

Inhibition of Wood Rotting Fungi by Prokaryotic Consortium under saline stress

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Abstract:

The advancing sea water under high tide condition damages the *Casuarina* plantation under saline stress due to tidal waves. The fragments of wood displaced often shows wood rotting activity by terrestrial wood rotting basidiomycetes fungi. In the given investigation, such infected wood infested with fungi similar in characteristics to *Coriolus* spp.(Isolate3) was isolated but in one of the samples, the growth of prokaryotic consortium including *actinomycetes* spp.(Isolate 1) and gram negative bacteria(Isolate 2) was also observed. All these organism were grown in vitro and were found to be saline tolerant to 25%. These organisms were characterized and it was observed that the fungi could not develop a sporocarp in presence of the prokaryotic competitors. This antagonism was clearly observed when grown in tryptic soy agar. The wood degradation activity was reduced by 10 % of previous decay in presence of the actinomycetes and gram negative bacilli. So these potential organisms can be utilized in protecting *Casuarina* plantation in the sea beaches and also in wood industries for biocontrol of wood rotting fungi where woods from *Casuarina* and other trees are used extensively.

Introduction:

Casuarina is a genus of 17 species in the family *Casuarinaceae*, native to Australasia, southeast Asia, and islands of the western Pacific Ocean. They are evergreen shrubs and trees growing to 35 m tall. It is common in tropical and subtropical areas the tree has delicate slender branches and scale like leaves, looking like a conifer and planted as wind breaks. The roots of these trees have nitrogen fixing abilities and hence naturally increase the fertility of soil. Even the leaves are rich in nitrogen content and can be used for mulching. Wood industries extensively use *Casuarina* wood for building-timber, furniture, tools and also as firewood. However, fungi and other microorganisms decay wood by releasing enzyme that digests specific wood components such as cellulose, hemicelluloses and lignin. Fungi that only grow on wood but actually cause it to decay are called lignicolous fungi. White Rot Fungi degrade all the major components i.e, cellulose, hemicelluloses and lignin. *Coriolus* spp is an example of such wood rotting fungi which is found throughout the world. It is a common polypore mushroom and one of the most effective lignolytic basidiomycetes[1].

Wood decay due to fungal activity, especially basidiomycetes, is known to be a major cause of concern for the wood and timber industries throughout the world. They damage forest wood more than insects or any other microbes [2]. The chemical preservatives used in these industries are often harmful to health and environment. Biochemical and biological control of wood-rotting fungi might prove helpful in avoiding these adverse effects and at the same time will facilitate the preservation of wood and timber in wood industries and conservation of natural plantations.

Our purpose was to study the characteristics of the potential salt tolerant wood rotting fungi and other microorganisms isolated from *Casuarina* wood, collected from the Golden beach of Chennai. Salt tolerant wood rotting fungi can be characterized by their decolonization, delignification and biobleaching abilities and tolerance to sea salt.

Samples from the wood sample obtained from the seashore, when cultured, showed that it was under the action of the fungi, bacteria and actinomycetes. All these organisms were grown in vitro in and were found to be tolerant to 25% NaCl concentration. Our study also revealed a reduced rate of lignolysis by the wood rotting fungi. This is suggestive of some kind of antagonism among the microorganisms growing in the wood sample. It was observed that the fungi could not produce a rhizophore in presence of the other prokaryotic competitors. The wood degradation activity was reduced by 10 % of previous decay in presence of the gram negative bacilli & the actinomycetes. Such biological systems might be effective in preservation of wood in industries and in protecting *Casuarina* plantation near the seashores.

Materials and method:

1) Sample collection:

Casuarina wood was collected from the Golden beach located on the Bay of Bengal, India, on the way from Chennai to Cuddalore via Puducherry.

2) Isolation of pure culture:

Fungal, bacterial and actinomycetes colonies were obtained from the culture of the wood sample in tryptic soy agar. Pure culture of each of these microbes were obtained and preserved for further investigations. The actinomycetes, bacterial and fungal isolates were termed as “isolate 1”, “isolate 2” and “isolate 3” respectively.

3) Determination of salt tolerance of bacteria:

To check the maximum concentration of salt (NaCl or KCl) that supports the growth of the bacteria obtained, samples from the pure culture were inoculated in nutrient broths with varying NaCl concentrations (5%, 10%, 15%, 20%, 25%, 30%) and incubated at 37°C for 24h and checked for growth. After 24 h the O.D of the inoculated test tubes were measured and compared with that of the control to check the growth of the isolates in media containing different concentrations of NaCl. From the O.D, we were able to estimate the amount of growth of the isolates and the concentration of NaCl that inhibits bacterial growth.

4) Determination of salt tolerance of fungi:

Similarly, the salt tolerance of the fungal isolate was determined by inoculating them separately in Czapek dox agar medium with salt concentrations upto 30%, as before and then we checked the growth after 36h of incubation at room temperature.

5) Characterization of isolates:

A) Gram's staining:

Bacterial culture was taken on the centre of a grease free glass slide and a thin smear was drawn with a glass rod. The smear was air dried and heat fixed. The film was stained with crystal violet stain and kept for 30 seconds and then the extra stain was washed off by holding the slide under running tap water. Lugol's Iodine solution was added and kept for 30 seconds. The slide was then washed with alcohol-acetone solution till the violet colour flows off. The slide was washed under running tap water and then safranin stain was added and kept for 30 seconds. The extra stain was removed by holding the slide under tap water. The slide was dried and observed under low and high power of microscope.

B) Enzyme characterization:

i) Catalase:

A drop of 3% H₂O₂ was taken on a clean glass slide and with the help of a sterile glass rod growth from an isolated colony was transferred onto the drop of H₂O₂.

ii) Oxidase:

A strip of filter paper was soaked with 1% of oxidase reagent and then at once used by rubbing a speck of culture on its surface with a sterile glass rod.

iii) Nitrate reductase

● **Composition of nitrate broth: pH=7**

Beef extract -3gm.
KNO₃ - 5gm,
Peptone - 5gm.
Distilled Water =1000ml

● **Composition of nitrate reagents:**

a) Nitrate reagent A:

α-Naphthylamine=5gm,
Acetic acid =30%
Distilled Water =1000ml

b) Nitrate reagent B:

Sulfanilic acid=3gm,
Acetic acid=30%
Distilled Water =1000ml

c) Zn dust.

Sterile nitrate broth was inoculated separately with each organism and incubated in BOD incubator for 24h. After the incubation period, 1 ml of reagent A and 1ml of reagent B was added to the medium in that order. If there's no red coloration within 30 seconds, Zn dust is added.

Results:

Isolation and characterization of organisms from the sample

Bacterial colonies along with fungal and actinomycetes colonies were isolated from the wood sample, which were then selected and streaked onto nutrient agar plates to obtain pure culture for further studies (**Fig 1**). Only one of the bacterial colonies was selected -(Isolate 2) - based on maximum salt tolerance and all other experiments were performed with the same. The said bacteria showed round regular circular off-white translucent flat colonies (**Table 1B**) on nutrient agar plates while Gram staining showed that morphologically the isolated bacteria were Gram negative bacilli (**Table 2**). Isolate 2 was found to be oxidase negative and catalase and nitrate reductase positive (**Table 2**). Fungal colonies (Isolate 3) were found to be off white cottony in appearance and peroxidase positive. The fungal growth was found to be inhibited by the actinomycetes and bacterial colonies, as is evident from the zone of inhibition of fungal growth around the actinomycetes and bacterial colonies on agar plate, and the fungus failed to develop sporocarp in the presence of these microbes. The actinomycetes obtained (Isolate 1), showed similar characteristics to that of *Streptomyces* spp. when stained with Gram's stain while, the

fungus showed a pattern similar to *Coriulus* spp. under the microscope when stained with lactophenol cotton blue stain. The fungus could also grow in liquid fungal media.

Table 1A: Characteristics of fungi

Colour	Form	Peroxidase	Salt tolerance
Off white	Cottony	Positive	25% of KCl

Table1B: Colony characteristics of bacteria

Isolate	Form	Shape	Edge	Color	Opacity	Elevation
Isolate 2	Regular	Round	Circular	Off white	Translucent	Flat
unnamed	rhizoid	Round/irregular	serrate	white	opaque	flat

Table1C: Colony characteristics of actinomycetes

Form	shape	Edge	Color	opacity	elevation
rhizoid	Round/irregular	serrate	white	opaque	flat



Figure 1 – After inoculation of the *Casuarina* wood, the bacteria was isolated which was further examined salt tolerance.

Table 2: Characterization of isolate

Morphological features		Biochemical features		
Gram character	Shape of cells	Enzyme characterisations		
		Oxidase	Catalase	Nitrate reductase test
Gram negative	Bacilli	negative	positive	positive

Determination of rate of lignolysis

The degradation of the wood was found to be occurring at a slower rate than it usually does under normal conditions due to the activity of lignolytic fungi, probably, owing to the unique microbial consortium that was found to exist in the *Casuarina* wood (**Table 3**)

Table 3: Lignolysis of the wood sample

Normal lignolysis under the action of the fungus			Lignolysis in presence of bacteria and actinomycetes		
Initial weight of the wood sample	Final weight	Percentage decay	Initial weight of the wood sample	Final weight	Percentage decay
18 g	13.95 g	22.5%	18 g	14.35g	20.27%

Determination of salt tolerance of isolated bacteria

We checked the salt tolerating capacity of the isolated bacteria by inoculating the bacteria in nutrient broth media of different salt (NaCl) concentrations upto 30%. On 24hour incubation at 37°C in BOD incubator, appreciable growth was observed in 25% NaCl concentration while growth was fairly reduced in 30% (Table 4).

Table 4: Salt tolerance of the bacteria

Concentration of salt	Mean OD values observed after 24hrs	Growth after 36hrs
5%	0.440	Growth observed
10%	0.525	Growth observed
15%	0.610	Growth observed
20%	0.410	Growth observed
25%	0.031	Growth observed

Determination of salt tolerance of isolated fungi

In order to check the salt tolerance of the isolated fungi, we inoculated the fungi in Czapek-dox agar media having different salt (KCl) concentrations. Fungal growth was observed in concentrations upto 25% after 36h incubation at room temperature (Table 5)

Table 5: Salt tolerance of fungi

Concentration of salt (KCL)	Growth after 36hrs
5%	Growth observed
10%	Growth observed
15%	Growth observed
20%	Growth observed
25%	Growth observed

Discussions:

Casuarina plant, which was under constant saline stress due to the advancing sea water of the high tide waves showed wood rotting activity by the fungi that shows similarity to *Trametes* (*Coriolus*) in its characteristics. Wood rotting fungi like *Trametes* produces significant amounts of laccase and lignin peroxidase, carboxymethyl cellulase, and avicelase which plays an important role in wood degradation [3]. Degradation of wood due to wood rotting activity of fungi is a cause of concern for the wood industries. The chemical preservatives used in these industries include pentachlorophenol (penta or PCP), creosote, copper, zinc, chromium, arsenic, and other compounds as the active ingredients. These compounds are extremely toxic to human health and the environment at large. Creosote, PCP and CCA cause activated T cell autoimmunity, B cell dysregulation and functional immunosuppression in workers of wood industries[4-5]. Hence is the necessity of introducing safe and nontoxic preservatives in these industries. Biological control of wood decay is expected to prevent wood rot while reducing the health and environmental hazards of using chemical preservatives at the same time.

The major interest of our work was to isolate wood rotting microorganisms from the wood sample and determine their salt tolerance as the sample was collected from a beach with high levels of salinity. In doing so, we observed that the growth of the wood rotting fungi was being restricted by the growth of actinomycetes and bacterial colonies on tryptic soy agar plates. The comparative study of normal lignonolysis under the action of fungi and lignonolysis in presence of bacteria and actinomycetes showed that the latter resulted in lesser percentage decay of the wood.

It has been observed in several studies that certain actinomycetes and bacterial species have an antagonistic effect on the growth of different wood rotting fungi. The most effective biocontrol

antagonists belong to genera *Gliocladium*, *Bacillus*, *Pseudomonas* and *Streptomyces*[6-14]. Antibiosis and mycoparasitism are the major mechanisms by which the biological control of wood decay might be achieved. Biochemicals including enzymes that degrade fungal cell wall (β -1,3-glucanases ,chitinases), siderophores, chelating iron and a wide variety of volatile and non-volatile antibiotics are probably responsible for such antagonistic activity. Competition for an ecological niche, consumption of available nutrients might also be another mechanism of inhibition of the fungal pathogens.

Thus it can be concluded that the presence of bacterial species and actinomycetes might have an antagonistic effect in the growth and wood-rotting activity of white rot fungi, and this findings can be effectively applied for large scale preservation of wood without any damage to health and environment.

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