium carbonate as well as ammonia from the use of sodium citrate and ammonium salts result in alkaline pH. This results in change of medium's colour from green to blue- positive test. Green colouration indicates negative result.

Urease test

This test detects the ability of bacteria to produce urease for the breakdown of urea which is a diamide of carbonic acid.
This test utilizes Christensen's Urea agar which contains urea solution. If the bacterium possesses urease enzyme, the urea is hydrolyzed with the release of ammonia and carbon dioxide. The ammonium reacts in solution to form ammonium carbonate resulting in alkalinization of the medium which can be detected by the addition of phenol red which acts a pH indicator. A pink colouration indicates alkaline environment and hence positive result.

Test to establish the antagonistic relationship between the algae and the bacteria which inhibits or decelerates the chitinase activity of the bacteria

BG-11 medium and the nutrient broth were prepared. Collateral medium was prepared by mixing BG-11 medium and nutrient broth in 1:1 ratio (by volume).Now, only BG-11 medium was taken in two different culture tubes; one was inoculated with only algae, while in the other, algae and the bacteria (identified by biochemical tests) were inoculated; the set up was kept under sunlight for 3 days. The room temperature was 37°C. Now the collateral medium was taken in 3 different culture tubes and separate tubes were inoculated by the algae, bacterium and a mixed inoculated with both. These 3 culture tubes were also kept under sunlight for 3 days. The room temperature was 37°C. All the culture tubes were kept at the same place at the same time so that even if there was some temperature variation, the manifestations would be the same for all. A replica of each culture tube was maintained. The algae after the time span, was filtered and the optical density of each culture tube was measured after filtering the algae. The chitinase activity of the bacteria was also determined.

RESULTS

Morphology of the algae

Algae are composed of 8 cells held together surrounded by mucilage. The cells are ovoid or slightly narrowed at one end to appear pear shaped. Each cell has 2 flagella.

Bacterial characteristics

The bacterial colonies that were isolated were harmful for the fungus, as they produced the enzyme chitinase (Table 1).

The work was carried out further by isolating the bacteria producing the maximum amount of chitinase, because the aim was to establish the antagonistic relationship with the algae which was supposedly decelerating the chitinase activity. It can be understood that if the chitinase activity of the bacteria producing the maximum amount of chitin is decelerated, all other bacterial chitinase activity will also be affected. The bacteria showed lactose fermentation ability through gas formation in all likelihood the bacterium may be coliform one.

Table 1 : Characteristic of bacterial isolates

Sample No.	Gram Chara-cter	Form/ Shape	Optical Density	Concentration of Enzyme Activity
1.	Negative	Coccobacilli	0.12	0.00006 M/min
2.	Negative	Coccobacilli	0.36	0.0002 M/min
3.	Negative	Cocci	0.05	0.27*10^-4 M/min
4.	Negative	Cocci	0.09	0.05*10^-4 M/min

Colony characteristics on EMB agar

Large, mucoid, pinkish colonies were observed

The test bacterium was Gram negative rods, showing positive results only for catalase, Nitrate reduction, Voges-Proskauer and Citrate tests and negative results in oxidase,methyl red, indole, urease and gelatin liquefaction tests.

It can be concluded that the test organism isolated from the given sample may be *Enterobacter* sp.

The bacterium was grown along with the algae in BG11/Co-lateral medium and the results are expressed in Table 2.

Optical density increased with increase in absorbance of light while passing through the medium. Absorbance increased with increase in cloudiness in the medium. With increase in number of bacterium, the cloudiness in the liquid medium increases. Thus, with increase in number of bacterium in liquid medium, the optical density increased.

Thus it can be concluded: that in case of BG-11 medium, the optical density is same in presence and absence of bacterium Thus it can be understood that bacterium cannot grow in BG-11 medium.

Table 2 : Growth of bacterium in media

Medium	Innoculum	Optical Density
BG-11	Algae :culture tube1	0.1
	culture tube2	0.1
	Algae + Bacteria :	
	culture tube1	0.1
	culture tube2	0.1
Collateral medium	Algae :culture tube1	0.1
	culture tube2	0.1
	Bacteria :	
	culture tube1	0.47
	culture tube2	0.49
	Algae + Bacteria :	
	culture tube1	0.40
	culture tube2	0.39

When collateral medium was inoculated with only algae, its optical density was found to be same as that of the BG-11 medium inoculated with algae.

However, the optical density of the collateral medium was more when inoculated with bacteria only, rather than when it was inoculated with both bacteria and algae. This meant that the bacterial growth was being inhibited in the presence of the algae, or in other words, algae had an antagonistic relationship with the bacterial colonies.
Chitinase assay of the bacteria in presence algae is shown in Table 3.

Table 3: Chitinase activity of bacteria

Medium	Innoculum	Chitinasw Activity
BG-11	Algae : culture tube 1	0.0 microM/min
	culture tube 2	0.0 microM/min
	Algae + Bacteria :	
	culture tube 1	0.0 microM/min
	culture tube 2	0.0 microM/min
Collateral Medium	Algae : culture tube 1	0.0 microM/min
	culture tube 2	0.0 microM/min
	Bacteria :	
	culture tube 1	0.2 microM/min
	culture tube 2	0.2 microM/min
	Algae + Bacteria :	
	culture tube 1	0.12 microM/min
	culture tube 2	0.18 microM/min

The chitinase activity of the bacteria decreased in the presence of the algae. So it can be understood that the algal growth was responsible for the inhi-

bition of chitinase enzyme production. Thus it can be concluded that the algae may have an antagonistic relationship with the chitinase producing bacterium which inhibits or decelerates its production of enzyme chitinase helping in the survival of the woodrotting fungi.

DISCUSSION

Ganoderma is of immense importance in the field of medical science - a potent anticancer drug (the fungus contains Beta-D-Glucan), antioxidant, possesses immunoregulatory, liver-protecting effects (Kenneth, 1992) and anti-inflammatory effects. *Ganoderma* is commonly prescribed for the treatment of chronic hepatitis in countries, such as China. The medicinal importance of *Ganoderma* has prompted researches to grow *Ganoderma* under artificial vigilance. However it was found that the medicinal properties were of greater magnitude in the wild type. Thus, proper maintenance of the wild type was considered leading to emergence of the concept of increasing the shelf life of the fungus.

Certain bacteria like *Burkholderia cepacia, Enterobacter* spp and *Pseudomonas aeruginosa* have antagonistic relationship with *Ganoderma* (Sapak *et al*, 2008.) which has to be overcome in order to ensure the fungal existence. In the present investigation, the fungal sample was intact even in the presence of bacterium producing enzyme chitinase, responsible for degradation of chitin of fungal cell wall. Biochemical tests suggested that the chitinase producing bacteria was one belonging to family *Enterobacteriaceae*. Further, the collected fungal conk was covered with a unique algal consortium which supposedly protected the fungal sporocarp from chitinase activity of the bacterium. As, viewed under the microscope, the algae was identified to be *Pandorina* sp. A widely distributed freshwater alga, *Pandorina* has cells that are ovoid or slightly narrowed at one end to appear pear shaped. It is composed of 8 cells held together surrounded by mucilage (Smith, 1920). The area of collection of sample was near a pond, i.e., a semi terrestrial habitat. The collected sample was used to isolate and study the bacterium, the algae and the fungus. The bacteria isolated from the sample showed chitinase activity as detected by chitinase enzyme assay. However, the fungal cell wall was not affected by the bacterial chitinase. Furthermore, the bacterial chitinase production was limited in the presence of the algae, i.e., a unique relationship existed between the algae and the chitinase producing bacterium which has to be investigated. The chitinase activity of the bacteria was reduced when grown together with the *Pandorina* as compared to when grown separately away from algal interference. The presence of the algal colony thus was able to increase the lifespan of the fungus in its natural environment without compromising on its medicinal properties. Thus, an antagonistic relationship between the algae and the chitinase producing bacteria was established, which could explain the fungal existence even in the presence of the bacterium producing chitinase. From our investigation it can be concluded that in the presence of a protective algal consortium, the *Ganoderma* was not damaged even when chitinase producing bacteria grew on it. This unique antagonistic relationship between the algae and chitinase producing bacteria can be employed in increasing the lifespan of medically and industrially important fungi wherein, the algae protects the fungal sporocarp against the degrading action of the bacterial chitinase enzyme.

The unique detection of algal consortium preventing bacterial invasion of *Ganoderma* opens up future studies for ultimate protection of *Ganoderma*, a very important fungus.

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