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Use of Bioprotectant Against Fungal Deterioration of Rubber Wood
(Final report of project KFRI 461/2004)

E.J. Maria Florence
M. Balasundaran

Kerala Forest Research Institute
(An Institution of Kerala State Council for Science, technology and Environment)
Peechi – 680 653, Thrissur, Kerala, India
KFRI

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CONTENTS

PROJECT PROPOSAL

Project No.	KFRI 461/04
Title	Use of Bioprotectant Against Fungal Deterioration of Rubber Wood
Principal Investigator	E.J Maria Florence
Associate	M. Balasundaran
Project fellow	K.L Sajitha
Objectives	1. Isolation of various microorganisms (fungi, bacteria and actinomycetes) antagonistic against the common fungal growth on rubber wood and other softwood species 2. Laboratory evaluation of different biocontrol organisms against major wood degrading organisms 3. Testing the efficacy of different bioprotectant during different seasons 4. Extraction and characterization of the antagonistic principle produced by the biocontrol organism
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ABSTRACT

Rubber wood is one of the widely used timber in wood-based industries in Kerala. One problem of utilization of rubber wood is its susceptibility to moulds, sapstain and decay fungi, and insect borers. Among various fungi, *Botryodiplodia theobromae* Pat. is the dominant fungus causing sapstain on rubber wood. Among the several microorganisms screened, a bacterium, *Bacillus subtilis* B2 and an actinomycete *Streptomyces* sp. SA18 were identified as possible bioprotectants against sapstain caused by *B. theobromae*. The inhibition of *B. theobromae* by the *Streptomyces* sp. SA18 was by producing chitinase enzyme and thereby degradation of the fungal cell wall chitin. The antibiotic, Iturin A produced by *B. subtilis* B2 was also inhibiting the fungal growth. When compared to actinomycete, the bacterium was more effective against the sapstain fungus in the field. Open stacking reduced the fungal infection than close stacking.

1. Introduction

The demand for timber is increasing day by day and one way of achieving this growing demand is to use timber species from sources other than forests. Due to scarcity of wood, various perishable timber species are currently being utilized as a substitute for conventional species.

Rubber wood is one of the most important resources that is currently in great demand as raw material for many wood-based industries in Kerala. India is the fourth largest natural rubber producing country in the world. The current average production of rubber wood/ha is 150 and 180 m^3 in rubber smallholdings and estates respectively. Kerala State accounts for 86 per cent of the total area under rubber cultivation (Mannothra, 1993; George and Joseph, 1993). After the extraction of latex, the wood is mainly used for making plywood, packing cases, low cost furniture, etc. However, the susceptibility to various biodegrading agents such as borers and microorganisms is a serious problem for the successful utilization of this timber.

The hot and humid climate offers a congenial environment for luxuriant growth of fungi in Kerala. Fungi cause different types of damages to wood such as decay, soft rot, moulds and sapstain/blue stain. Sapstain fungi, the primary colonizers of freshly felled lumber are a great concern in timber industry, which has attracted wide attention during the last few years.

Protection of wood and wood products from fungal deterioration is achieved mainly through chemical wood preservatives. The health hazards and environmental pollution caused by the application of various wood preservatives have brought out the necessity of adopting new approaches in the field of wood protection. In order to reduce the use of chemical wood preservatives, one alternative method is to adopt biological control measures employing antagonistic microorganisms. In agriculture, biological control is well

established to prevent pests (Jatala, 1986; Tian *et al*., 2007) and plant pathogens (Weller, 1988; Pal and Gardener, 2006). The present investigation was to evaluate suitable biological control agents for prevention of sapstain on rubber wood.

2. Review of literature

Wood deterioration is mainly due to the activity of biotic agents like microorganisms and insects. The decay and staining caused by fungi and infestation by insects are the main causes of timber loss. Fungi are unique organisms, which penetrate, invade, digest and absorb soluble constituents from complex wood tissues with the help of enzymes (Zabel and Morrell, 1992).

Wood inhabiting fungi are classified into three categories depending on the type of damage such as moulds and stains, decay and soft rot (Subramanian, 1983). Of these, staining due to superficial moulds and internal discoloration by sapstain fungi is the most serious problem in humid tropics as well as temperate climatic regions. Zabel and Morrell (1992) reported that fungal stains are world wide in distribution and sapstain becomes a serious problem when wood with high sapwood content is harvested in humid conditions, which is favourable for fungal growth.

Kaarik (1980) compiled a useful comprehensive list of all staining fungi. *Ceratocystis spp*. was the dominant stain fungi in the temperate regions (Kaarik, 1980; Miller and Goodell, 1981). In tropical hardwoods, the staining is caused mainly by the *Diplodia* sp. and in particular *Botryodiplodia theobromae* Pat. (Cartwright and Findlay, 1958; Olofinboba, 1974; Florence and Sharma, 1990).

Many reports were published based on the sapstain infection of *Botryodiplodia theobromae* on various tropical woods like poplar (Pinheiro, 1971), jelutong

(Hong, 1976) and pines (Masuka, 1991). Rubber wood, which is one of the major alternatives for the conventional timber resources utilized for the manufacture of furniture, *Botryodiplodia theobromae* is the main threat (Hong *et al*., 1980; Sujan *et al*., 1980; Florence and Sharma, 1990).

There is an urgent need to protect the timber from the blue stain fungi. Usually chemical fungicides are used to protect colonization of wood and wood products from sapstain fungi, but some of these may have considerable adverse health hazards (Dickson, 1980). Little research has been conducted on the biological control of sapstain fungi. Microorganisms such as fungi and bacteria, including actinomycetes were tried as biocontrol agents against various wood inhabiting fungi including sapstain fungi.

Croan and Highley (1991, 1996) reported the antagonistic effect of various brown rot and white rot fungi against the sapstain fungus *Ceratocystis coerulescens*. The efficiency of albino strains of *Ophiostoma* spp. and nonpigmented isolates of *Ceratocystis* sp. as biological control agents against sapstain fungi on wood was studied by Croan (1996), White-Mcdougall *et al.* (1998) and Held *et al.* (2003). The studies of Payne and Bruce (2001) showed the antagonistic property of the yeast *Debaryomyces hansenii* against the common wood inhabiting moulds and blue stain fungi. Effect of the *Trichoderma* against the decay and sapstain infection on wood (Highley and Ricard, 1988; Brown and Bruce, 1998) and the antagonistic volatiles produced by *Trichoderma* (Bruce *et al.,* 2000) were investigated previously.

Reports of Seifert *et al. (*1987); Benko and Highley, (1990); Kim *et al. (*2003); Feio *et al. (*2004) and Motia *et al. (*2005) have revealed the antagonistic ability of *Bacillus subtilis* against various stain and decay fungi. The effects of volatiles from various bacteria and yeast on staining fungi were reported by Fiddaman and

Rossall, (1993) and Payne *et al. (*2000) and Bruce *et al. (*2003, 2004). The significance of various substrates on the production of antifungal volatiles by *Bacillus subtilis* was studied in detail by Fiddaman and Rossall (1994). The antifungal properties of the volatiles produced by *Streptomyces* against various fungi were discussed in detail by Gupta and Tandon (1977) and Herrington *et al.* (1987).

The detailed report on various antibiotics and control of antibiotic biosynthesis (Martin and Demain, 1980) has given a clear idea about the antibiotic production from *Bacillus subtilis* (Rossall, 1991). Gueldner *et al. (*1988) and Gong *et al. (*2006) has observed the antifungal ability of *Bacillus subtilis* and the extraction and identification of its secondary metabolite Iturin A using HPLC. The isolation and characterization of various antibiotics from *Bacillus subtilis* was conducted by Besson and Michel (1987), Vanittanakom and Loeffler (1986), Mhammedi *et al.* (1982). The antibiotic properties of various lipopeptides from different bacterial strains were tested by Eshita and Roberto (1995) and Neilsen *et al. (*2002).

The efficiency of *Streptomyces* spp. as biocontrol agent was studied well by Croan and Highley (1992, 1994), Yuan and Crawford (1995) and Croan (1997). The chitinolytic activities of actinomycetes on fungal cell wall were experimented by Beyer and Diekmann (1985) and Gomes *et al. (*2000). The purification and antifungal activity of chitinase was explained by Wang and Chang (1997), Vaidya *et al. (*2001), Gomes *et al. (*2001), Harjono and Widyastuti (2001) and Hoster *et al.* (2005). Trudel and Asselin, 1989; Zou *et al.* (2002) and Gohel *et al.* (2005) have reported the staining method on chitin agar plate for the detection of chitinase. Saito *et al.* (1998) has described how glucose inhibits the chitinase production in *Streptomyces lividans*.

The literature search on biological control of sapstain using bacteria and actinomycetes suggests that there is ample scope for finding out suitable biological control organisms for preventing the growth of stain fungi on rubber wood.

3. Materials and Methods

3.1. Isolation and identification of sapstain fungi

In order to isolate the sapstain fungi, the stained rubber wood pieces were collected from various sawmills in Thrissur. Small pieces of stained wood were surface sterilized and inoculated on Potato Dextrose Agar (PDA) medium and incubated at 28 $\pm$ 2 ^{o}C for 7 days. The fungal cultures grown from the wood pieces were isolated and maintained on PDA slants for further studies.

The dominant fungal cultures isolated from different rubber wood pieces were tested for their ability to stain the fresh rubber wood. Rubber wood blocks (7 X 5 X 1cm), steam sterilized at 15 lb/in^2 for 20 minutes were used for testing in the laboratory. An 8 mm dia disc taken from the edge of an actively growing culture of the dominant stain (*Botryodiplodia theobromae)* was placed aseptically over each wood block and the Petri plates were sealed and incubated at room temperature for 2 weeks. After the incubation period, the wood blocks were observed for staining. Each wood block was split open to see the internal stain.

3.2. Isolation of test microorganisms

Different sources such as compost, soil and rubber wood were selected for isolating various antagonistic microorganisms. Soil Extract Agar, Starch Casein Agar and Rose Bengal Agar were used to isolate bacteria, actinomycetes and fungi respectively. Isolation was done by serial dilution and spread plate method and incubated for microbial growth. All isolated microbial cultures were purified and maintained in respective media for further studies.

3.3. Screening microorganisms for antagonism

For testing the antagonistic ability, microbes isolated using dilution plate techniques from different source materials were tested individually using agar plate assay (Johnson and Curl, 1972) against *Botryodiplodia theobromae* in pure culture. In the first method, the potential antagonistic microbe was streaked at the periphery of the Petri plate having PDA medium and a 6 mm dia disc taken from the edge of an actively growing culture of *B. theobromae* was placed on the opposite side of the streaked microbe and incubated at 28 $\pm$ 2^{o}C for 7 days. In the second method, the antagonist was placed at three positions on the PDA plate and *B. theobromae* was inoculated in the center. Microorganisms, which inhibited the growth of the test fungi by developing the inhibition zone, were observed and sub cultured for future studies. The inhibition zone developed between the cultures was measured.

3.4. Testing the antagonistic property of selected isolates

To understand the effectiveness of the antagonists, the selected isolates were tested against another important sapstain fungus on temperate timbers such as *Ceratocystis fimbriata*. The antagonistic ability was also tested against common decay fungi such as *Trametes hirsuta, Trametes versicolor* and *Pycnoporus sanguineus* and common pathogens such as *Colletotrichum gloeosporioides, Fusarium solani*, *Cylindrocladium quinqueseptatum*, *Phomopsis* sp. and *Phoma* sp. For testing, the test fungi were placed in the center and the antagonists inoculated at three places on the PDA plate.

3.5. Antifungal property by producing volatiles

Antifungal property of volatiles produced by the effective antagonistic bacteria and actinomycetes against the stain and decay fungi were studied using dual sealed plate technique. In this technique, the selected antagonistic bacteria were grown in nutrient agar for 2 days and actinomycetes in Starch Casein Agar for

one week. After removal of the Petri dish lids, plates of freshly inoculated test fungi were inverted over the plates that contained the grown antagonistic actinomycetes and bacteria. Controls were also maintained using plates inoculated with test fungi except that the bottom plate contained no actinomycetes and bacteria. Five replicates were maintained for all tests and controls. All dual plates were sealed using cling film and incubated at 30˚C until the growth of the test fungus in the controls had reached the edge of the plate.

3.6. Antagonistic activity against *B. theobromae* in liquid culture

The effect of the antagonists in liquid media was studied using broth culture. A loop full of the bacterial antagonist was inoculated into the nutrient broth and incubated for 48 hours. To the bacterial culture an 8 mm dia disc taken from the edge of an actively growing culture of *B. theobromae* was inoculated and incubated at 28 $\pm$ 2 ^{o}C for two weeks. For growing the actinomycete, a loop full of the actinomycete was inoculated into starch casein broth and incubated at 28 $\pm$ 2 ^{o}C. After one week the test fungus of 8 mm dia disc was inoculated into the broth and incubated for two weeks. Controls were also maintained without inoculating the antagonistic microbes. The growth of *B. theobromae* in both liquid media was observed.

3.7. Effect of culture filtrate on the growth of *B. theobromae*

To test the antagonistic ability using culture filtrate, the selected bacteria were grown in nutrient broth and the actinomycete in starch casein broth for 2 weeks. The following two methods were adopted for testing the antagonistic ability.

Well method: The antagonistic activity was tested directly after removing the potential antibiotic producing organism. The crude culture filtrate was concentrated through 5000 cut off Amicon ultra membrane filter. The concentrated culture filtrate was filtered using Millipore (0.22µm) filters. To check the antifungal activity of the extract, 0.5 ml of the filtrate was poured into

the well (10 mm) made in Potato dextrose agar medium using a cork borer. The test fungus, *B. theobromae* was inoculated on the opposite side of the well at the periphery of the Petri plate. The plates were incubated for one week and the growth of the fungus observed. Controls adding sterile distilled water in the well were also maintained.

Mixing of culture filtrate in growth medium: The filter sterilized culture filtrate (5 ml) was mixed with 15 ml of molten PDA medium at bearable warmth. After solidifying the agar, a 6 mm dia disc of *B. theobromae* was inoculated in the centre of the Petri dish and incubated for one week at room temperature.

3.8. Selection of the antagonist

Among the different antagonists screened based on the agar assay, volatile production and culture filtrate assay, two efficient microbes (one bacterium and one actinomycete) were selected for further studies.

3.9. Microscopic examination of the fungal hyphae

The reaction of the test fungus against the efficient antagonists was studied by observing the fungal mycelium microscopically from the inhibition zone. The mycelium of the test fungus from the inhibition zone of both the antagonists was stained in Cotton Blue and observed under light microscope.

3.10. Biochemical tests

Various biochemical tests were carried out for genus level identification of the selected actinomycete and the bacterium.

Carbohydrate fermentation: Fermentative degradation of various carbohydrates by the two selected antagonists under anaerobic condition is carried out in a fermentation tube (a culture tube containing Durham's tube) for the detection of

gas production at the end of the reaction. The fermentation broth contains ingredients of nutrient broth along with a specific carbohydrate (glucose, sucrose, lactose etc.) and a pH indicator (Phenol Red), which is red at neutral pH and turns yellow below a pH of 6.8 due to the production of an organic acid.

Starch hydrolysis: This is for testing the production of amylase enzyme by the microbe. Both antagonists were grown in the starch agar medium for 24 hours in a Petri dish. Gram's iodine was added to the Petri dish having the microbial growth. Iodine binds with the starch and give deep blue coloration. If the amylase enzyme degrades starch that portion appear colorless.

Gelatin hydrolysis: Hydrolysis of gelatin is carried out to understand the capability of the antagonistic microorganisms to produce a proteolytic exoenzyme such as gelatinase. The bacterium and the actinomycete were grown in nutrient gelatin medium. Degradation of gelatin was detected by observing liquefaction even at low temperature.

Casein hydrolysis: Casein agar medium is opaque due to the casein in colloidal suspension. Casein agar medium was prepared in Petri plates and the microbes were grown for one week and results were observed. Formation of a clear zone adjacent to the growth of the microorganism in the agar medium is an evidence of casein hydrolysis.

Carboxymethyl cellulose (CMC) hydrolysis: The test is to understand the production of cellulase by the antagonists. CMC agar is used for inoculating the microbes. This test deals with assaying the production of CMCase (endoglucanase), an extracellular enzyme. Evidence for the microbial utilization of CMC was detected by a dye, Congo Red. Congo Red will bind to the intact CMC and will stain red. The portion where CMC was degraded appeared as colorless.

Hydrogen sulfide production test: The hydrogen sulfide production can be detected by incorporating a heavy metal salt containing (Fe_2+) ion as H_2S indicator to a nutrient culture medium of the antagonists containing cysteine and sodium thiosulfate as the sulfur substrates. H_2S when produced reacts with Fe_2+ forming visible insoluble black ferrous sulfide precipitates.

Indole production test: This test is performed by inoculating the antagonists into nutrient broth containing tryptone (10g/l) broth; the indole produced during the reaction can be detected by adding Kovac's Reagent, which produces a cherry red layer.

Methyl Red and Voges-Proskauer tests: Both these tests are performed simultaneously because they are physiologically related and are performed on the same MR-VP broth. The antagonists were inoculated into MR-VP broth and incubated for 24 h. In MR test if an organism produces large amount of organic acids from glucose, the medium will remain red (positive result) after the addition of methyl red, a pH indicator. In others, Methyl Red will turn yellow. In the VP test after the addition of VP reagent 1 and 2 to the culture, development of ruby pink colour indicates the positive VP test.

Citrate utilization tests: This test was performed by inoculating the antagonists on an organic synthetic medium, Simmon's citrate agar. Bromothymol blue is used as an indicator. When the citric acid is metabolized, the CO_2 generated combines with sodium and water to form sodium carbonate, which changes the colour of indicator from green to blue, which is a positive result.

Urease test: Urease is a hydrolytic enzyme, which attacks the carbon and nitrogen bond amide compounds (urea) with the liberation of ammonia. The test is conducted by growing both the bacteria and actinomycete on urea agar

medium with pH indicator as phenol red. As the pH increases due to production of ammonia the colour changes from yellow to pink. This is a positive indication of urease production.

Catalase test: Catalase breaks down the lethal H_2O_2. Catalase test is performed by adding H_2O_2 to grown culture on agar slant. Release of free oxygen is a positive catalase test.

Oxidase test: The test is done by placing grown antagonistic cultures on oxidase discs. Development of pink, then maroon and finally black colouration on the microbial colonies indicates the positive result.

Triple sugar iron agar: The test carried out to identify the fermentation of lactose, sucrose and glucose. It is used to detect the H_2S and gas producing ability of the microbes. The selected antagonists were streaked on agar slants and inserted to the butt portion of the media. The cultures were incubated for two days at $28\pm2^{o}C$. The colour change of the slant and butt portion indicates the fermentative capacity of the microbes. Bubbles and dark colouration in the medium denotes gas and H_2S production.

3.11. Identification of the selected actinomycete (SA18)

The genus level identification of the actinomycete was done using morphological characters under microscope. The selected actinomycete SA18 was streaked on agar media and a cover slip was inserted under the streaked portion so that the culture would grow over the cover slip. After two weeks this cover slip was taken out and observed under microscope.

3.12. Molecular characterization of the actinomycete (SA18)

*Genomic DNA preparation (*Ausubel *et al.,* 1994*):* Actinomycete culture was grown to saturation and spun 1.5 ml for 2 minutes at 3000 rpm in a microcentrifuge.

Resuspended the pellet in 567µl TE buffer, 3µl of 20 mg/ml proteinase k, 10µl RNase, mixed well and incubated at 37˚C for one hour. After incubation 100µl of 5M NaCl and 50µl CTAB /NaCl solution was added, mixed thoroughly and incubated at 65˚C for 10 minutes. To this added equal volume of chloroform: isoamylalcohol (24: 1), spun at 5000 rpm for 5 minutes. The aqueous phase was transferred to a fresh tube and equal volume of chloroform was added, spun at 5000 rpm for 5 minutes. Again the aqueous phase was transferred and the DNA was extracted with 0.6 volume of isopropanol. Kept at -20˚C overnight and centrifuged at 5000 rpm for 5 minutes. Washed the precipitate with 70% alcohol, drained the supernatant and air-dried the pellet. The pellet was dissolved in sterile double distilled water and stored at -20˚C for further use.

***Amplification of the 16S rRNA of SA18*:** To characterize the SA18 strain, the small ribosomal RNA unit was amplified by PCR using the conserved primers (forward StrepB 5'- ACAAGCCCTGGAAACGGGGT 3' and reverse Strep F 5'- ACGTGTGCAGCCCAAGACA 3') (Rintala *et al.,* 2001). The PCR reaction mixture (25µl) was prepared by mixing the following components. 15-30 ng of template DNA, 0.05 µl of 0.2 µmol/l of each primers, 200µM of each dNTPs, 5µl of 5XHF buffer, 0.02u/ µl of Phusion DNA polymerase and 17.4 µl of sterile distilled water. Amplification was carried out as follows – a preliminary denaturation step was done at 98˚C for 5 min, followed by 30 cycles of denaturation (1 min at 95˚C), primer annealing (40 s at 58˚C) extension (2 min at 70˚C). After 30 cycles, a final extension of 10 minutes at 72˚C was done. The PCR product was checked by electrophoresis.

Sequencing of amplified portion: The identification of the species was further confirmed by sequencing. The amplified 16S rDNA was sequenced at MWG BIOTECH, Bangalore. Out of 1070 bp, a partial sequencing of 990 bp was done.

3.13. Characterization of the active principle produced by actinomycete (SA18)

The microscopic examination of the mycelia from the zone of inhibition emphasized the presence of an inhibitory metabolite, which is responsible for the malformation of fungal mycelia. Several reports indicated that the actinomycetes produce chitinase enzyme, which is inhibitory to the chitin in the fungal cell wall. Therefore tests for chitinase production were carried out.

Preparation of substrates - colloidal chitin (Hsu and Lockwood, 1975)*:* Crude chitin was treated with conc. HCl in the ratio 1:10 (w/l) and the mixture was stirred well for an hour and cold water was added to the mixture. To change the pH of the acidic colloidal chitin mixture, it was washed with several changes of water until the pH became 6. The chitin was collected by filtering, dried and used for detecting the chitinase activity of the culture and culture filtrate.

Reduction of turbidity in colloidal chitin broth: A loop full of the selected actinomycete (SA18) was inoculated into a conical flask containing 100 ml of the colloidal chitin broth (0.5% colloidal chitin, 0.02% yeast extract, 0.07% KH_2PO_4, 0.03% K_2HPO_4, 0.4% NaCl, 0.05% $MgSO_4$, 0.001% $FeSO_4$, 0.0001% $ZnSO_4$ and $MnSO_4$) with pH 7 and incubated at 37˚C for two weeks at 100 rpm.

Plate assay for chitinase production: To check the chitinase production a 6 mm dia disc of the actinomycete was inoculated in the center of the Petri dish containing colloidal chitin agar medium containing 0.5% colloidal chitin and incubated at 30˚C for two weeks. The plate was then stained with the dye, Calcofluor white (0.01 %) and viewed under ultra violet light.

3.14. Identification of the selected bacterium (B2)

Gram staining: A loopful of the selected bacterial culture grown for 24 hours in nutrient agar was taken and a uniform smear was made on a clean slide. The

heat fixed smear was covered with crystal violet stain for 30 seconds. The slide was washed and flooded with Gram's iodine solution. After 30 seconds the slide was washed and counter stained with saffranine solution for 30 seconds. After drying it was observed under microscope.

3.15. Molecular characterization of the selected bacterium (B2)

***Genomic DNA preparation from B2**:* The genomic DNA preparation of B2 was done adopting the protocol of Ausubel *et al.,* (1994) described in section no. 3.12.

***16S rRNA amplification of bacterial DNA**:* To characterize the bacterial strain, the small ribosomal RNA unit was amplified by PCR using the conserved primers (forward 8F-F 5' AGTTGATCCTGGCTCAG 3' and Reverse 1492 – R 5' ACCTTGTTACGACTT 3') (Sacchi *et al.,* 2002). The PCR reaction mix (25µl) was made by mixing the following components. 15-30 ng template DNA, 0.5µM of each primer, 200µM of each dNTPs, 5µl of 5XHF buffer, 0.02u/µl of Phusion DNA polymerase and 17µl of sterile distilled water. Amplification was carried out as follows: A preliminary denaturation step was done at 98˚C for 30 s, followed by 35 cycles at 98˚C for 8 s (denaturation), 20 s at 40.4˚C (annealing) 25 s at 72˚C (extention) after 35 cycles, a final extention of 10 minutes at 72˚C was done. The PCR product was checked by electrophoresis. The product was sequenced at MWG BIOTECH, Bangalore for species identification.

3.16. Antifungal antibiotic assay of bacterial culture filtrate (B2)

To check the antifungal activity of the bacterial extract, 1 ml of the filter sterilized bacterial filtrate was poured to a well bored in the PDA medium. An 8 mm dia mycelial disc taken from the edge of an actively growing culture *of B. theobromae* was inoculated at the opposite side. The plate was incubated at 28° C for one week for observing the antagonism.

3.17. Extraction of antifungal antibiotics of bacterium (B2)

The bacterium was grown in 100 ml chemically defined medium (2% glucose, 3% peptone, 0.5% yeast extract, 0.1% KH_2PO_4, 0.05% $MgSO_4$, 0.01% $CaCO_3$, 0.01% NaCl) at 37°C, 100 rpm for 7 days. After incubation, the culture was centrifuged at 12000 rpm for 15 minutes at 4°C. The cell free culture broth was acidified by HCl to lower the pH to 2. The precipitate was collected by centrifugation and the solid was air dried overnight. It was then extracted with ether for 6 h in a soxhlet extractor to remove nonpolar biologically inactive impurities. The remaining solid was dissolved in methanol and the solution was chromatographed on C-18 reversed-phase absorbent column.

Chemicals and solvents: Authentic sample of Iturin A from Sigma Aldrich, US and methanol, water and acetonitrile of HPLC grade from Merck were obtained.

HPLC Analysis: Sample was analyzed by reversed-phase C-18 HPLC. Chromatography was performed on a SCL-10AVP controller (Shimadzu) equipped with a SPD-M 10AVP photo diode array detector (Shimadzu). The column used was CTO-10 ASVP (Schimadzu), Luna 5u C18 (2) 100A, 250 X 4.6mm. The mobile phase was 65% water and 35% acetonitrile. The flow rate was 0.75 ml/minute. The range of detection wavelength was set from 190 to 800 nm.

3.18. Testing the antagonists in the laboratory

Fresh rubber wood blocks (7 X 5 X 1cm), steam sterilized at 15 lb/in^2 for 20 minutes were used for testing in the laboratory. The bacterial culture (B2) grown in nutrient broth and actinomycete (SA18) in Yeast Glucose Broth for two weeks were used. The wood blocks were dipped in the culture (10^7 cfu/ml) for 10 minutes, drained and kept in sterile Petri dish with a moist filter paper. An 8 mm dia disc taken from the edge of an actively growing culture of *B. theobromae* was placed aseptically over each wood block; the plates were sealed and incubated.

For testing the microbes, 25 wood blocks each were maintained for both the cultures. Wood blocks dipped in sterile water and inoculated with the test fungus were kept as control and incubated at room temperature. After the incubation period of one month the wood blocks were observed for the fungal growth.

3.19. Testing the culture filtrates of the antagonists

The liquid culture of both bacterium and actinomycete were filtered using Millipore filters. Rubber wood blocks (7 X 5 X 1cm) were dipped in culture filtrate of both microbes separately and kept inside sterile Petri plates and inoculated with the test fungus. The plates were incubated for two weeks and observed for the fungal growth. For each culture filtrate 10 wood blocks were maintained. Controls dipped in sterile distilled water were also maintained.

3.20. Testing the antagonists in the field

Testing of both the bacterium and the actinomycete was conducted separately in the field during the northeast monsoon and bacterium alone was tested in southwest monsoon season. The medium used for preparing the bacterial culture was nutrient broth and yeast glucose broth for actinomycete.

The field-testing was done during the month of November (north east monsoon) and July (south west monsoon) at Evershine Packing Industries, Ollur, Thrissur. Fresh rubber wood planks of 500 X 100 X 15 mm thickness were used. After removing the sawdust, wood planks were dipped in the bacterial and actinomycete culture (10^7 cfu/ml) for 10 minutes. The excess solution from the wood planks was drained by keeping them in slanting position and stacked closely and openly. For both seasons, the same pattern of stacking was followed.

Close stacking: In a single stack, there were a total of 30 planks. For close stacking 3 replicate stacks were maintained for both treatment and control. In each stack, one row consisted of 5 planks and in each stack there were six rows.

Open stacking: Open stacking was made in such a way that a space of 50 mm was left in between planks. All the planks were arranged in criss-cross pattern. The control planks were also stacked in the same manner. Observations for the growth of fungi in both the stacks were recorded after one month. All the planks were arranged on the floor under a shed. At the end of the treatment period, the planks were observed for fungal growth on the surface and assessed visually using the rating index given in Table 1. The planks were planed for observing the internal stain.

Table 1. Rating index for mould/stain/decay

Rating	Mould/stain & decay
0	No fungal growth
1	<10%
2	10-25%
3	26-50%
4	51-75%
5	76-100%

4. Results

4.1. Isolation and identification of sapstain fungi

Several fungi were isolated from the stained wood pieces collected from various sawmills. *Botryodiplodia theobromae* was the dominant stain fungus growing from all the wood blocks. (Fig. 1a). The surface of the inoculated wood blocks was bluish black in colour after two weeks. The internal portions of the blocks were also black in colour (Fig. 1b).

Fig. 1a. Culture of *B. theobromae*

Fig. 1b. Stained rubber wood

4.2. Isolation of test microorganisms

The microorganisms isolated for screening against *B. theobromae* from various source materials include: 30 actinomycetes, 10 bacteria and 16 fungi from compost, 14 actinomycetes, 5 bacteria and 7 fungi from rubber wood and 9 actinomycetes, 4 fungi and 9 bacteria from soil.

4.3. Screening microorganisms for antagonism

Inhibition of growth of *B. theobromae* by the microorganism is observed in both methods of inoculation (Figs. 2a, 2b, 3a & 3b). It is observed from the figures that

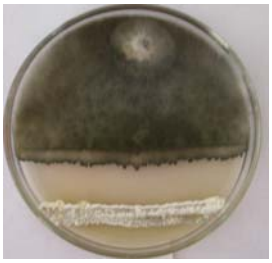


Fig. 2a.Actinomycete at the periphery

Fig. 3a. Actinomycete at three portions

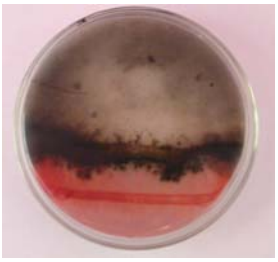


Fig. 2b. Bacteria at the periphery

Fig. 3b. Bacteria at three portions

the fungus *B. theobromae* could not grow towards the test microbe thus leaving a space in between the two cultures indicating the antagonistic nature of the isolates.

Among the different microbes tested 11 actinomycetes, 3 bacteria and 1 fungus showed various degrees of antagonism. The inhibition zone developed in between two cultures was measured (Table 2). The maximum inhibition zone was observed in the culture SA4. Three actinomycetes isolated from soil (SA4, SA14, and SA18) and 2 bacteria isolated from compost

Fig. 4. Effective antagonists
Upper 3 Cultures: Actinomycetes
Lower 2 cultures: Bacteria

(B2, B8), which had shown inhibition zone of > 73 mm were used for the further studies (Fig. 4).

Table 2. Inhibitory zone produced by various microorganisms against *B. theobromae*

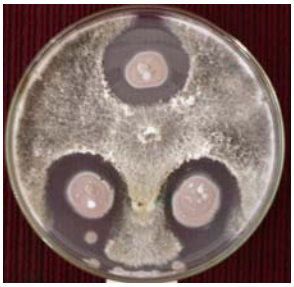
Sl. No	Cultures tested	Width of inhibition zone (mm)
1	CA1*	53
2	CA3	70
3	CA4	60
4	CA10	63
5	CA15	30
6	CA19	40
7	CA38	50
8	SA18*	100
9	SA19	53
10	SA14	80
11	SA4	120
12	B2*	90
13	B8	73
14	B10	20
15	F20*	69

CA- Actinomycete from compost; SA - Actinomycete from Soil; B - Bacteria; F - Fungi

4.4. Testing the antagonistic property of selected isolates

Figs. 5 a-f show the inhibition of different fungi due to antagonism by the selected microorganisms. All the bacterial and actinomycete isolates were very effective in inhibiting the growth of sap stain fungi, *Ceratocystis fimbriata* and pathogenic fungi such as *Cylindrocladium quinqueseptatum*, *Phomopsis* sp., *Fusarium solani*, *Phoma* sp., and *Colletotrichum gloeosporioides*.

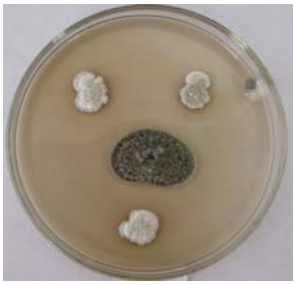
a. *Cylindrocladium quinqueseptatum* and antagonist SA14



b. *Phomopsis* sp. and antagonist SA18

c. *Ceratocystis fimbriata* and antagonist B2

d. *Fusarium solani* and antagonist SA18



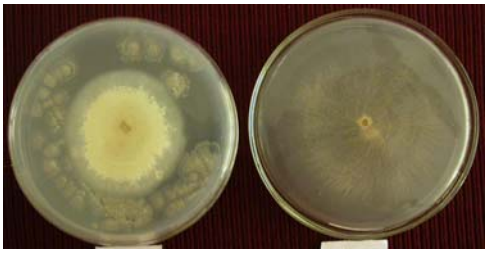
e. Phoma sp. and antagonist SA14

f. *Colletotrichum gloeosporioides* and antagonist B8

Fig. 5 Inhibition of fungal growth by the selected antagonists

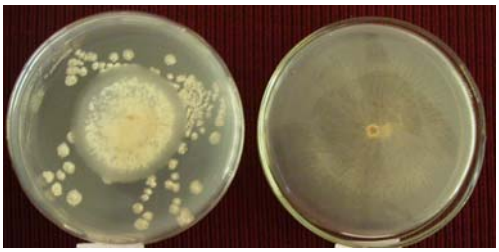
4.5. Antifungal property by producing volatiles

The inhibition of the growth of the decay fungi such as *Trametes hirsuta* and *T. versicolor* by the volatiles produced by the antagonists SA4 and SA18 is clearly seen in the Figures 6a & 6b. Not much inhibition of the growth of the test fungi was observed in the plate inoculated with actinomycete SA 14. Among the three, actinomycete SA 4 was more effective in inhibiting the growth of decay as well as stain fungi by producing volatiles. In the plates inoculated with bacterial isolates, no inhibition of fungal growth was observed assuming that bacteria are not found to produce volatiles.



Decay fungi and SA18 Control

Fig. 6a. Inhibition of *T. hirsuta* due to volatiles produced by actinomycete SA18



Decay fungi and SA4 Control

Fig. 6b. Inhibition of *T. versicolor* due to volatiles produced by actinomycete SA4

4.6. Antagonistic activity against *B. theobromae* in liquid culture

The growth inhibitory activity of the antagonists was seen in Figs. 7a & 7b. In

Treated broth Control

Fig 7a. Inhibition of *B. theobromae* in broth by bacteria B2

Treated broth Control

Fig. 7b. Inhibition of *B. theobromae* in broth by actinomycete SA18

the treated broths the test fungus could not grow whereas in the control the fungus grew luxuriantly and the liquid culture became black in colour.

4.7. Effect of culture filtrate on the growth of *B. theobromae*

Well method: In the Figure 8a, the inhibition of fungal growth was observed. *B. theobromae* could not grow near the well filled with culture filtrate

Fig. 8a. Inhibition of *B.theobromae* due to culture filtrate of SA18 poured inside the well (left) Control (right)

of actinomycete SA18, whereas in the control plate it was growing and covering the entire surface within one week. Clear zone of inhibition was also observed around the well indicating that the growth was arrested due to the inhibitory activity of the culture filtrate. No inhibition of the growth of the fungus was observed in the culture filtrate extracted from SA 4.

Mixing of culture filtrate in growth medium: Results indicated that B. *theobromae* could not grow in the plates mixed with the culture filtrates due to the antagonistic activity whereas in control plates the fungus grew profusely (Figs. 8b & 8c).

Fig. 8b. Inhibition of *B.theobromae* by the culture filtrate of actinomycete mixed with the medium

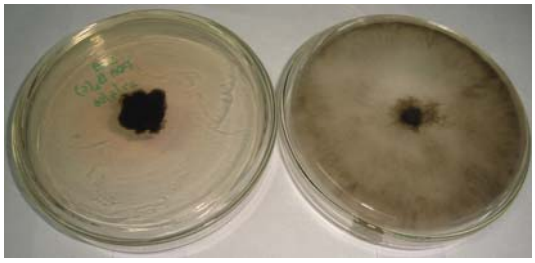


Fig. 8c. Inhibition of *B. theobromae* by the culture filtrate of bacterium mixed with the medium

4.8. Selection of the antagonist

After ascertaining the efficacy of all antagonists using agar plate assay, liquid culture assay, volatile production and culture filtrate assay, a bacterium (B2) and an actinomycete (SA 18) showing the maximum inhibition in all the tests were selected for further studies.

4.9. Microscopic examination of the fungal hyphae

The microscopic study in the zone of inhibition of the effective antagonists revealed that many portions of the hyphae were malformed. Hyphal lysis and bulging of mycelia was observed. (Figs. 9b & 9c).

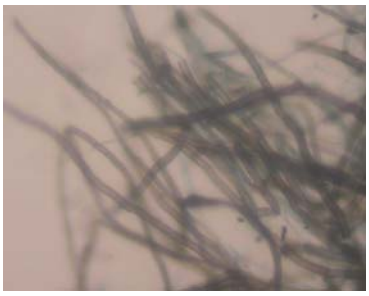
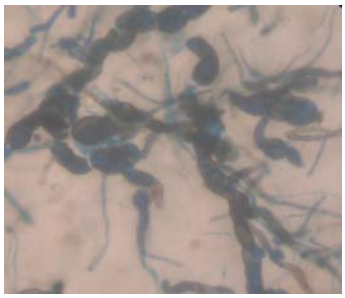
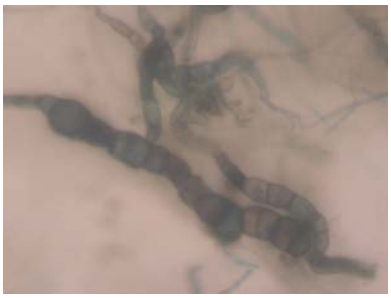


Fig. 9a. Normal mycelia





Figs. 9b. & 9c. Malformed mycelia

The bulging of mycelium may be due to the production of some secondary metabolite by the antagonist, which damaged the cell wall of *B. theobromae*. The normal mycelium was found slender and elongated without any bulging (Fig. 9a).

4.10. Biochemical tests

The results of the various biochemical tests carried out for the two antagonists are given in the Table 3.

Table 3. Biochemical tests carried for the antagonists

No.	Tests	Results- B2	Results - SA18
1.	Carbohydrate fermentation		
	1. Glucose	Colour changed from red to yellow, gas produced	Gas production observed
	2. Sucrose	Colour changed from red to yellow, gas produced	Colour changed from red to pink
	3. Lactose	Colour changed from red to pink, gas produced	Colour changed from red to pink
2.	Starch hydrolysis	+*	+
3.	Gelatin hydrolysis	+	+
4.	Casein hydrolysis	+	+
5.	CMC hydrolysis	+	+
6.	H2S production	-*	-
7.	Indole production	-	+
8.	Methyl red test	+	-
9.	VP test	-	+
10.	Citrate utilization test	+	+
11	Urease test	-	+
12	Catalase test	+	+
13	Oxidase test	-	-
14.	Triple sugar iron agar test	Yellow butt, red slant, gas produced	Not much change observed

* +Positive, *- Negative

4.11. Identification of the selected actinomycete (SA18)

From the microscopic examination the actinomycete, SA18 was identified as a *Streptomyces* sp. (Fig. 10). Both aerial and branched mycelium was present and the conidial chain arises from the branched aerial mycelium. The conidial chain was differentiated into *Rectiflexibles* carrying spores.

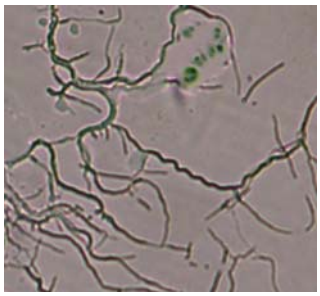


Fig. 10. Aerial mycelium of actinomycete (SA18)

4.12. Molecular characterization of the actinomycete (SA18)

The partial sequence of 16S rDNA of SA18 genome was amplified (1070 bp) using the specific forward and reverse primers for *Streptomyces* genus (Fig. 11). When the sequenced 16S rDNA was subjected to BLAST sequence similarity search, it showed 99% sequence similarity to *Streptomyces* sp 16S rDNA sequence deposited in the nucleotide library (NCBI Accession numbers FJ200398.1, FJ481057.1, FJ406045.1 EU841573.1) with 100% query coverage. The isolated actinomycete antagonist was identified as a *Streptomyces* sp. SA18 (NCBI Accession number FJ643450).

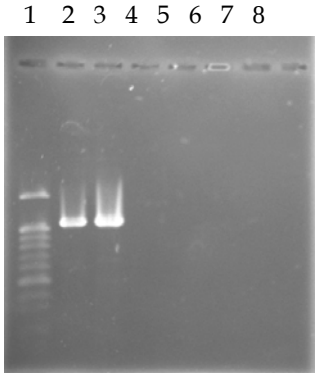


Fig. 11. Amplified product of 16S rDNA of SA18 genome

Lane 1: 1.5 kb ladder

2, 3: amplified product of 16 S rDNA

Amplified product is around 1070 bp in length

4.13. Characterization of the active principle produced by actinomycete (SA18)

Reduction of turbidity in colloidal chitin broth (CCB): The turbidity of the liquid culture inoculated with the actinomycete disappeared and the solution became clear with sedimentation in the bottom (Fig. 12b). In the control, the broth was turbid (Fig. 12a). It is indicated that the actinomycete, SA 18 degraded the colloidal chitin in the broth and the colony mass sedimented at the bottom.

Fig. 12a. Control (CCB)

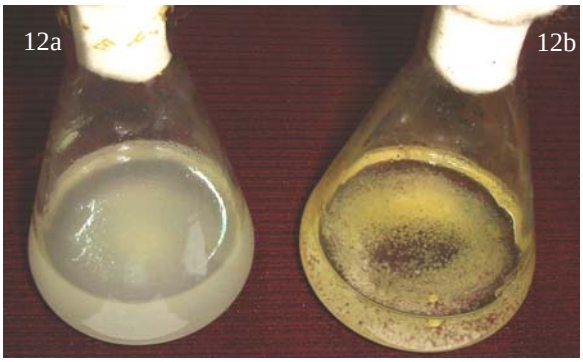


Fig. 12b. *Streptomyces* SA 18 inoculated in CCB

Fig. 12.. Turbidity reduction in colloidal chitin broth caused by *Streptomyces* SA18

Plate assay for chitinase production: After the incubation period, when the growth of the *Streptomyces* SA 18 culture in colloidal chitin agar was viewed under normal light, a zone of inhibition was observed around the fungal growth (Fig. 13). When the plate stained with Calcofluor White was viewed under ultra violet light, the clear zone around the fungal growth appeared dark and the remaining portion fluoresced (Fig. 14). The property of the dye is that, it will bind with the chitin and will fluoresce under UV light. The *Streptomyces* SA 18 degraded the chitin around the colony by the production of chitinase and showed a clear zone in the medium. Since there is no chitin present in the clear zone around the colony, the dye could not fluoresce.

Fig. 13. Clear zone developed by the degradation of colloidal chitin agar by *Streptomyces* SA18 (viewed under normal light)

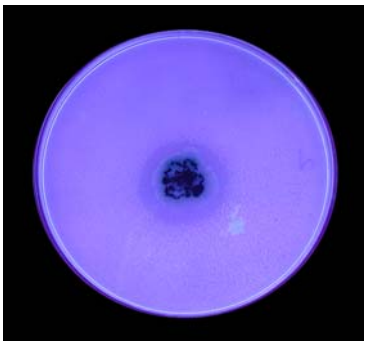


Fig. 14. Dark zone developed by the degradation of colloidal chitin agar by *Streptomyces* SA18 (viewed under uv light)

4.14. Identification of the selected bacterium (B2)

Gram staining: After gram staining, the bacterial cells appeared as long purple rods. The bacterial cells retained the purple colour even after decolourization with ethanol and addition of saffranine (Fig. 15). Hence the observed bacterium was confirmed as gram-positive bacilli.

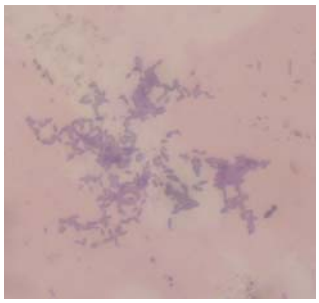
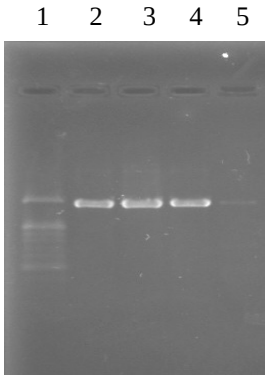


Fig. 15. Gram-positive rods

4.15. Molecular characterization of the selected bacterium (B2)

From the gram staining test and PCR amplification, the genus of B2 was identified as *Bacillus*. The partial sequence of 16S rDNA of *Bacillus* B2 genome was amplified (1400 bp) using the specific forward and reverse primers for *Bacillus* genus (Fig. 16). When the sequenced 16S rDNA was subjected to BLAST sequence similarity search, it showed 99% sequence similarity to *Bacillus subtilis* 16S rDNA sequence deposited in the nucleotide library (NCBI Accession numbers EU869255.1, EU869247.1, EU869230.1, EU869223.1) with 100% query coverage. The isolated bacterial antagonist *Bacillus* B2 was identified as *Bacillus subtilis* strain B2 (NCBI Accession number FJ445405).



Lane 1 - 1.5 kb DNA ladder
Lane 2,3,4 & 5 - amplified product of 16S rDNA gene

Fig. 16. Amplified product of 16S rDNA of B2 genome

4.16. Antifungal antibiotic assay of bacterial filtrate (B2)

The culture filtrate added in the well inhibited the growth of *B. theobromae* in the Petri dish. The fungus could not grow towards the well. A definite zone of inhibition was developed around the well (Fig. 17).

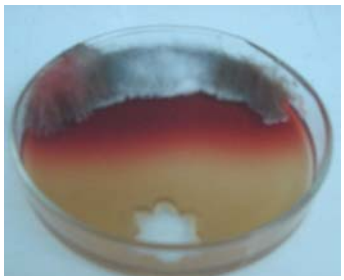


Fig. 17. Zone of inhibition of *B. theobromae* developed by the *Bacillus subtilis* B2 culture filtrate

4.17. Extraction of antifungal antibiotics of bacterium B2

The antibiotics produced by *Bacillus subtilis* were extracted and characterized. One of the antibiotics extracted using HPLC was identified as Iturin A comparing with an authentic standard (Fig. 18). The retention time of the standard was 4.878 minutes and the maximum absorbance was noticed at 254 nm. In the sample, at the same retention time, 4.837 minutes, a peak was observed (Fig. 19) and the UV spectra of both peaks (Figs. 20, 21) were similar and proved to be the same compound. The extra peaks observed in the chromatogram represented other antibiotics, which play efficient role in the inhibition of sapstain fungus *Botryodiplodia theobromae.*

Fig. 18 **Chromatogram of Iturin A standard**

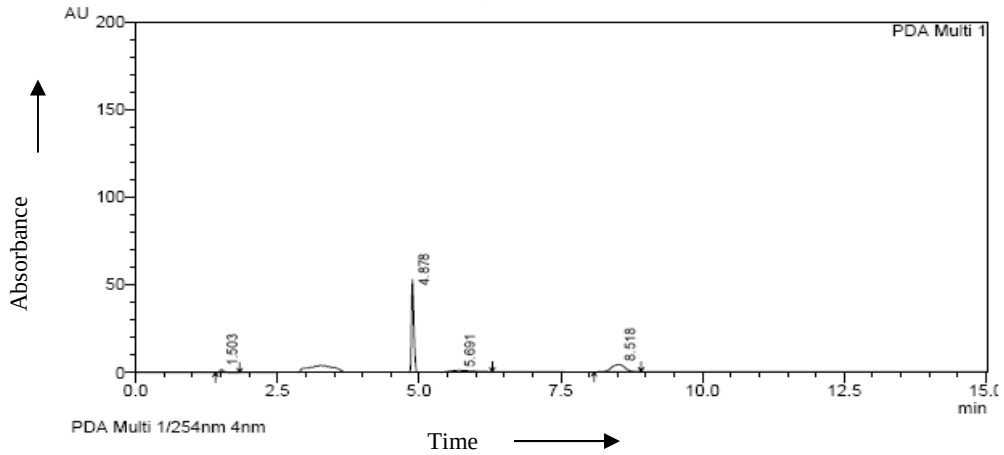


Fig. 19 Chromatogram of Bacillus subtilis Antibiotics

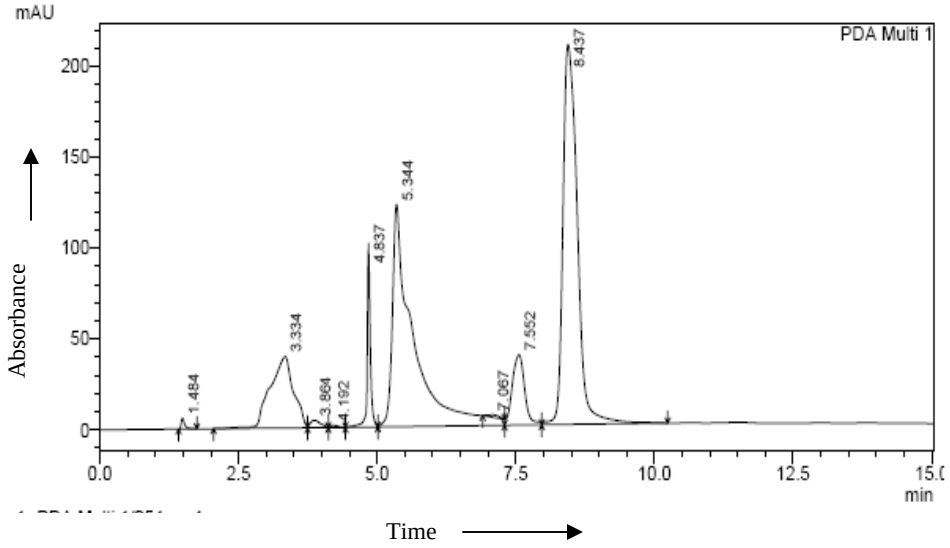


Fig. 20 **UV spectrum of Iturin A standard**

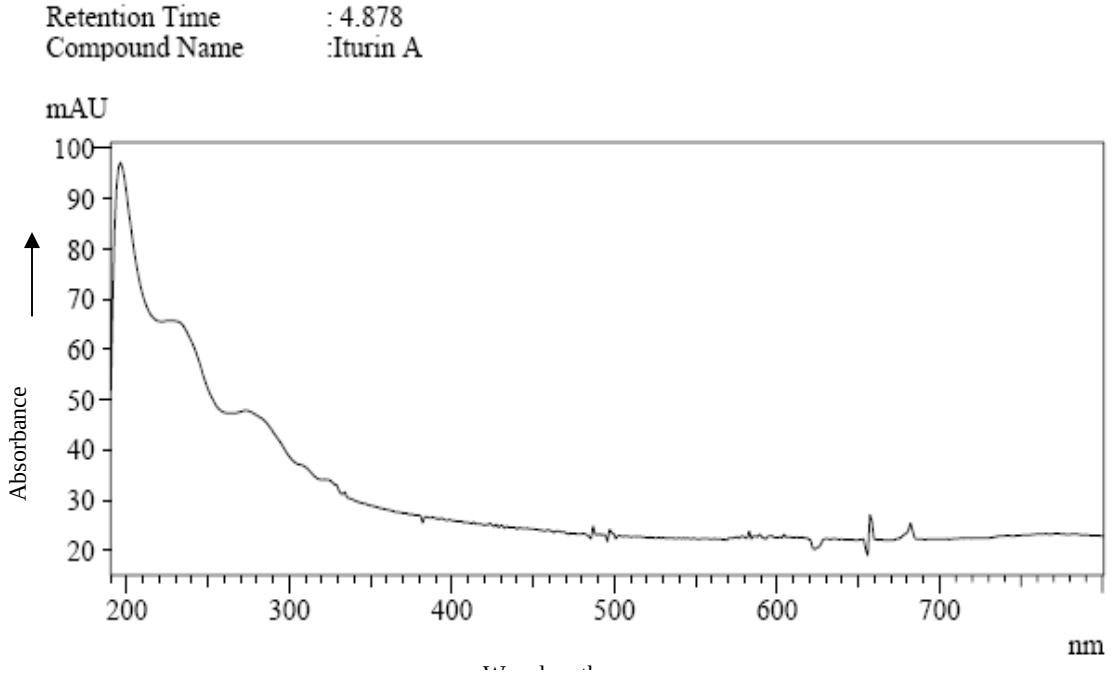
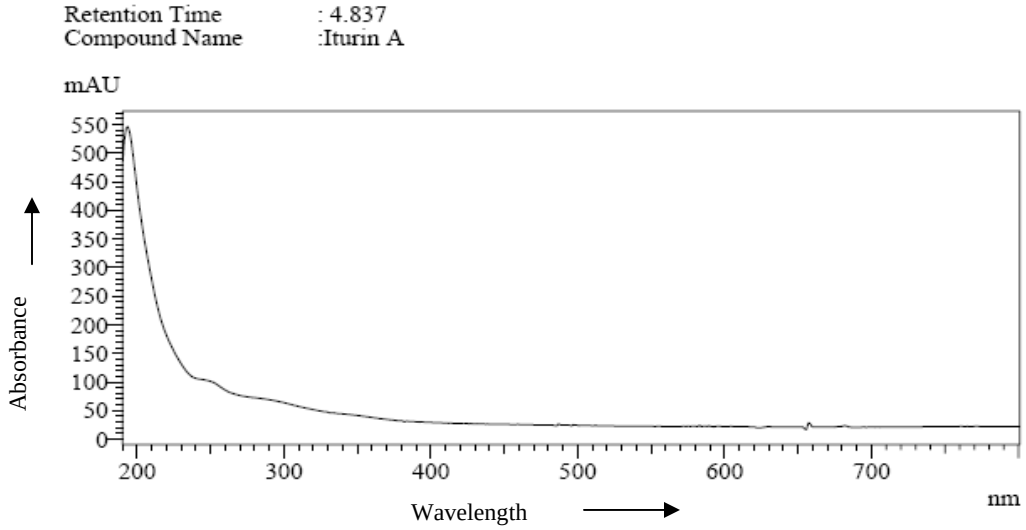


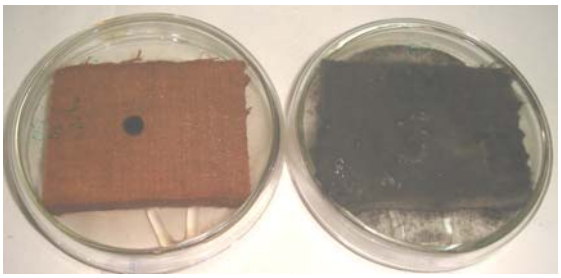
Fig. 21 **UV spectrum of Iturin A in Sample**



4.18. Testing the antagonists in the laboratory

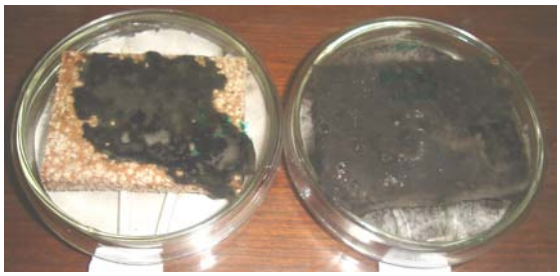
No growth of the *B. theobromae* was observed on the wood blocks dipped in bacterial culture (Fig. 22). On the wood blocks dipped in actinomycete culture, the fungal growth was inhibited on the surface of the wood block where the

tested actinomycete itself was growing (Fig. 23). In both controls, *B. theobromae* was growing profusely over the wood blocks.



Treated control

Fig. 22. Wood blocks treated with *Bacillus subtilis* B2

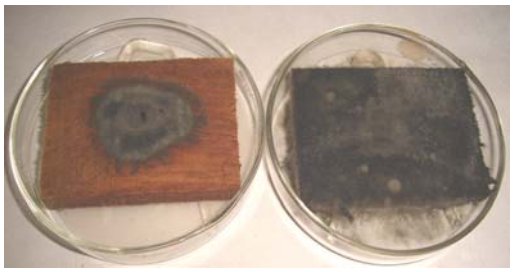


Treated control

Fig. 23. Wood blocks treated with *Streptomycete*

4.19. Testing the culture filtrates of the antagonists

The wood blocks dipped in culture filtrate of bacterium, B2 and the control is shown in the Fig. 24. In the wood blocks treated with the bacterial culture filtrate, the growth of *B. theobromae* was partially inhibited. No inhibition of fungal growth was observed in the wood blocks dipped in the culture filtrate of actinomycete. On control blocks thick growth of the test fungus was recorded.



Treated control

Fig. 24. Wood block treated with culture filtrate of *Bacillus subtilis* B2

4.20. Testing the antagonists in the field:

4.20.1. Treatment with bacterium during north east monsoon

Close stacking: The results presented in Table 4 revealed that at the end of one month during the northeast monsoon season, the rating of surface growth of fungi on the bacterial treated planks was 1 whereas it was 4 on control (Fig. 25). After planing, it was found that the internal stain and decay was only 1.7 - 2.5 %

on treated planks. On control planks after planing, the internal staining and decay was 22.5-24.7% and appeared grayish black inside (Fig. 26).

Table 4. Rating of the open and close-stacked planks treated with bacterial culture

Close stacking				Open stacking			
Bacillus subtilis B2 treated		Control		*Bacillus subtilis* B2 treated		Control	
Surface growth % (Rating)	Inner staining & decay % (Rating)	Surface growth % (Rating)	Inner staining & decay % (Rating)	Surface growth % (Rating)	Inner staining & decay % (Rating)	Surface growth % (Rating)	Inner staining & decay % (Rating)
8 (1)	1.7 (1)	57 (4)	24.7 (2)	2 (1)	0	15 (2)	9 (1)
9 (1)	2.5 (1)	55.3 (4)	24 (2)	2.5 (1)	0	12.7 (2)	7.7 (1)
9.6 (1)	2.5 (1)	51.5 (4)	22.5 (2)	1.5 (1)	0	13.5 (2)	8 (1)

Control *Bacillus subtilis* B2 treated

Fig. 25. Surface growth of *B. theobromae* on the close-stacked planks treated with *Bacillus subtilis* B2

Control *Bacillus subtilis* B2 treated

Fig. 26. Internal stain & decay on the close-stacked planks treated with *Bacillus subtilis* B2 (after planing)

Open stacking: In the open stacked treated planks during northeast season, the percentage of fungal growth on the surface was only 1- 2.5% whereas on control it was 12.7 –15% (Fig. 27). In the treated planks there was no internal stain or decay after planing. The internal fungal growth on the control planks was

7.7 – 9% (Table 3 and Fig. 28). Compared to close stacking there was a reduction in the surface growth as well as internal stain and decay for open stacking.

Control B2 Treated

Fig. 27. Open-stacked planks *Bacillus subtilis* B2 treated (before planing)

Control B2 treated

Fig. 28. Open-stacked planks *Bacillus subtilis* B2 treated (after planing)

4.20.2. Treatment with actinomycete

On the planks treated with actinomycete, in both type of stacking, the treated actinomycete was growing on the surface of planks and thus giving an unpleasant appearance on the surface. Since the surface of the planks was covered with actinomycetous growth, there was no space for the growth of other fungi. After planing there was no internal stain on the planks. On control planks profuse growth of moulds, stain and decay fungi was observed.

4.20.3. Treatment with bacterium during southwest monsoon

When compared to northeast monsoon the percentage of control of sapstain was less on bacterial treated planks than the planks treated during the southwest monsoon. The reason for the low percentage of control may be the congenial atmospheric condition for the growth of fungi. Kerala gets maximum amount of rains during southwest monsoon. During this season, fungal growth on wood will be high due to the high humidity and moisture content. In order to get

maximum protection from fungal growth, the antibiotic produced must be increased.

5. Discussion

The peculiar climatic conditions prevailing in Kerala State appear to be very congenial for the development of sapstain on rubber wood. Among the stain causing fungi, *B. theobromae* is the major one causing bluish-black discoloration on rubber wood. *B. theobromae*, a wound pathogen causing diseases of various tropical and subtropical plants is also a blue stain causing fungi of wide occurrence (Thapa, 1971; Kaarik, 1980; Sujan *et al*., 1980).

Among the antagonists isolated, *Streptomyces* sp. SA18 and *Bacillus subtilis* B2 are found to be very effective in preventing the growth of *B.theobromae* on rubber wood. Antagonistic phenomena against fungi may be due to nutritional competition, production of antibiotics, or secondary metabolites or volatiles.

Antagonistic activity of several *Streptomyces* sp. against a number of fungal pathogens has been known for a long time (Crawford *et al*., 1993). Light microscopic observations of the test fungus from the inhibition zone of the SA18 culture have revealed the morphological changes of the mycelium. Abnormal swelling and hyphal lysis were noticed. This might be due to the enzymic digestion of the terminal region of the hyphae, which is rich in chitin. In this study it has been proved that *Streptomyces* SA18 produced chitinase, which degraded the chitin present in the fungal cell wall and stopped the further growth of the fungus. Chitinases are well known to lyse fungal cell walls (Ordentlich *et al*., 1988; Cheranin *et al*., 1995**;** Gupta *et al*., 1995). *Streptomycete* SA18 has a good chitinolytic potential, which is evident from its growth and chitin hydrolysis in colloidal chitin broth and colloidal chitin agar.

B. subtilis has been proved as a promising biocontrol agent against sapstain fungi (Bernier *et al*., 1986; Seifert *et al*., 1987). *Bacillus* species are well known producers of antibiotics (Gueldner, *et al*., 1988; Rossall, 1991; Gong, *et al*., 2006). A wide range of antibiotics is produced by *Bacillus* sp. such as Iturin A, Mycosubtilin, Fengycin and Bacillomycin by *B. subtilis* (Rossall, 1991), Bacitracin by B. *licheniformis*, and Polymixin by *B. polymyxa* (Katz and Demain, 1977). Among the antibiotics, the major role of Iturin was studied by Gueldner *et al*. (1988) and Gong *et al*. (2006). Iturins are a group of similar cyclic lipopeptides with high antifungal activity, which can modify the membrane permeability and lipid composition and inhibit the mycelium growth and sporulation of fungi (Latoud *et al*., 1987). Various studies have been carried out on the isolations and antifungal activities of the iturin group antibiotics (Peypoux *et al*., 1985; Besson and Michel, 1987; Eshita *et al*, 1995). In the present study also *B. subtilis* B2 was found inhibiting the growth of *B. theobromae* by producing antibiotic Iturin A. The effect of antifungal antibiotics, Iturin A on sapstain fungus *B. theobromae* was studied for the first time in this trial.

Though the culture of *Streptomyces* SA18 was not acceptable as a bioprotectant for the sapstain on the rubber wood in the field since the actinomycete itself was colonizing on the surface and giving an unpleasant appearance to the wood the application of chitinase enzyme produced by the actinomycete in inhibiting the fungal growth can be explored.

In this study *B. subtilis* strain B2 has been proved as a biocontrol agent for preventing the sapstain on rubber wood. But in Kerala, during southwest monsoon due to the heavy load of the fungal inoculum the quantity of bacterial biomass or the antibiotics produced by the *B. subtilis* B2 was insufficient for the total control of sapstain infection. A further study is required for mass production of *B. subtilis* B2 and enhanced production of antifungal antibiotics.

6. Conclusion

Among the several isolates of bacteria and actinomycetes screened against the sapstain fungus *B. theobromae,* a bacterium and an actinomycete were found very effective in inhibiting the growth. Through morphological, physiological and molecular tests, the bacterium was identified as *Bacillus subtilis* B2 and actinomycete as *Streptomyces* sp. SA18. The culture filtrate of the two antagonists was found inhibiting the fungal growth in the culture medium. On wood blocks only the bacterial cultural filtrate was inhibiting the fungal growth. Microscopic observations revealed the bulging and malformation of fungal mycelia in the inhibition zone. Inhibition of growth of *B. theobromae* by the *Streptomyces* sp. SA18 may possibly due to the degradation of chitin present in the cell wall of the fungus by chitinase enzyme. The antibiotic produced by *B. subtilis* was identified using HPLC as Iturin A. When compared to *Streptomyces* SA18, *Bacillus subtilis* B2 was found more effective against the sapstain fungus under field condition. The bacterium proved to be efficient in controlling the fungal growth on rubber wood planks except in the heavy rainy season. Open stacking was found to reduce the infection of fungi on rubber wood. During heavy rains, due to the rich fungal microflora on wood, the quantity of antibiotic produced by the bacterial mass was insufficient for the total control of sapstain infection.

7. **References**

Ausubel, M.F., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. 1994. Preparation of genomic DNA from bacteria, Current protocols in molecular biology Vol. 1, John Wiley and Sons. Inc, 2.4.1 – 2.4.2.

Benko, R. and Highley, T.L. 1990. Biological control of wood attacking fungi using bacteria. Biodeterioration Research, 3: 327-332.

Bernier, R., Desrochers, M. and Jurasek, L. 1986. Antagonistic effect between *Bacillus subtilis* and wood staining fungi. Journal of the Institute of Wood Science, 10 (5): 214-216.

Besson, F. and Michel, G. 1987. Isolation and characterization of new iturins: Iturin D and Iturin E. The Journal of Antibiotics, XL (4): 437-442.

Beyer, M. and Diekmann. 1985. The chitinase system of *Streptomyces* sp. ATCC 11238 and its significance for fungal cell wall degradation. Applied Microbiology and Biotechnology, 23 (2): 140-146.

Brown, H.L. and Bruce, A. 1998. Assessment of the biocontrol potential of a *Trichoderma viride* isolate in a field. IRG/WP/10252. The International Research Group on Wood Preservation, Stockholm.

Bruce, A., Stewart, D., Verrall, S. and Wheatley, R.E. 2003. Effect of volatiles from bacteria and yeast on the growth and pigmentation of sapstain fungi. International Biodeterioration & biodegradation, 51: 101-108.

Bruce, A., Verrall, S., Hackett, C.A. and Wheatley, R.E. 2004. Identification of volatile organic compounds (VOCs) from bacteria and yeast causing growth inhibition of sapstain fungi. Holzforschung, 58: 193-198.

Bruce, A., Wheatley, R.E., Humphris, S.N., Hackett, C.A. and Florence, E.J.M. 2000. Production of volatile organic compounds by *Trichoderma* in media containing different amino acids and their effect on selected wood decay fungi. Holzforschung, 54 (5): 481-486.

Cartwright, K. St. G. and Findlay, W.P.K. 1958. Decay of timber and its prevention. Chemical Publishing Co., HMSO, London. Pp 282-320.

Cheranin, L., Ismailov, Z., Haran, S. and Chet, I. (1995). Chitinolytic *Enterobacter agglomerans* antagonistic to fungal pathogens. Applied and Environmental Microbiology, 61: 1720-1726.

Crawford, D,L., Lynch, J.M., Whipps, J.M. and Ousley, M.A. (1993). Isolation and characterization of actinomycetes antagonists of a fungal root pathogen. Applied and Environmental Microbiology, 59: 3899- 3905.

Croan, S.C. 1996. Biological control of sapstain fungi in wood. IRG/ WP/ 10158. The International Research Group on Wood Preservation, Stockholm.

Croan, S.C. and Highley, T.L. 1991. Biological control of the blue stain fungus *Ceratocystis coerulescens* with fungal antagonists. Material und Organismen, 25 (4): 255-266.

Croan, S.C. and Highley, T.L. 1992. Synergistic effect of boron on *Streptomyces rimosus* metabolites in preventing conidial germination of sapstain and mold fungi. IRG/WP/1565. The International Research Group on Wood Preservation, Stockholm.

Croan, S.C. and Highley, T.L. 1994. Biological treatment for controlling wood deteriorating fungi. Patent No: 5356624.

Croan, S.C. and Highley, T.L. 1996. Fungal removal of wood sapstain caused by *Ceratocystis coerulescens.* Material und Organismen, 30: 45-56.

Croan, S.C. 1997. Environmentally benign biological wood preservatives by *Streptomyces rimosus*, SC-36. IRG/ WP/ 10196. The International Research Group on Wood Preservation, Stockholm.

Dickson, D. 1980. PCP dioxins found to pose health risks. Nature, 283: 418.

Eshita, S.M. and Roberto, N.H. 1995. Bacillomycin L_c, a new antibiotic of the iturin group: isolations, structures and antifungal activities of the congeners. The Journal of Antibiotics, 48 (11): 1240-1247.

Feio, S.S., Cabrita, A.B.M., Nunes, L., Esteves, A., Roserio, J.C. and Curto, M.J.M. 2004. Antifungal activity of *Bacillus subtilis* 355 against wood surface contaminant fungi. Journal of Indian Microbiology & Biotechnology, 31: 199-203.

Fiddaman, P.J. and Rossall, S. 1993. The production of antifungal volatiles by *Bacillus subtilis*. Journal of Applied Bacteriology, 74: 119-126.

Fiddaman, P.J. and Rossall, S. 1994. Effect of substrates on the production of antifungal volatiles from *Bacillus subtilis*. Journal of Applied Bacteriology, 76: 395-405.

Florence, E.J.M. and Sharma, J.K. 1990. *Botryodiplodia theobromae* associated with blue staining on commercially important timbers of Kerala and its possible biological control. Material und Organismen, 25: 193-199.

George, T.K and Joseph, T. (1993). Untapped market potential and comparative advantage of a by-product; the case of rubber wood processing industry in India. Wood News, 3 (3): 6-10.

Gohel,V., Vyas, P. and Chhatpar, H.S. 2005. Activity staining method of chitinase on chitin agar plate through polyacrylamide gel electrophoresis. African Journal of Biotechnology, 4(1): 87-90.

Gomes, R.C., Semedo, L.T.A.S., Soares, R.M.A., Linhares, L.F., Ulhoa, C.J., Alviano, C.S. and Coelho,R.R.R. 2001. Purification of a thermostable endochitinase from *Streptomyces* RC1071 isolated from a cerrado soil and its antagonism against phytopathogenic fungi. Journal of Applied Microbiology, 90: 653-661.

Gomes, R.C., Semedo, L.T.A.S., Soares, R.M.A., Alviano, C.S., Linhares, L.F. and Coelho, R.R.R. 2000. Chitinolytic activity of actinomycetes from a cerrado soil and their potential in biocontrol. Letters in Applied Microbiology, 30: 146-150.

Gong, M., Wang, J.D., Zhang, J., Yang, H., Lu, X.F., Pei, Y. and Cheng, J.Q. 2006. Study of the antifungal ability of *Bacillus subtilis* strain PY-1 in vitro and identification of its antifungal substance (Iturin A). Acta Biochimica et Biophysica Sinica, 38(4): 233-240.

Gueldner, R.C., Reilly, C.C., Pusey, P.L., Costello, C.E., Arrendale, R.F., Cox, R.H., Himmelsbach, D.S., Crumley, F.G. and Cutler, H.G. 1988. Isolation and identification of Iturins as antifungal peptides in biological control of peach brown rot with *Bacillus subtilis*. Journal of Agriculture and Food Chemistry, 36: 366-370.

Gupta, R., Saxena, R.K., Chaturvedi, P. and Virdi, J.S. (1995). Chitinase production by *Streptomyces viridificans*: its potential in fungal cell wall lysis. Journal of Applied Bacteriology, 78: 378-383.

Gupta, R.C. and Tandon, I.N. 1977. Growth inhibition of fungi by volatiles from *Streptomyces.* Transactions of the British Mycological Society, 68 (3): 438-439.

Harjono. and Widyastuti, S.M. 2001. Antifungal activity of purified endochitinase produced by biocontrol agent *Trichoderma reesei* against *Ganoderma philippii*. Pakistan Journal of Biological Sciences, 4(10): 1232-1234.

Held, B.W., Thwaites, J.M., Farrell, R.L. and Blanchette, R.A. 2003. Albino strains of *Ophiostoma* species for biological control of sapstaining fungi. Holzforschung, 57 (3): 237-242.

Herrington, P.R., Craig, J.T. and Sheridan, J.E. 1987. Methyl vinyl ketone: a volatile fungistatic inhibitor from *Streptomyces griseoruber*. Soil Biology &. Biochemistry, 19 (5): 509-512.

Highley, T.L. and Ricard, J. 1988. Antagonism of *Trichoderma* spp. and *Gliocladium virens* against wood decay fungi. Material und Organismen, 23 (3): 157-169.

Hong, L.T. 1976. A blue stain organism of jelutong (*Dyera costulata* H.k.f). The Malay Forester, 39: 177-188**.**

Hong, L.T., Tam, M.K., Daljeet Singh, K. and Arshad Omar.1980. The effectiveness of preservatives in the control of sapstain in rubber wood (*Hevea brasiliensis)* logs. The Malay Forester, 43: 522-527.

Hoster, F., Schmitz, J.E. and Daniel, R. 2005. Enrichment of chitinolytic microorganisms: isolation and characterization of a chitinase exhibiting antifungal activity against phytopathogenic fungi from a novel *Streptomyces* strain. Applied Microbiology and Biotechnology, 66(4): 434-442.

Hsu, S.C and Lockwood, J.L. 1975. Powdered chitin agar as a selective medium for enumeration of actinomycetes in water and soil. Applied Microbiology, 29: 22-426.

Jatala, P. 1986. Biological control of plant parasitic nematodes. Annual Review of Phytopathology, 24: 453-89.

Johnson, L.F. and Curl, E.A. 1972. Testing for antibiotic activity, Methods for research on the ecology of soil-borne plant pathogens, Burgess Publishing Company, Minneapolis, 153-155.

Kaarik, A. 1980. Fungi causing sapstain in wood. The International Research Group on Wood Preservation Document No. IRG/WP/199.

Katz, E. and Demain, A.C. 1977. The peptide antibiotics of *Bacillus*, Chemistry, biogenesis and possible functions. Biological Review, 41: 449-474.

Kim, H.S., Park, J., Choi, S.W., Choi, K.H., Lee, G.P., Ban, S.J., Lee, C.H. and Kim, C.S. 2003. Isolation and characterization of *Bacillus* strains for biological control. The Journal of Microbiology, 41 (3): 196-201.

Latoud, C., Peypoux, F., Michel, G. 1987. Action of Iturin A, an antifungal antibiotic from *Bacillus subtilis*, on yeast *Saccharomyces cerevisea*: Modifications of membrane permeability and lipid composition. The Journal of Antibiotics (Tokyo), 40: 1588-1595.

Mannothra, J.M. (1993). Rubber wood production and utilisation in India. Wood News, 3(3): 21-28.

Martin, J.F. and Demain, A.L. 1980. Control of antibiotic biosynthesis. Microbiological Reviews, 44 (2): 230-251.

Masuka, A.1991. Laboratory evaluation of the efficacy of antiblu and biocide in the control of sapstain and mold fungi on *Pinus elliotti.* European Journal of Forest Pathology, 21: 185-188.

Mhammedi, A., Peypoux, F., Besson, F. and Michel, G. 1982. Bacillomycin F, a new antibiotic of iturin group; isolation and characterization. The Journal of Antibiotics, XXXV (3): 306-311.

Miller, D.J. and Goodell, B. 1981. Blue staining in ponderosa pine sapwood at moderate and low temperatures. Forest Products Journal, 31(2): 54-59.

Moita, C., Feio, S.S., Nunes, L., Curto, M.J.M. and Roseiro, J.C. 2005. Optimisation of physical factors on the production of active metabolites by *Bacillus subtilis* 355 against wood surface contaminant fungi. International Biodeterioration & Biodegradation, 55: 261-269.

Nielson, T.H., Sorensen, D., Tobiasen, C., Andersen, J.B., Christopherson, C., Givskov, M. and Sorensen, J. 2002. Antibiotic and biosurfactant properties of cyclic lipopeptides produced by fluorescent *Pseudomonas* spp. from the sugar beet rhizosphere. Applied and Environmental Microbiology, 68 (7): 3416-3423.

Olofinboba, M.O. 1974. Sap stain in *Antiaris africana*, an economically important tropical white wood. Nature, 249: 860.

Ordentlich, A., Elad, Y. and Chet, I. 1988. The role of chitinase of *Serratia marcescens* in biocontrol of *Sclerotium rolfsii*. Phytopathology, 78: 84-88.

Pal, K. K. and Gardener, B.M. 2006. Biological control of plant pathogens. The Plant Health Instructor DOI: 10.1094/PHI-A-1117-02.

Payne, C. and Bruce, A. 2001. The yeast *Debaromyces hansenii* as a short-term biological control agent against fungal spoilage of sawn *Pinus sylvestris* timber. Biological control, 22: 22-28.

Payne, C., Bruce, A. and Staines, H. 2000. Yeast and bacteria as biological control agents against fungal discolouration of *Pinus sylvestris* blocks in laboratory – based tests and the role of antifungal volatiles. Holzforschung, 54 (6): 563-569.

Peypoux, F., Marion, D., Maget-Dana, R., Ptak, M., Das, B.C., Michel, G. 1985. Structure of Bacillomycin F, a new peptidolipid antibiotic of the iturin group. European Journal of Biochemistry, 153: 335-340.

Pinheiro, A.C.A. 1971. Blue stain in poplar wood. Material und Organismen, 6: 93-100.

Rintala, H., Nevalainen, A., Ronka, E and Suutari, T. 2001. PCR primers targeting the 16 S rRNA and gene for the specific detection of *Streptomycetes*. Molecular and cellular probes, 15: 337-347.

Rossall, S. 1991. Antibiotic derived from Bacillus subtilis. US Patent No: 5061495.

Sacchi, C.T., Whitney, A.M., Mayer, L.W., Morey, R., Steigerwalt, A., Boras, A., Weyant, R.S and Popvic, T. 2002. Sequencing of 16S rRNA gene: a rapid tool for identification of *Bacillus anthracis*. Emerging Infectious diseases, 8: 1117-1123.

Saito, A., Fujii, T., Yoneyamma, T. and Miyashita, K. 1998. glkA is involved in glucose repression of chitinase production in *Streptomyces lividans*. Journal of Bacteriology, 180(11): 2911-2914.

Seifert, K.A., Hamilton, W.E., Breuil, C. and Best, M. 1987. Evaluation of *Bacillus subtilis* C186 as a potential biological control of sapstain and mould on unseasoned lumber. Canadian Journal of Microbiology, 33: 1102-1107.

Subramanian, C.V. 1983. Hyphomycetes. Taxonomy and Biology. Academic Press, London, 502 PP.

Sujan, A., Tan, A.G. and Stevens, M. 1980. Some studies on fungal deterioration of rubber wood. IRG/WP/2140. The International Research Group on Wood Preservation, Stockholm.

Thapa, R.S. 1971. Results of preliminary investigation on black stains in commercial trees in Dipterocarp forests of Sabah. The Malay Forester, 34: 53-58.

Tian, B., Yang, J and Zhang, K. (2007). Bacteria used in the biological control of plant parasitic nematodes: populations, mechanisms of action and future prospects. FEMS Microbiology Ecology, 61(2): 197.

Trudel, J. and Asselin, A. 1989. Detection of chitinase activity after polyacrylamide gel electrophoresis. Analytical Biochemistry, 178(2): 362-366.

Vaidya, R.J., Shah, I.M., Vyas, P.R. and Chhatpar, H.S. 2001. Production of chitinase and its optimization from a novel isolate *Alcaligenes xylosoxydans*: potential in antifungal biocontrol. World Journal of Microbiology and Biotechnology, 17(7): 691-696.

Vanittanakom, N. and Loeffler, W. 1986. Fengycin- a novel antifungal lipopeptide antibiotic produced by *Bacillus subtilis* F-29-3. The Journal of Antibiotics, XXXIX (7): 888-901.

Wang, S. and Chang, W. 1997. Purification and characterization of two bifunctional chitinases/ lysozymes extracellularly produced by *Pseudomonas aeruginosa* K-187 in a shrimp and crab shell powder medium. Applied and Environmental Microbiology, 63(2): 380-386.

Weller, D.M. (1988). Biological control of soil borne plant pathogens in the

rhizosphere with bacteria. Annual Review of Phytopathology, 26: 379-407.

White-Mcdougall, W.J., Blanchette, R.A. and Farrell, R.L. 1998. Biological control of blue stain fungi on *Populus tremuloides* using selected *Ophiostoma* isolates. Holzforschung, 52(3): 234-240.

Yuan, W.M. and Crawford, D.L. 1995. Characterization of *Streptomyces lydicus* WYEC108 as a potential biocontrol agent against fungal root and seed rots. Applied and Environmental Microbiology, 61 (8): 3119-3128.

Zabel, R.A. and Morrell, J.J.1992. Wood Microbiology: Decay and its Prevention. Academic Press, San Diego, CA. 476 pp.

Zou, X., Hiroyuki, N. and Gregory, W.E. 2002. A gel diffusion assay for visualization and quantification of chitinase activity. Molecular Biotechnology, 22 (1): 19-23.