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MASS PRODUCTION OF *BACILLUS SUBTILIS* FOR BIOCONTROL OF SAPSTAIN ON RUBBERWOOD

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ABSTRACT OF THE PROJECT PROPOSAL

Project Number	:	KFRI RP 630/2011
Title	:	Mass production of *Bacillus subtilis* for biocontrol of sapstain on rubber wood
Objectives	:	1. Mass production of the antagonist bacteria, *Bacillus subtilis* in a bioreactor. 2. Enhanced production of antibiotic iturin A and its qualitative and quantitative determination by HPLC 3. Testing the effectiveness of the mass produced bacterial biocontrol agent against the stain fungus on rubber wood during different seasons.
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CONTENTS

1. INTRODUCTION

Rubberwood, *Hevea brasiliensis* Muell. Arg. belonging to the family Euphorbiaceae, is one of the tropical softwood timber species mainly cultivated in the countries like Indonesia, Malaysia, Thailand, India and Sri Lanka. Rubberwood is a raw material for many of the wood based industries in India. The light cream colour, strength, mechanical attributes and wood working properties contribute to its industrial demand. India is the fourth largest natural rubber producing country in the world. Kerala State accounts for 78 % of the area and 90 % of total rubber produced in India (George and Chandrashekar, 2014). The wood is mainly used for making plywood, packing cases, low cost furniture and flooring, among others.

The hot and humid climate prevailing in this part of the country offers a congenial environment for the luxuriant growth of various fungal pathogens including moulds, stain, decay fungi, etc. in the felled rubberwood (Subramanian, 1983). Sapstain fungi, the primary colonizers of freshly felled rubberwood are a great concern and development of an appropriate control strategy has thus become the need of the hour. Economic value of rubberwood is heavily affected by the discolouration of wood due to the infection of *Lasiodiplodia theobromae* Pat. as in Canadian softwoods (Uzunovic *et al*., 1999). Chemical protection of the wood from fungal deterioration through application of preservatives like chromate copper arsenate (CCA), sodium pentachlorophenates (Na-PCP), inorganic arsenicals and borax boric acid solutions is being practiced (Greg Kidd, 2001). Health hazards and pollution caused by the application of chemical wood preservatives have necessitated the adoption of environment friendly approaches in the field of wood protection. One alternative method is to take up biological control measures employing antagonistic microorganisms. Biological control, a process of managing pests (including insects, mites, weeds and microbial plant diseases) using other living organisms is a substitute to chemical pesticides and results in disease diminution or decrease in inoculum potential to cause infection of a pathogen.

The feasibility of controlling sapstain infection on timber using bacterial species like *Bacillus subtilis* (Bernier *et al.,* 1986; Seifert *et al.,* 1987; Florence and Sharma, 1990) and certain fungal species (Croan and Highley, 1996; Held *et al.,* 2003) has been attempted in the past. In India, the biological control of sapstain fungi has not been experimented widely.

Recently, Florence and Balasundaran (2009) evaluated the efficacy of *Bacillus subtilis* as a biological control agent for the prevention of sapstain on rubberwood after screening various microbes from different sources. The antifungal antibiotics, especially iturin A, produced by *B. subtilis* (Florence and Balasundaran, 2009) against the stain fungus was also reported in that study. The present study aims at large-scale production of *Bacillus subtilis* in a bioreactor, production and quantification of the antifungal secondary metabolite, iturin A, by *B. subtilis* during fungal inhibition and field efficacy testing of the biocontrol agent.

2. REVIEW OF LITERATURE

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 are the most serious problems in humid tropics as well as in temperate climatic regions. Sapstain infections are more predominant in humid tropics which are conducive for their growth, mostly on wood with high sapwood content (Zabel and Morrell, 1992). In tropical hardwoods, staining is caused mainly by *Diplodia* sp. and in particular *Lasiodiplodia theobromae* Pat. (Cartwright and Findlay, 1958; Olofinboba, 1974; Florence and Sharma, 1990). Many published reports exist on the sapstain infection of *L. theobromae* on various tropical woods like poplar (Pinheiro, 1971), jelutong (Hong, 1976), pines (Masuka, 1991) and rubberwood (Hong *et al.,* 1980; Sujan *et al.,* 1980; Florence and Sharma, 1990).

Biological control employing antagonistic microorganisms offers an alternative to the chemical treatments generally employed for the control of plant pathogens. Biological control agents of plant diseases are most often referred to as antagonists (Suprapta, 2012). Biocontrol agents are commonly isolated from various sources like soil (Gil *et al*., 2009) and compost (Yuliar *et al*., 2011). Aerobic and anaerobic composts have attracted wide attention previously for its disease suppressive effect as well as for its rich biota of bacteria (Phyla Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria and Deinococcus-Thermus) with biocontrol probability (Pasi *et al*., 2010; Gaind, 2012). Biological control measures employing antagonistic microorganisms against pathogens and pests have been previously reported as an eco-friendly approach in many of the agricultural crops (Tian *et al*., 2007; Mishra *et al*., 2011). *Trichoderma* sp. and *Bacillus* sp. are the widely used biocontrol agents for the control of plant pathogens (Junaid *et al*., 2013).

Some of the first flourished biocontrol agents used against insects and pathogens were members of the genus Bacillus (Powell and Jutsum, 1993; Prashar *et al*., 2013) and commercial strains of Bacillus subtilis were applied for fungal diseases in crops (Warrior *et al*., 2002; Ashwini and Srividya, 2014). *B. subtilis* collectively with other *Bacillus* species represent about half of the industrially available biopesticides in the world market (Fravel, 2005). *B. subtilis*, a

representative of gram positive microbes, can antagonize pathogens by competing for niche and nutrients, by stimulating the defensive capacities of the host plant and more directly by producing low molecular weight compounds toxic to fungi (Compant *et al*., 2005). The potential of *B. subtilis* is based on its ability to produce a broad array of low toxic lipopeptides with powerful antifungal properties, high biodegradability and eco-friendly features as compared to chemical pesticides (Benko, 1988).

Iturins are non-ribosomally synthesized cyclic lipoheptapeptides linked by a β-amino fatty acid. Based on the variation of aminoacids in the peptide moiety iturin has been classified as iturin A, C, D, E, bacillomycin D, F, L, L_c and mycosubtilin (Pecci *et al*., 2010). Amongst all iturins, iturin A has been found to be predominantly secreted by most strains of *B. subtilis* (Romero *et al*., 2007). Iturin A shows a strong antibiotic activity against a broad antifungal spectrum, making it an ideal biological control agent and can assist in reducing the use of chemical pesticides in agriculture (Delcambe *et al*., 1977). In addition, clinical trials on humans and animals have also shown iturin A to be a valuable drug due to its broad antifungal spectrum, low toxicity and allergic effects (Maget-Dana and Peypoux, 1994; Lucca and Walsh, 1999; Yao *et al*., 2003). Iturin A is constituted by a cyclic lipopeptide with seven α-amino acids, and one unique β-amino fatty acid (βAA). The fatty acid chain of lipopeptide varies from 13 to 17 carbon atoms (Delcambe *et al*., 1977). In nature, iturin A is produced as a mixture of up to eight isomers named as iturin A1−A8 (Peypoux *et al*., 1978). The structure of iturin A is shown in Figure 1.

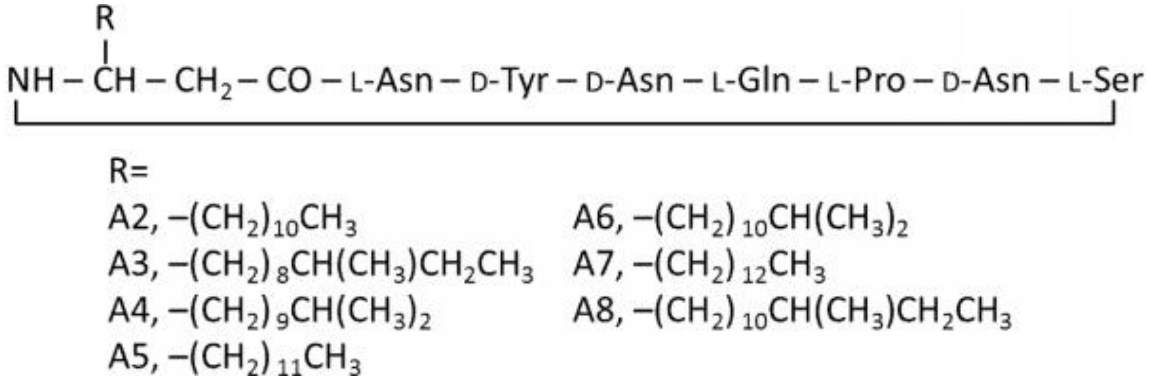


Fig. 1. Chemical structure of Iturin A

Since 1980's, many bacterial strains have been tested for their biocontrol abilities towards various fungal infections on woods. The biocontrol potential of various bacteria such as *Pseudomonas cepacia, Streptomyces rimosus, Bacillus subtilis*, *Serratia* sp*., Lactobacillus plantarum, Bacillus amyloliquefaciens,* etc. against general wood decay and stain fungi like *Ophiostoma* sp., *Ceratocystis* sp., *Fusarium* sp., *Lasiodiplodia* sp., (Benko, 1988, 1989; Benko and Highley, 1990a, 1990b; Florence and Sharma, 1990; Payne *et al*., 2000; Shekhar *et al*., 2006; Schubert *et al*., 2008; El-Fouly *et al*., 2011; Yuan *et al*., 2012; Zhang *et al*., 2014) as well as against the stain, brown and white rot fungi in the pine wood blocks has been previously demonstrated (Behrendt *et al*., 1995; Moita *et al*., 2005; Melentev *et al*., 2006). Biological control of stain fungi using *Streptomyces* sp. SA18 was investigated by Sajitha and Florence (2013). Even though the biocontrol potential of *Streptomyces* sp. SA18 against sapstain fungus *L. theobromae* was high, its uneven growth on wood surface couldn't control the sapstain effectively. The industrial potential of a biocontrol agent can be best exploited only by scaling up the production employing bioreactor technique in a cost effective manner (Mendizabal *et al*., 2012).

Bioreactor or fermentor plays a major role in the mass production of either the biocontrol organism or their commercial by-products (Matar *et al*., 2009; Chenikher *et al*., 2010). *Bacillus subtilis* strain B1, isolated from the market available compost was effective against the bluish black sapstain fungus, *L. theobromae* infecting the rubberwood (Sajitha *et al*., 2014). However, its large scale production in a bioreactor using any of the synthetic media is expensive. An ideal medium for the microbial growth requires adequate amount of both macro and micro nutrients. Natural media like coconut water, potato extract, barley, corn flour, cassava flour, rice flour, brown sugar, citrus juice waste, corn soybean meal, corn steep liquor, among others had prolonged use in microbial production (Muis, 2006; Sen *et al*., 2011). Molasses is a globally available residue from sugarcane industry which plays a major role in microbial growth as well as commercial production of their by products (Yu-Peng *et al*., 2008; Younis *et al*., 2010). A cost effective medium in a bioreactor to scale up the production of any biocontrol agent can enhance the broad spectrum use of the antagonist.

3. OBJECTIVES

The objectives of the present study are

1. Mass production of the antagonistic bacteria, *Bacillus subtilis* in a bioreactor using cost effective medium
2. Enhanced production of antibiotic iturin A and its qualitative and quantitative determination by HPLC
3. Testing the effectiveness of the mass produced bacterial biocontrol agent against the stain fungus on rubberwood during different seasons

4. MATERIALS AND METHODS

4.1. Isolation of test fungus - *Lasiodiplodia theobromae*

Isolation, identification, morphological and microscopic description of the sapstain fungus, *L. theobromae* had previously been carried out in Kerala Forest Research Institute (KFRI), Peechi, Kerala, India (Florence, 1991; Sharma and Florence, 1996; Florence and Balasundaran, 2009). In the present study, to isolate the sapstain fungus, *L. theobromae* infected rubberwood samples were collected from various sawmills of Thrissur District, Kerala, India. Wood pieces were washed thoroughly in running tap water and cleaned with 70% alcohol. Small pieces for inoculation were taken from the interior portion of the infected area and were surface sterilized with 0.01 % of $HgCl_2$ for 50 seconds, washed thrice in sterile distilled water, dried and inoculated on PDA (Johnson and Curl, 1972) medium supplemented with streptomycin (300 mg/ l) and incubated at 28 $\pm$ 2°C for 7 days. The isolated cultures were confirmed as *L. theobromae* by the morphological and microscopical descriptions provided. Isolated pure fungal cultures were maintained on PDA slants. The cultures of sapstain fungus *(L. theobromae*), thus obtained were stored and used for further studies.

4.2. Bacterial isolation from composts

B. subtilis isolated from the compost was found to be an effective biocontrol agent for the sapstain fungus (*L. theobromae*) in a previous study conducted at KFRI (Florence and Balasundaran, 2009). For the present study, three different aerobic composts *viz.* weed compost produced by Kerala Forest Research Institute (KFRI), vermicompost by Kerala Agricultural University (KAU) and market available compost were selected for the isolation of biocontrol agent. Completely randomized design (CRD) was adopted as experimental design for the bacterial isolation. Samples were collected from the three composts and the methodology used for bacterial isolation was serial dilution and plating on to nutrient agar (NA) plates (Johnson and Curl, 1972). For each dilution, three replicates were maintained. The inoculated plates were incubated at 37°C for 24 hours and the results were observed. The plates were observed and colonies counted on the next day and morphologically different and distinct bacterial colonies were selected and subcultured on nutrient agar slants for further screening.

4.3. Antagonistic screening

The antagonistic activities of the isolated bacteria were carried out on PDA plates using dual culture technique (Johnson and Curl, 1972). The isolated bacteria were streaked on one side of the PDA plate and a 6 mm disc of an actively growing culture of *L. theobromae* was placed on the opposite side. Triplicates were maintained for each bacterial screening. The plates were incubated at 28 $\pm$ 2°C for 7 days. The inhibition zone developed as a result of inhibition of the fungal growth was observed and measured. Growth inhibition of the stain fungus in the dual culture was analyzed by two parameters: the percentage inhibition (% I) of radial growth and the width of the zone of inhibition (ZI) measured at the smallest distance between both colonies. Degree of antagonism was expressed based on the visual rating given for the growth of the fungus (Benko, 1988) (Table 1).

Table 1. Visual ratings of percentage inhibition of the fungus (Benko, 1988)

Degree of antagonism	Description
0	Normal growth of the blue stain fungus
1	Partial obstruction of growth of the blue stain fungus
2	Obstruction of growth of the blue stain fungus
3	Stoppage of the blue stain fungus and appearance of a wider or narrower inhibitory zone
4	Very strong and fast stoppage of growth of the blue stain fungus and appearance of a rather wide inhibitory zone.

4.4. Identification of the antagonistic bacteria

The bacteria which showed antagonistic effect towards the test fungus were identified. Colony morphology, staining techniques and various biochemical tests including carbohydrate disc fermentation tests (Himedia, India) were adopted for the bacterial identification. A definite chromogenic method for *Bacillus* identification has been done by growing the bacterial cultures in HiCrome Bacillus agar (Himedia, India). 16S rRNA-encoding gene amplification and sequencing was used as confirmatory tool for the bacterial species identification.

4.4.1. Colony morphology and staining

The seven bacteria were grown in nutrient agar plates for 24 hours and various colony morphological features like size, form, margin, elevation, texture, among others were noted. The staining techniques like Gram staining and endospore staining were also carried out. The test results were correlated with the identification flow chart for further identification (Bergey's manual).

4.4.2. Biochemical tests (Aneja, 2001)

Biochemical tests such as starch hydrolysis, casein hydrolysis, cellulose hydrolysis, indole production test, methyl red- Vogues proskauer test, citrate utilization test, urease test, catalase test, oxidase test, triple sugar iron (TSI) test and carbohydrate fermentation tests were carried out.

4.4.3. Chromogenic method

HiCrome Bacillus Agar was used for isolation and differentiation of various species of *Bacillus*. To one half of the sterilized HiCrome Bacillus agar medium (Himedia, India), polymyxin B, the selective supplement was added aseptically at 50°C. It was mixed well and poured into sterile petriplates. The other half was poured into the plates without the selective supplement. All the isolates were streaked onto HiCrome Bacillus agar medium plates and incubated at 37°C for 24 hours and the results were noted.

4.4.4. PCR amplification and sequencing

The genomic DNA of the isolates was extracted using SDS method (Ausubel *et al*., 1994). To characterize the strains, the 16S rRNA-encoding gene unit was amplified by polymerase chain reaction (PCR) using the universal primers 8F and 1492R (Lane, 1991; Turner *et al*., 1999). The PCR reaction mixture (20 µl) has 15-30 ng of each template DNA, 0.01 µM of each primers, 200 µM of each dNTPs, 2 µl of 5X Taq buffer and 3 U of Taq DNA polymerase. Amplification was carried out with an initial denaturation step at 94˚C for 5 minutes followed by 35 cycles of denaturation (94˚C for 1 min), primer annealing (55˚C for 1 min) and extension (72˚C for 2 min). After 35 cycles of PCR amplification, the PCR products were screened by electrophoresis and the eluted products were subjected to Sanger's dideoxy sequencing for species identification.

The similarity search for the sequences was carried out using the *BLAST (N)* option and the sequences were deposited to NCBI Genbank (http://www.ncbi.nlm.nih.gov/genbank).

4.4.5. Statistical analysis

Data were statistically analyzed using analysis of variance, followed by Duncan's multiple range test ($P = 0.05$), using SPSS version 17 (SPSS Inc., Chicago, Illinois, U.S.A).

4.5. Selection of antagonist

Based on the antagonistic ability of the various screened bacteria, *B. subtilis* strain B1 was selected for the further studies.

4.6. Effect of standard iturin A on *Lasiodiplodia theobromae*

Iturin A standard (Sigma, USA) stock was prepared by dissolving 1mg of iturin A in 1 ml of methanol. 100 µl (1 mg/ ml) of this standard was poured into a well on the PDA medium with the test fungus *L. theobromae* on the opposite side. Control plate was kept with sterile water and test fungus. Effect of iturin A was observed after one week.

4.7. Identification of iturin A biosynthetic gene in *Bacillus subtilis* B1

The genomic DNA of *B. subtilis* strain B1 was extracted according to Ausubel *et al*., (1994). Itu C gene, which is responsible for the production of Iturin A synthatase enzyme during the biosynthesis of iturin A, was PCR amplified using specific primers *itu C* F1 and *itu C* R1 (Baysal *et al*., 2008). The PCR reaction mixture (20 µl) has 15-30 ng of each template DNA, 0.01 µM of each primers, 200 µM of each dNTPs, 2 µl of 5X Taq buffer and 3 U of Taq DNA polymerase. Amplification was carried out with an intitial denaturation step at 94˚C for 5 minutes followed by 35 cycles of denaturation (94˚C for 1 min), primer annealing (60.1˚C for 1 min) and extension (72˚C for 1 min). After 35 cycles of PCR amplification, the PCR products were screened by electrophoresis.

4.8. Enhanced production of antibiotic iturin A and its qualitative and quantitative determination by HPLC

The enhanced production of iturin A was studied with different antibiotic production media, pH and temperature and the metabolite effect was noted by measuring the inhibition percentage of *L. theobromae* by agar well diffusion method (Sen *et al*., 1995).

4.8.1. Effect of media on antibiotic production

Twelve different broths, with various formulations (Table 2) were selected for the enhanced production of antibiotics. Bacterial strain B1 was grown in all media and the effect of the culture filtrate on the stain fungus was checked by agar well diffusion method. A modified method of Hassan *et al*. (2010) was used to assess the inhibitory effect of the strain B1 culture filtrate by culturing it in different medium, at 30˚C for 7 days. Each day, the culture was filtered through 0.22 µm membrane and was used to test its antagonism against *L. theobromae* by agar well diffusion method (Sen *et al*., 1995). Different quantities (200 µl, 500 µl and 1000 µl) of the culture filtrate were used in the agar well diffusion method to assess the inhibitory efficiency of the filtrate. Triplicates were maintained for each experiment.

Table 2. Different media used in antibiotic production test

Media	Components (g/l)	References
No. 1	Glucose – 10, $FeSO_4$ – 0.01, KH_2PO_4 – 2, Glutamic acid – 5, $MgSO_4$ – 0.5, KCl – 0.5, $MnCl_2$ – 0.01	Rossall, 1991
No. 2 YGB	Yeast Extract – 4, Dextrose – 2, NaCl – 5, K_2HPO_4 – 2.5, KH_2PO_4 – 0.5	Moita *et al*., 2005
No. 3 (LB)	Glucose-20 g, Glutamic acid- 5 g, $MgSO_4$- 0.5 g, KCl- 0.5 g, KH_2PO_4- 1 g, $FeSO_4$- 0.15 mg, $MnSO_4$- 5 mg, $CuSO_4$- 0.16 mg, L-phenylalanine- 2 mg	Hassan *et al*., 2010
No. 4	Sorbitol – 20, $FeSO_4$ – 0.01, KH_2PO_4 – 1, Glutamic acid – 2.5, $MgSO_4$ – 0.5, Asparagine – 2.5, KCl – 0.5, $MnCl_2$ – 0.01	Rossall, 1991
No. 5	Glucose -3, Peptone – 5, Beef extract – 3, $NaNO_3$ – 1, KH_2PO_4 – 0.1,KCl - 0.5, $MgSO_4$ – 0.02, $FeSO_4$ – 0.01	Modified medium of Benko and Highley, 1990c
No. 6 (TSB)	Trypticase Soya Broth, Himedia	Moshafi *et al*.,2011
No.7	Glucose – 5, Beef extract – 5, Yeast extract – 2.5, Peptone – 5, NaCl – 2.5, KH_2PO_4 – 0.25, KNO_3 – 1, $FeSO_4$ – trace Instead of glucose in the above medium, lactose, sucrose and mannitol was also tried	Modified medium of Benko and Highley, 1990c
No. 8	To medium No.7, 2.5g sucrose was added	Modified medium of Benko and Highley,1990c
No. 9	Glycerol – 11.5, Beef extract – 8, NaCl – 2.5, KH_2PO_4 – 0.25, KNO_3 – 1, Ammonium sulphate – 0.25, $FeSO_4$ – trace	Modified medium of Benko and Highley, 1990c
No. 10	Peptone – 30, Dextrose – 20, Yeast extract – 7, KH_2PO_4 – 1.9, $MgSO_4$ – 0.45, $CuSO_4$ – 0.001mg, $FeCl_3$ -0.005 mg, Na_2MoO_4 – 0.004 mg, KI – 0.002 mg, $MnSO_4$ – 3.6mg, $ZnSO_4$ – 0.14mg	Hbid *et al*., 1996
No.11 KMB	Tryptone – 20, Glycerin – 10, K_2HPO_4 – 15, $MgSO_4$ - 15	Gong *et al*., 2006
No. 12	Sucrose – 20, Peptone – 10, Yeast extract – 1, $CaCO_3$ – 0.1, NaCl – 0.1	Modified medium of Benko and Highley,1990c

4.8.2. Effect of temperature and pH on antibiotic production

The bacterial culture in the selected medium was incubated at different temperatures (20, 25, 30, 35, 40, 45°C) and pH (4 - 10) and the effect of culture filtrate was tested by agar well diffusion method mentioned previously. Different quantities (200 µl, 500 µl and 1000 µl) of the culture filtrate were used in the agar well diffusion method to assess the inhibitory efficiency of the filtrate. Triplicates were maintained for each experiment.

4.8.3. Antibiotic extraction (Rossall, 1991)

B. subtilis strain B1 was grown in 500 ml antibiotic production medium Landy Broth [LB - Glucose (20 g), Glutamic acid (5 g), $MgSO_4$ (0.5 g), KCl (0.5 g), KH_2PO_4 (1 g), $FeSO_4$ (0.15 mg), $MnSO_4$ (5 mg), $CuSO_4$ (0.16 mg), L-phenylalanine (2 mg), distilled water (1000 ml)] at 30°C, pH 7, 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 concentrated HCl to lower the pH to 2. The precipitate was collected by centrifugation and dissolved in distilled water and pH was adjusted to 7 using 1N NaOH. This was partitioned thrice with butanol and the organic phase was taken and evaporated to dryness in a rotary vacuum evaporator. The powder obtained was then purified with diethyl ether for 1 h in a soxhlet extractor (Soxtech^{TM} 2043 Extraction unit, FOSS Analytical AB, Sweden) to remove non-polar biologically inactive impurities. The remaining solid was dissolved in methanol and the solution was chromatographed on C-18 reversed-phase absorbent column.

4.8.4. HPLC analysis

Sample was analyzed by 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 4.6 mm. The method used for HPLC analysis was a slightly modified version previously reported (Florence and Balasubdaran, 2009). The mobile phases used were 60 % water and 40 % acetonitrile. The flow rate was 1 ml/ minute and oven temperature was maintained at 40°C. The detection wavelength was set at 200 nm.

For this purpose, 1 mg/ ml stock of iturin A (Sigma, USA) was diluted to obtain different concentrations (50, 100, 150, 200 mg/ l) with methanol. Then 20 µl of the standard solution

was injected into the chromatographic system sequentially using the same chromatographic conditions described above, and the linear regression equation was obtained.

4.9. Shake flask study for the growth standardization of *Bacillus subtilis* B1

In order to standardize the medium and physical parameters like pH and temperature for the optimum growth of *B. subtilis* B1, a preliminary shake flask culturing was conducted.

4.9.1. Selection of medium

For standardizing the mass production medium, different growth media like Nutrient Broth (NB) (Benko and Highley, 1990c), Luria Bertani (LB) (Bozic *et al*., 2011), Potato Dextrose Broth (PDB) (Benko and Highley, 1990c), modified Molasses Peptone Broth (MPB) (Younis *et al*., 2010), modified Coconut water Glucose Peptone Broth (CGPB) (Muis, 2006) and Beef Extract Peptone Broth (BEPB) (Younis *et al*., 2010), were inoculated with *B. subtilis* B1 and incubated at 37˚C. The effective growth was tested by serial diluting and spread plating the *B. subtilis* B1 culture grown for 24 hours in each test media.

4.9.2. Selection of temperature and pH

For the optimization of temperature, the selected medium was inoculated with *B. subtilis* culture and the flasks were incubated at different temperatures like 23˚C, 30˚C 37˚C, 44˚C and 51˚C based on the previous report (Moita *et al*., 2005). The pH was standardized by culturing the bacterium in the selected medium with varying pH from 4 to 10.

4.10. Mass production of *Bacillus subtilis* B1 in bioreactor

The batch fermentations were performed in a 3L bioreactor (Scigenics, India), equipped with an agitator shaft with three impellers and connected to a digital control unit. Temperature and pH were measured using the probes and the pH was maintained by automatic feeding of 1N NaOH and 1N HCl. Compressed air was supplied through the sterile filter. The agitation and aeration could be manually controlled in parallel to the dissolved oxygen. The dissolved oxygen level and pH were controlled and measured. Foaming was eliminated by using silicone oil (Himedia, India).

4.10.1. Batch cultivation

The batch process was started with an initial volume of sterile 2.5 L Molasses peptone Broth (MPB) (Molasses 10 %, peptone 0.25 %) at pH 7 and inoculated with 25 ml shake flask

pre-inoculated *B. subtilis* B1 culture and the temperature was maintained at 30˚C. In bioreactor, the optimum aeration and agitation were standardized using a range from 0.5 to 3 lpm (liquid per minute) and 0 to 350 rpm (rotation per minute), respectively. The dissolved oxygen set at the beginning was 100 % and it was maintained above 20 % (Matar *et al*., 2009) manually by controlling the agitation. After every 24 hours of growth, the optical density (OD) of the samples at 600 nm was taken and viable cells count as well as cell dry weight were calculated.

4.10.2. Viable cells count

The plate count method was used to determine the number of viable cells during *B. subtilis* B1 growth in the bioreactor (Postgate, 1969). In this method, 1 ml of the fermentation broth was serially diluted in sterile distilled water to form different dilutions. From this, 100 µl of several dilutions (10^{-6} – 10^{-9}) were spread plated on nutrient agar plate in triplicates. The plates were incubated at 37˚C for 24 hours and the colonies were counted. The number of colony forming units (CFU) per ml was estimated from the colonies counted with the dilution factor.

4.10.3. Biomass concentration

The biomass concentration was determined by the dry weight method explained (Rivera, 1998). 10 ml samples of fermentation broth was collected after 24 hours and filtered on a dried, previously weighed 0.45 µm membrane filter under vacuum. The filtered mass was kept at 100˚C for 24 hours in an oven, and the dry weight (g/ l), was calculated by deducting the filter dry weight from the added weight of mass and filter.

4.11. Testing the effectiveness of the mass produced *Bacillus subtilis* B1 against the stain fungus on rubberwood

Two ways of laboratory evaluation trials were carried out with the mass produced *Bacillus subtilis* B1.

4.11.1. Complete treatment in the controlled environment (aseptic conditions) (Sajitha and Florence, 2013).

Thirty fresh rubberwood blocks (7 x 5 x 1 cm) were cleaned, steam sterilized at 121˚C, 100 kPa pressure for 20 min. These blocks were dipped in the mass produced *B. subtilis* B1 culture (2.7 x10^{9} CFU/ ml) obtained in the sixth day for 10 min, drained and kept in sterile petridishes with moist filter paper. An 8 mm disc of an actively growing *L. theobromae* culture was inoculated on

the wood blocks aseptically and incubated at 28˚C for one month and were observed for fungal growth. Thirty sterile wood blocks dipped in the plain medium, inoculated with the fungus were kept as control and incubated in the same condition as above.

4.11.2. Treatment conducted in the lab (non-aseptic condition) (Behrendt et al., 1995)

The design used for the experiment was completely randomised design without replacement. Thirty fresh rubberwood blocks (7 x 5 x 1 cm) were cleaned, and dipped in the mass produced *B. subtilis* B1 culture (2.7 $x10^9$ CFU/ ml) for 10 min and the excess culture was drained. Three sets of treated wood blocks, each with 10 planks were kept open in room temperature. In each set, wood blocks were inoculated on the top with 8 mm disc of actively growing *L. theobromae*. The blocks with the fungus were kept one over the other in such a way that, each fungal disc was in contact with the treated wood blocks. In the same way, thirty blocks dipped in the plain medium and inoculated with the test fungus were kept open in three sets as control. 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 3 (Florence and Balasundaran, 2009).

Statistical analysis

Independent t-test was conducted for mean comparison between the fungal infection in the treated and control wood blocks using SPSS version17 (SPSS Inc., Chicago, Illinois, U.S.A).

4.11.3. Treatment conducted in the field

Testing the effectiveness of the bacterium B1 on rubberwood in the field during the three seasons, Northeast monsoon, Summer and Southwest monsoon were conducted. Molasses peptone broth was the medium used for preparing bacterial inoculum. The field testing was done during the month of March (Summer), July (Southwest monsoon) and November (Northeast monsoon) at Evershine Packing Industries, Ollur, Thrissur. The design used for the experiment was completely randomised design without replacement. Fresh rubberwood planks of 500 x 100 x 10 mm thickness were used (Florence and Balasundaran, 2009). After removing the sawdust, wood blocks were painted with bacterial culture (2.7 $x10^9$ CFU/ ml), using a standard sized painting brush. The excess inoculum from the wood planks was drained by keeping them in slanting position and stacked closely and openly. For all seasons, the same

pattern of stacking was followed. Control wood blocks were painted with plain molasses peptone medium.

4.11.3.1. Close stacking

For close stacking, three stacks were maintained for both treatment and control. In a single stack, there were a total of 30 planks. Each stack consisted of six rows with five planks. The planks were stacked in a criss-cross manner (Fig. 2).

Fig. 2. Close stacking of rubberwood blocks

4.11.3.2. 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. Three stacks were maintained for both the treatment and control (Fig. 3).

Fig. 3. Open stacking of rubberwood blocks

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 3. The planks were plained for observing the internal stain.

Table 3. Rating index for mould/ stain/ decay (Florence and Balasundaran, 2009)

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

Statistical analysis

Data was statistically analyzed using analysis of variance, followed by Duncan's multiple range test (P = 0.05), using SPSS version17 (SPSS Inc., Chicago, Illinois, U.S.A).

5. RESULTS

5.1. Isolation of test fungus

The test fungus *Lasiodiplodia theobromae* (Figs. 4 a & b) was isolated from the bluish black stained rubberwood on to the PDA plate and identified based on the colony morphology.

Fig. 4a. *Lasiodiplodia theobromae* infected rubberwood block

Fig. 4b. Pure culture of *Lasiodiplodia theobromae* isolated from the infected wood block

5.2. Isolation, screening and identification of bacteria

The total bacterial count from all the three composts was tabulated in Table 4.

Table 4. Total number of bacteria present in 1 g of each compost

Sl. No.	Composts	No. of bacteria/ g of compost (mean of 3 replicates) Standard error as subscripts
1	Weed Compost	$1.35 \times 10^{7}_{\pm 2.31}$
2.	Market Compost	$1.05 \times 10^{7}_{\pm 1.33}$
3.	Vermi Compost	$1.14 \times 10^{7}_{\pm 0.84}$

From the three aerobic compost samples, 17 visually different bacteria were isolated. The eight different bacteria isolated from weed compost were denoted as A1, A2, A3, A4, A5, A6, A7 and A8. A3 and A8 inhibited the growth of stain fungus *L. theobromae* (Fig. 5 b). Similarly, the five visually different bacteria from market available compost were named B1, B2, B3, B4 and B5. Of these, B1, B2 and B4 were found to be effective against the sapstain fungus (Fig. 5 b). The four

dissimilar bacteria from agricultural vermicompost were indicated as C1, C2, C3 and C4 and among these C3 and C4 were effective against test fungus (Fig. 5 b). The degree of antagonism and percentage of inhibition is provided in Table 5.

Out of the total isolates only 41 % showed inhibition towards the test fungus. Among these, the bacteria from market available compost (B1 and B2) were very effective in inhibiting the *L. theobromae*. The percentage of inhibition of bacterium B1 (64.44 %) was significantly different from others at p= 0.05.

Fig. 5a

Fig. 5b

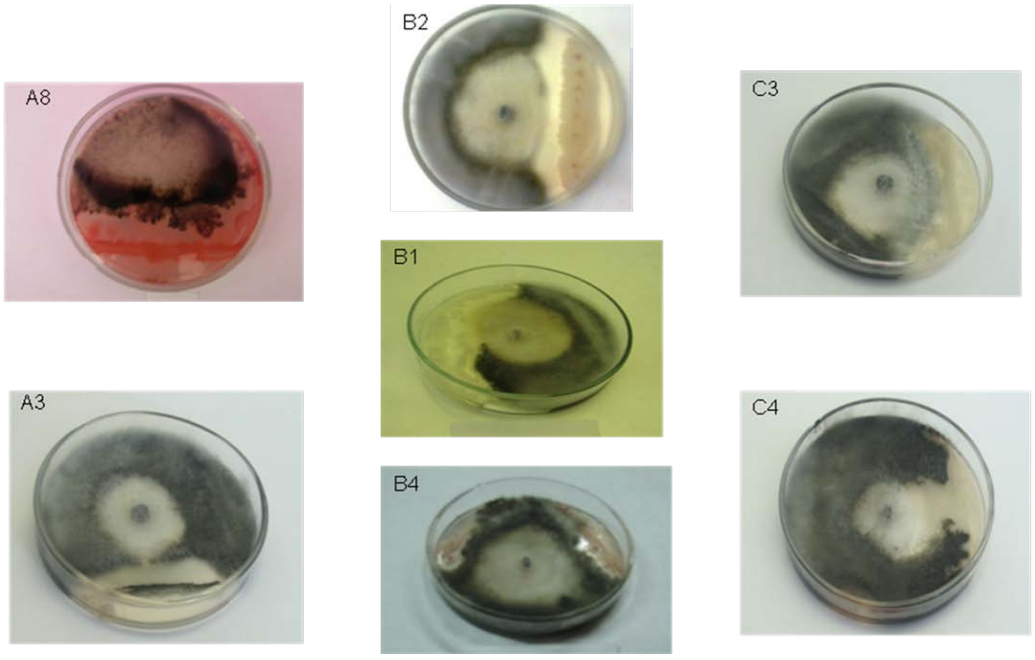


Fig. 5a. The control fungus *Lasiodiplodia theobromae* **5b.** Inhibitory effect of the 7 bacterial isolates co-cultured with sapstain fungus *Lasiodiplodia theobromae.*

Table 5. Degree of antagonism and percentage inhibition of the isolated bacteria from three different composts

Sl. No.	Compost	Bacteria	Antagonism	% Inhibition [x]	Inhibition(degrees) [y] Standard error is denoted as subscripts
1.	Weed compost	A1	0	0	$0.08^{a}_{\pm 0.00}$
2	,,	A2	0	0	$0.08^{a}_{\pm 0.00}$
3	,,	A3	1	22.6	$13.02^{d}_{\pm 0.15}$
4	,,	A4	0	0	$0.08^{a}_{\pm 0.00}$
5	,,	A5	0	0	$0.08^{a}_{\pm 0.00}$
6	,,	A6	0	0	$0.08^{a}_{\pm 0.00}$
7	,,	A7	0	0	$0.08^{a}_{\pm 0.00}$
8	,,	A8	2	25.9	$15.02^{d}_{\pm 0.41}$
9	Market compost	B1	3	64.44	$40.13^{g}_{\pm 1.72}$
10	,,	B2	3	60.74	$37.26^{f}_{\pm 0.57}$
11	,,	B3	0	0	$0.08^{a}_{\pm 0.00}$
12	,,	B4	1	11.13	$4.78^{b}_{\pm 0.83}$
13	,,	B5	0	0	$0.08^{a}_{\pm 0.00}$
14	Vermi compost	C1	0	0	$0.08^{a}_{\pm 0.00}$
15	,,	C2	0	0	$0.08^{a}_{\pm 0.00}$
16	,,	C3	2	35.87	$21.02^{e}_{\pm 1.37}$
17	,,	C4	1	17.77	$8.21^{c}_{\pm 1.63}$

[x] The inhibition percentage calculated from the plates using the equation % I = 100 x (r1-r2) / r1.

[y] The degree of inhibition calculated after arcsine transformation. The treatment means with different alphabetic superscripts differ significantly at p=0.05 according to Duncan's multiple range of comparison test.

5.2.1. Bacterial Identification

The seven antagonists from the various composts were identified using different morphological, biochemical and molecular techniques.

5.2.1.1. Colony morphology studies

The colony morphology of A3, A8 and B2 were similar but the colony size of A8 and B2 were larger than A3. Similarly, the colony colour of B2 was different from A3 and A8. The morphological characters of the seven antagonists are summarized in Table 6.

5.2.1.2. Staining and biochemical tests

All the seven isolates were Gram positive, endospore bearing, motile rods. The test results of staining and biochemicals were summarized in Table 6. A3, A8 and B1were showing same result except for TSI test, where A8 and B1 were producing gas along with the fermentation while A3 was not producing gas during the triple sugar fermentation. Among the seven isolates, C3 alone produced indole and hydrogen sulphide gas and couldn't utilize the citrate carbon source.

Comparing the results of colony morphology and biochemical tests with the identification flowchart of Bergey's Manual of Determinative Bacteriology, all the Gram positive isolates showed the features of the genera *Bacillus* and hence HiCrome Bacillus agar was tried for the species level identification.

Table 6. Colony morphology, staining tests and biochemical tests of seven screened antagonists

Identification techniques	**A3**	**A8**	**B1**	**B2**	**B4**	**C3**	**C4**
Colony morphology							
Size	L	L	L	L	L	M	L
Form	Ir	Ir	Ci	Ir	Ci	Ci	Ir
Margin	U	U	E	U	E	E	U
Elevation	F	F	Co	F	F	Ra	F
Texture	R	R	S	R	R	S	R
Pigment	NP	NP	NP	NP	NP	NP	NP
Opacity	O	O	TL	O	TL	O	TL
Consistency	D	D	M	D	D	M	D
Colour	White	White	White	Light brown	Light brown	Cream	White
Gram staining	+	+	+	+	+	+	+
Endospore staining	With spore	With spore	With spore	With spore	With spore	With spore	With spore
Motility	Motile	Motile	Motile	Motile	Motile	Motile	Motile
Biochemicals							
Casein hydrolysis	+	+	+	+	+	-	-
Starch hydrolysis	+	+	+	+	+	+	+
CMC hydrolysis	+	+	+	+	+	+	+
H_2S production	-	-	-	-	-	+	-
Indole production	-	-	-	-	-	+	-
MR test	+	+	+	-	-	-	+
VP test	+	+	+	+	+	+	+
Citrate utilization	+	+	+	+	+	-	+
Urease	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+
TSI	YB,RS, no gas	YB,RS, gas	YB,RS, gas	YB,RS, gas	YB,RS, gas	YB,YS, gas	YB,RS no gas

L- large, M- medium, Ir- irregular, Ci- circular, U- undulate, E- entire, F- flat, Co- convex, Ra- raised, R- rough, S- smooth, NP- nonpigment, O- opaque, TL- translucent, D- dry, M- mucoid, CMC- Carboxy Methyl Cellulose, MR- Methyl Red, VP- Voges Proskauer, TSI- Triple Sugar Iron, YB- Yellow Butt, RS- Red Slant, YS- Yellow Slant

5.2.1.3. Identification using HiCrome Bacillus agar

Among the seven bacteria, A3, A8, B1, B2, B4 and C4 grew effectively on HiCrome Bacillus agar in the absence of selective supplement polymixin B and gave green colonies, which was the characteristic property of *B. subtilis*. C3 gave yellow colonies. Bacillus agar with polymixin B slightly inhibited their growth.

5.2.1.4. PCR amplification and sequencing

Molecular identification of the isolated strains was carried out based on 16S rRNA-encoding gene sequence analysis. The partial 16S rRNA-encoding gene sequence of each isolate was subjected to *BLAST* sequence similarity search. Out of the seven isolates, A3, A8, B1, B2, B4 and C4 showed 100 % sequence homology to the 16S rRNA-encoding gene sequence of *B. subtilis* strain ZQ1101 (Genbank accession no. KJ 538550.1) and 99 % sequence similarity to *B. subtilis* strain LD181 (Genbank accession no. KJ564129.1). Bacterium C3 showed 100 % sequence similarity to *Paenibacillus polymyxa* strain 1096 (Genbank accession no. EU982487. 1).

Based on their morphological and biochemical characteristics, the isolated strains were identified to belong to the genus *Bacillus*. *Bacillus* species identification was performed by their growth in HiCrome Bacillus agar and species identity was confirmed using 16S rRNA-encoding gene sequence based phylogeny analyses. Accordingly, the isolated strains were named as *B. subtilis* A3, *B. subtilis* A8, *B. subtilis* B1, *B. subtilis* B2, *B. subtilis* B4, *B. subtilis* C4 and *Paenibacillus polymyxa* (*Bacillus polymyxa)* (Nemeckova *et al*., 2011) C3 and partial 16S rRNA-encoding gene sequences of the respective strains were deposited into NCBI Genbank as strain A3 (KJ652086), A8 (KJ652091), B1 (KJ652088), B2 (FJ445405), B4 (KJ652090), C4 (KJ652089) and C3 (KJ652087). The results revealed that, in the selected aerobic composts, *B. subtilis* was the major antagonistic microorganism against sapstain fungus, *L. theobromae*.

5.3. Selection of antagonist

The most effective antagonist, *B. subtilis* B1, which gave 64.44 % inhibition to the stain fungus *L. theobromae* was selected for the further studies.

5.4. Effect of standard iturin A on *Lasiodiplodia theobromae*

Standard iturin A could effectively inhibit the growth as well as the bluish black colouration of *L. theobromae* on PDA plate, while fungus was fully grown on the control plate (Figs. 6a and b).

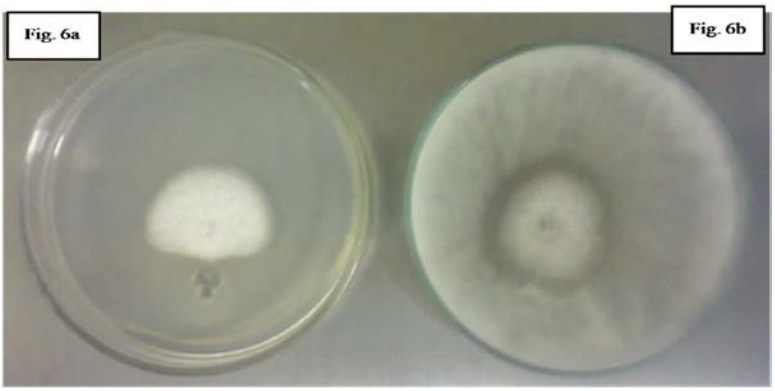


Fig. 6a Iturin A standard inhibiting the growth of *Lasiodiplodia theobromae*
Fig. 6b Fully grown fungus on the control plate

5.5. Identification of iturin A biosynthetic gene in *Bacillus subtilis* B1 by polymerase chain reaction

Iturin A synthetase gene of 594 bp was obtained by PCR amplification (Fig. 7), which shows the presence of iturin A biosynthetic gene in *B. subtilis* B1 genome.

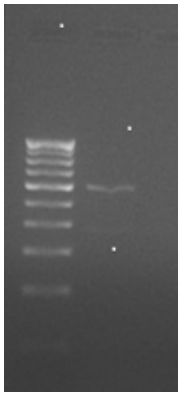


Fig. 7. PCR product of 594bp of iturin A biosynthetic gene in *Bacillus subtilis* B1 genome

5.6. Enhanced production of antibiotic iturin A and its qualitative and quantitative determination by HPLC

5.6.1. Effect of media on antibiotic production

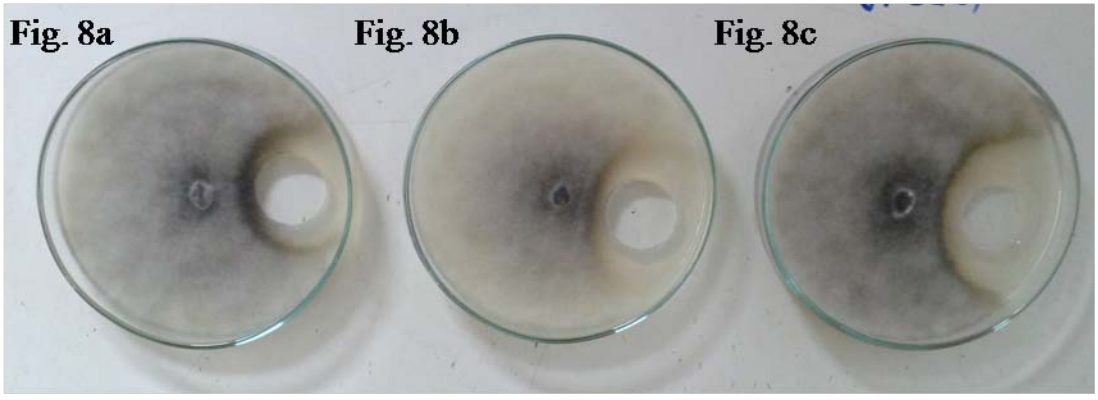
Among the twelve different media tested, landy broth (medium No. 3) was found to be very effective in antifungal antibiotic production which inhibited *L. theobromae* with an inhibition of 75.7 %. The inhibitory effect of the bacterial culture in various media is given in Table 7.

Table 7. Inhibitory effect of the bacterial culture filtrate grown in various media

Media	**Inhibition % (1 ml filtrate) mean of 3**
No. 1	55 %
No. 2 (YGB)	50 %
No. 3 (LB)	75.7 %
No. 4	65 %
No. 5	Nil
No. 6 (TSB)	58 %
No.7	Nil
No. 8	Nil
No. 9	Nil
No. 10	Nil
No.11 (KMB)	60 %
No. 12	27 %

5.6.2. Effect of temperature and pH on antibiotic production

Bacillus subtilis B1 culture grown in landy broth with pH 7, at 30ºC was effective in antifungal metabolite production. The effect of different pH and temperature on antifungal metabolite production is given in Table 8. The observations on the inhibitory effect of the culture filtrate were made from the first day onwards. No inhibition was noted in the first two days of the culture filtrate. From third day onwards inhibition was observed and the highest inhibition was noted on the sixth day. The inhibition percentage of 200 µl, 500 µl and 1000 µl of the culture filtrate were 68.14 %, 71.78 % and 75.7 % respectively (Figs. 8 a, b & c).



Figs. 8a, b & c Inhibitory effect of the bacterial culture filtrate (200 µl, 500 µl and 1000 µl) grown in LB

Table 8. Inhibitory effect of the bacterial culture filtrate grown in LB at different pH and temperatures

Sl. No	pH	Inhibition % (mean of 3 replicates)	Temperature	Inhibition % (mean of 3 replicates)
1	4	No inhibition	20	20
2	5	50	25	64
3	6	65	30	75.2
4	7	75	35	60
5	8	60	40	40
6	9	42	45	20
7	10	Very less	50	No inhibition

5.6.3. HPLC analysis

The antibiotics extracted from the culture filtrate were dissolved in methanol and analyzed in HPLC. The individual iturin A isomers were well separated on a RP-C18 analytical column. The peaks of iturin A isomers in the sample are identified by the retention time of standard iturin A (Sigma, USA). The standard curve (peak-area; Y, versus concentration; X (mg/ l) was constructed in the range of 50-200 mg/ l for iturin A. Regression analysis for HPLC provided a linear relationship of concentration to absorbance. The obtained linear regression equation was Y = 20234.246X + 440903.5 (where X is the concentration in mg/ l and Y is the peak area of the chromatogram), correlation coefficient (R) was 0.9972.

The proposed method was applied for the determination of the concentration of iturin A in a sample. The chromatograms of the standard iturin (200 ppm) and extracted solution are shown in Figs. 9 a & b. Five homologues of iturin A standard were detected in the HPLC. The retention times of five homologues were 9.423, 10.427, 14.817, 15.787 and 26.496 minutes.

Fig. 9a

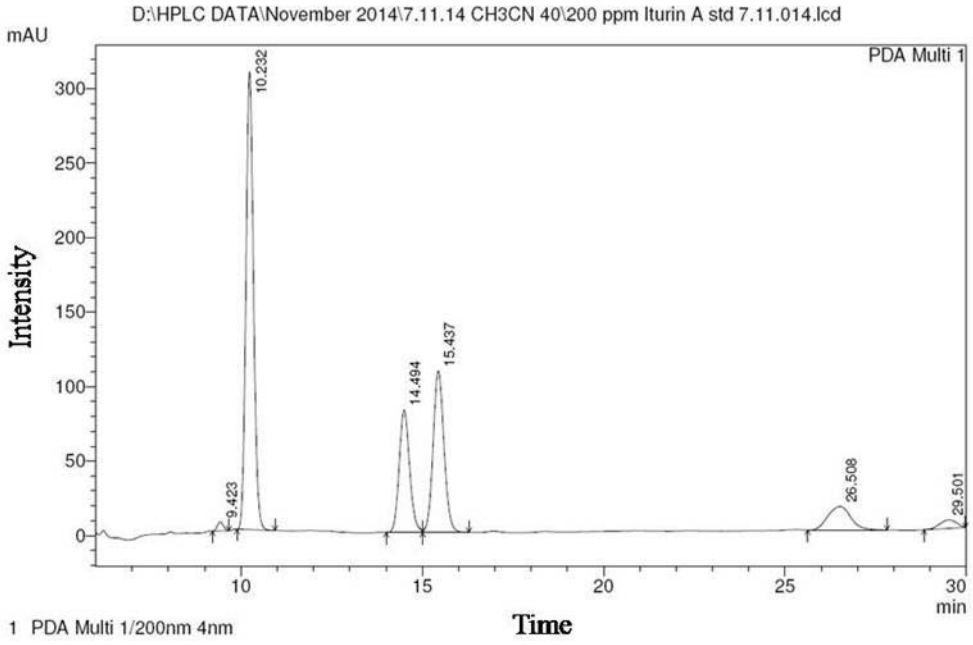


Fig. 9b

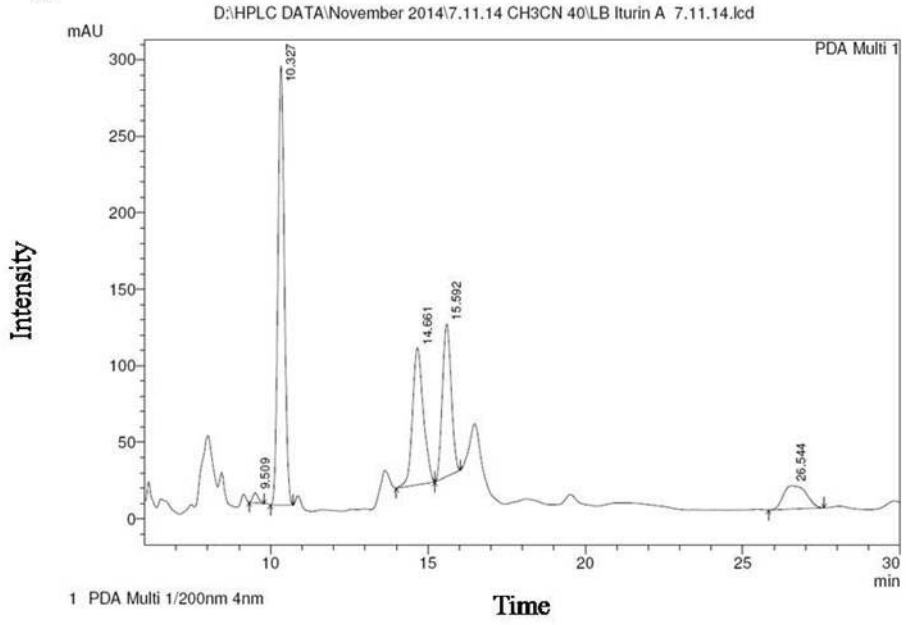


Fig. 9. HPLC chromatograms of (a) iturin A standard and (b) iturin A from *Bacillus subtilis* B1 grown in landy broth medium. Column: RP-C18 analytical column. Eluent: acetonitrile and water (40:60, v/ v). Flow-rate: 1.0 ml/ min. Temperature: 40^{o}C. Detection: 200 nm

The concentration of 178.499 mg/ l of iturin A was determined in the sample by the regression equation obtained from the chromatogram of peak areas and concentrations of standard solutions.

5.7. Mass production of *Bacillus subtilis* B1

In the preliminary shake flask study, the medium, pH and temperature for the optimum growth of *B. subtilis* B1 was standardized.

5.7.1. Shake flask study

Among the six different media tried, the growth of *B. subtilis* B1 was more in the CGPB medium. But due to the commercial non-availability of coconut water on a wider scale, MPB was selected as an ideal medium for further scale up production (Fig. 10 a). The effective pH and temperature for the optimum growth was standardized as 7 and 30 - 37˚C (Figs. 10 b & c).

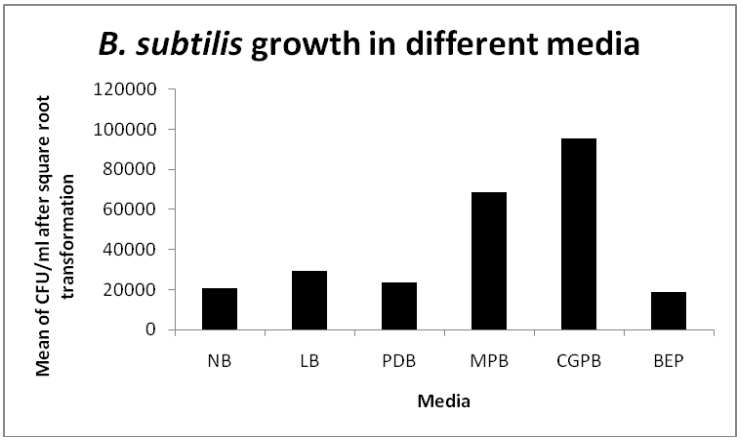


Fig. 10a. Effect of *Bacillus subtilis* B1 growth in different media after 24 hours of growth

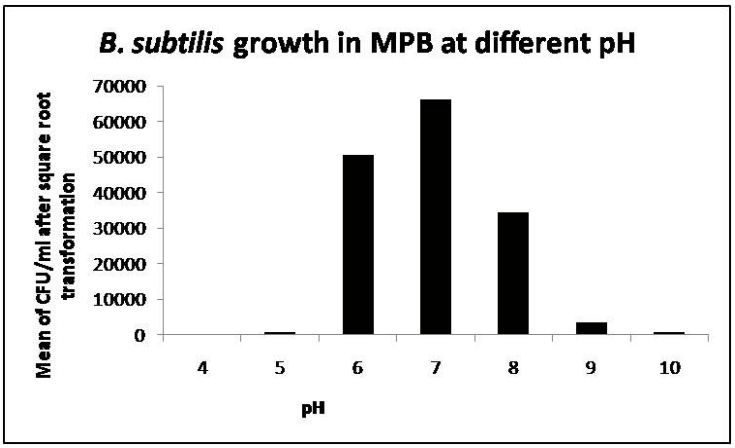


Fig. 10b. Effect of *Bacillus subtilis* B1 growth in MPB medium at different pH after 24 hours of growth

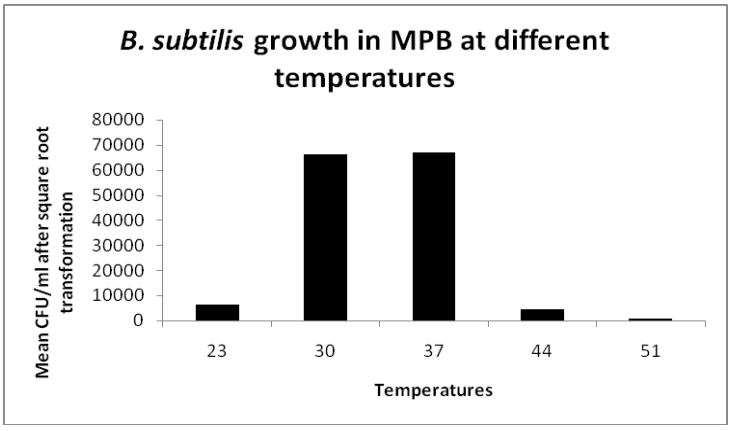


Fig. 10c. Effect of *Bacillus subtilis* B1 growth in MPB medium at different temperatures after 24 hours of growth

4.7.2. Mass production of Bacillus subtilis B1in the bioreactor

During standardization, higher aeration, agitation and temperature resulted in excess evaporation and medium loss. Considering the evaporation of the medium in the bioreactor, the aeration was maintained at 1 lpm and the temperature was maintained at 30˚C, throughout the fermentation reaction. The agitation was started at 250 rpm and was slightly adjusted to maintain the dissolved oxygen at 20 %. When batch cultivation of *B. subtilis* B1 was carried out in the 3 L bioreactor in the controlled conditions, the viable cell count was 1.13 x 10^{11} CFU/ ml and biomass achieved was 10.5 g/ l, after 24 hours (Table 9). The optical density values at 600 nm and viable cell counts after each 24 hours upto 168^{th} hour (7^{th} day) are given in Table 10. No difference was observed in the biomass attained after each 24 hours. *B. subtilis* B1 in the bioreactor using MPB medium produced antifungal metabolite from the third day onwards and the highest inhibition using metabolite filtrate was observed in the sixth day. When compared to the antibiotic production medium landy broth, the inhibition by culture filtrate was less for *B. subtilis* B1 in the MPB medium (44.44 %).

Table 9. Effect of different rotation per minute and liquid per minute on the growth of *Bacillus subtilis* in the bioreactor

Sl. No.	rpm	lpm	CFU/ ml after 24 hours of incubation
1.	No rpm	0.5	4.70×10^5
		1	2.00×10^6
		1.5	5.00×10^7
		2	5.12×10^7
		2.5	1.50×10^8
		3	5.36×10^8
2.	100	0.5	3.00×10^8
		1	1.50×10^9
		1.5	1.00×10^9
		2	1.00×10^{10}
		2.5	3.00×10^9
		3	Slight medium loss
3	150	0.5	4.93×10^8
		1	3.80×10^9
		1.5	1.80×10^{10}
		2	2.46×10^{10}
		2.5	4.05×10^9 (Slight medium loss)
		3	Medium loss
4.	200	0.5	2.00×10^9
		1	7.00×10^9
		1.5	5.00×10^{10}
		2	2.98×10^{10}
		2.5	3.50×10^9 (Slight medium loss)
		3	Medium loss
5.	250	0.5	5.15×10^{10}
		1	1.13×10^{11}
		1.5	8.40×10^{10}
		2	1.88×10^{10} (Slight medium loss)
		2.5	Medium loss observed
		3	Medium loss observed
6.	300	0.5	2.80×10^{10}
		1	1.00×10^{10}
		1.5	1.08×10^{10} (Slight medium loss)
		2	Medium loss observed
		2.5	Medium loss observed
		3	Medium loss observed
7.	350	0.5	Medium loss observed
		1	Medium loss observed
		1.5	Medium loss observed
		2	Medium loss observed
		2.5	Medium loss observed
		3	Medium loss observed

Table 10. Optical density and viable cell count of *Bacillus subtilis* B1 grown in Molasses Peptone Broth (MPB) medium in the bioreactor

Days	Optical Density (OD) at 600 nm (mean of 3 experiments)	Viable cell count (mean of 3 experiments) Standard error denoted as subscripts
1	2.65	$1.13 \times 10^{11}{}_{\pm 8.82 \times 10^{9}}$
2	2.25	$4.23 \times 10^{9}{}_{\pm 1.45 \times 10^{8}}$
3	2.21	$3.43 \times 10^{9}{}_{\pm 1.2 \times 10^{8}}$
4	2.21	$3.1 \times 10^{9}{}_{\pm 5.77 \times 10^{7}}$
5	2.15	$2.73 \times 10^{9}{}_{\pm 1.45 \times 10^{8}}$
6	2.07	$2.7 \times 10^{9}{}_{\pm 1.15 \times 10^{8}}$
7	1.75	$7.03 \times 10^{8}{}_{\pm 4.98 \times 10^{7}}$

5.8. Testing the effectiveness of the mass produced *Bacillus subtilis* B1 against the stain fungus on rubberwood

5.8.1. Complete treatment in the controlled environment (aseptic conditions)

Thirty rubberwood blocks, treated with the *B. subtilis* B1 culture and inoculated with the test fungus, efficiently inhibited the fungal growth. Inhibition of *L. theobromae* was 100 % in this experiment under controlled aseptic conditions. Whereas, the control wood blocks treated with plain medium were fully infected by the inoculated stain fungus *L. theobromae* (Figs. 11 a & b).

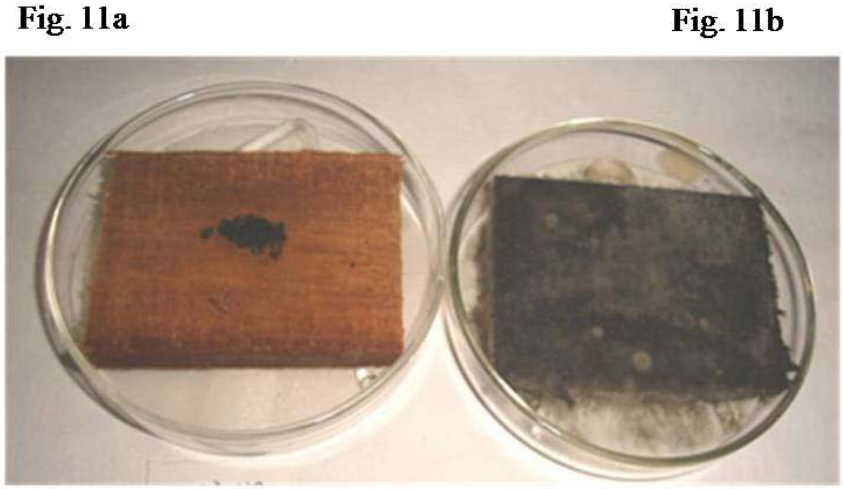


Fig. 11. (a) Inhibition of *Lasiodiplodia theobromae* infection on rubberwood blocks treated with *Bacillus subtilis* B1culture (b) Fully infected rubberwood blocks dipped in plain medium and inoculated with *Lasiodiplodia theobromae*.

5.8.2. Treatment conducted in the lab (non-aseptic conditions)

Thirty rubberwood blocks treated with *B. subtilis* and kept in three sets under non-asceptic conditions were found effective in controlling the inoculated test fungus, *L. theobromae* (Fig. 12 a). But profuse fungal infection was noted in the control wood blocks (Fig. 12 b). Visual rating was given for the fungal infection (Table 11). Independent t-test was conducted for mean comparison and significant difference was observed in the group means, between the fungal infection in the treated and control wood blocks (the value in "Sig. (2- tailed)" row is less than 0.05) (Table 12).

Table 11. Visual rating of control and *Bacillus subtilis* B1 treated rubberwood blocks in the open environment (Florence and Balasundaran, 2009)

	Control		***Bacillus subtilis* B1treated**	
Replica	Surface growth % (Mean of 10 planks)	Rating	Surface growth % (Mean of 10 planks)	Rating
1	$71_{\pm 1.19}$	4	$3.4_{\pm 0.16}$	1
2	$68_{\pm 0.76}$	4	$3.1_{\pm 0.19}$	1
3	$69.5_{\pm 0.56}$	4	$3.2_{\pm 0.2}$	1

Standard error is denoted as subscripts

Table 12. Independent t-test for mean comparison of the treated and control rubberwood blocks in the open laboratory condition

Independent Samples Test										
Levene's Test for Equality of Variances				t-test for Equality of Means						
									95 % Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Wood fungal infection	Equal variances assumed	29.02	.000	15.88	58	.000	66.27	4.17	57.91	74.62
	Equal variances not assumed			15.88	29.88	.000	66.27	4.17	57.74	74.79

Fig. 12a

Fig. 12b

Fig. 12. (a) Rubberwood blocks infected with *Lasiodiplodia theobromae* kept in the open laboratory environment as control (b) Inhibition of *Lasiodiplodia theobromae* infection on rubberwood blocks treated with *Bacillus subtilis* B1 culture kept in the open laboratory environment

5.8.3. Testing the antagonists in the field

The visual ratings are given for the treated and control wood blocks in all the three seasons- Summer, Southwest monsoon and Northeast monsoon.

5.8.3.1. Wood surface fungal growth and internal stain in close stacking conditions

The observations of fungal growth on wood surface and their internal stain on close stacked wood blocks in all the three seasons are tabulated in Table 13. The Duncan's multiple range tests for control and treated means were done.

Table 13. Comparison of wood surface growth (%) and internal stain (%) in closed stacked wood treatments in all 3 seasons using Duncan's multiple range tests

No.	Seasons	Treatments (closed stacked)	Wood surface growth in % (Mean of 3 replicas)	Visual rating (Surface)	Wood internal stain in % (Mean of 3 replicas)	Visual rating (stain)
1	Northeast monsoon	Control	$76.53^{e}_{\pm 0.76}$	5	$37.53^{d}_{\pm 1.66}$	3
2	,,	Treated	$9.28^{b}_{\pm 0.362}$	1	$2.2^{a}_{\pm 0.13}$	1
3	Summer	Control	$63.18^{d}_{\pm 1.6}$	4	$31.59^{c}_{\pm 0.8}$	3
4	,,	Treated	$5.9^{a}_{\pm 0.17}$	1	$0.48^{a}_{\pm 0.08}$	1
5	Southwest monsoon	Control	$95.87^{f}_{\pm 0.13}$	5	$73.08^{e}_{\pm 0.82}$	4
6	,,	Treated	$21.37^{c}_{\pm 1.63}$	2	$4.9^{b}_{\pm 0.15}$	1

Means with different alphabetic superscripts differ significantly.

Standard error is denoted as subscripts

5.8.3.1.1. Surface growth and internal stain in Northeast monsoon

In Northeast monsoon, after one month of incubation, the rating of surface growth of fungi on the bacterial treated planks was 1 whereas it was 5 for control (Table 13, Fig 13 a & b). On control planks after planing, the internal stain and decay was 31.59 % and appeared grayish black (Fig. 14 a). After planing, the internal stain and decay was only 2.2 % on treated planks (Fig. 14 b, Table 13).

Fig. 13a **Fig. 13b**

Fig. 13. (a) Surface growth on close stacked control rubberwood blocks in the Northeast monsoon after one month of incubation (b) Inhibition of fungal growth on close stacked rubberwood blocks treated with *Bacillus subtilis* B1 culture

Fig. 14a

Fig. 14b

Fig. 14. (a) Internal stain on close stacked control rubberwood blocks in the Northeast monsoon after one month of incubation (b) Absence of internal stain on close stacked rubberwood blocks treated with *Bacillus subtilis* B1culture after one month of incubation

5.8.3.1.2. Surface growth and internal stain in summer season

After one month of incubation in the summer season, the rating of surface growth of fungi on the bacterial treated planks was 1 whereas it was 4 on control (Table 13, Fig. 15 a & b). After plaining, it was found that the internal stain and decay was only 0.48 % on treated planks (Table 13, Fig. 16 b). On control planks after plaining, the internal staining and decay were 37.53 % and appeared grayish black (Table 13, Fig. 16 a). A reduction in the fungal growth was observed in the treated planks in the summer season.

Fig. 15a

Fig. 15b

Fig. 15. (a) Surface growth on close stacked control rubberwood blocks during the summer season after one month of incubation (b) Inhibition of fungal growth on close stacked rubberwood blocks treated with *Bacillus subtilis* B1culture during summer season

Fig. 16a

Fig. 16b

Fig. 16. (a) Internal stain on close stacked control rubberwood blocks during the summer season after one month of incubation (b) Absence of fungal growth on close stacked rubberwood blocks treated with *Bacillus subtilis* B1culture

5.8.3.1.3. Surface growth and internal stain in Southwest monsoon

During the Southwest monsoon season, the rating for surface growth of fungi on the closely stacked bacterial treated planks was 2 whereas it was 5 on control (Fig. 17 a & b, Table 13). When compared to other seasons, more infection was observed in this season. After plaining, the internal stain and decay on treated planks was 4.9 % (Fig.18 a, Table 13). On control planks after plaining, the internal staining and decay was high 73.08 % and appeared more grayish black (Fig. 18 b).

Fig. 17a

Fig. 17b

Fig. 17. (a) Surface growth on close stacked control rubberwood blocks in the Southwest monsoon after one month of incubation (b) Inhibition of fungal growth on close stacked rubberwood blocks treated with *Bacillus subtilis* B1culture

Fig. 18a

Fig. 18b

Fig. 18a. (a) Internal stain on close stacked control rubberwood blocks in the Southwest monsoon after one month of incubation (b) Slight internal stain on close stacked rubberwood blocks treated with *Bacillus subtilis* B1 culture in southwest monsoon

5.8.3.2. Wood surface fungal growth and internal stain in open stacking conditions

Observations of fungal growth on wood surface and their internal stain in open stacked wood blocks in all the three seasons are given in Table 14. The Duncan's multiple range test for control and treated means were done.

Table 14. Comparison of wood surface growth (%) and internal stain (%) in open stacked wood treatments in all 3 seasons using Duncan's multiple range test

No.	Seasons	Treatments (open stacked)	Wood surface growth in % (Mean of 3 replicas)	Visual rating (Surface growth)	Wood internal stain in % (Mean of 3 replicas)	Visual rating (internal stain)
1	Northeast monsoon	Control	$46.76^{d}_{\pm 0.89}$	3	$20.41^{c}_{\pm 0.59}$	2
2	,,	Treated	$1.64^{a}_{\pm 0.28}$	1	$0.00^{a}_{\pm 0.0}$	0
3	Summer season	Control	$34.18^{c}_{\pm 2.5}$	3	$23.45^{d}_{\pm 0.67}$	2
4	,,	Treated	$1.64^{a}_{\pm 0.33}$	1	$0.00^{a}_{\pm 0.00}$	0
5	Southwest monsoon	Control	$90.38^{e}_{\pm 1.38}$	5	$53.18^{e}_{\pm 0.84}$	4
6	,,	Treated	$10.37^{b}_{\pm 1.07}$	2	$1.79^{b}_{\pm 0.32}$	1

Means with different alphabetic superscripts differ significantly.

Standard error is denoted as subscripts

5.8.3.2.1. Surface growth and internal stain in Northeast monsoon

In the open stacktreated planks during Northeast monsoon, the percentage of fungal growth on the surface was 1.64 % whereas on control it was between 46.76 % (Fig. 19 a & b, Table 14). In the treated planks there was no internal stain or decay after plaining. The internal fungal stain on the control planks was 20.41 % (Table 14 and Fig. 20 a). Compared to close stacking there was much reduction in the surface growth as well as internal stain and decay in open stacking.

Fig. 19a **Fig. 19b**

Fig. 19. (a) Surface growth on open stacked control rubberwood blocks during the Northeast monsoon after one month of incubation (b) Inhibition of fungal growth on open stacked rubberwood blocks treated with *Bacillus subtilis* B1culture

Fig. 20a **Fig. 20b**

Fig. 20 (a) Internal stain on open stacked control rubberwood blocks during the Northeast monsoon after one month of incubation (b) Complete absence of fungal stain in open stacked rubberwood blocks treated with *Bacillus subtilis* B1culture

5.8.3.2.2. Surface growth and internal stain in summer season

In the open stacked treated planks during the month of March, the percentage of fungal growth on the surface was only 1.64 % whereas on control it was 34.18 % (Fig. 21 a & b). In the treated planks there was no internal stain or decay after plaining (Fig. 22 b, Table 14). The internal fungal stain on the control planks was 23.45 % (Table 14 and Fig. 22 a). As in the case of Northeast monsoon, compared to close stacking there was a reduction in the surface growth as well as internal stain and decay for open stacking.

Fig. 21a **Fig. 21b**

Fig. 21. (a) Surface growth on open stacked control rubberwood blocks in the summer season after one month of incubation (b) Inhibition of fungal growth on close stacked rubberwood blocks treated with *Bacillus subtilis* B1culture

Fig. 22a **Fig. 22b**

Fig. 22. (a) Internal stain on open stacked control rubberwood blocks in the summer season after one month of incubation (b) Complete inhibition of fungal growth on open stacked rubberwood blocks treated with *Bacillus subtilis* B1culture

5.8.3.2.3. Surface growth and internal stain in Southwest monsoon

In the open stacked treated planks during Southwest monsoon season, the percentage of fungal growth on the surface was only 10.37 % whereas on control it was high as 90.38 % (Fig. 23 a, Table 14). In the treated planks the internal stain or decay was rated as 1, after planing. The internal fungal growth on the control planks was rated as 4 (Table 14 and Fig. 24 a). Compared to close stacking there was a reduction in the surface growth as well as internal stain and decay for open stacking.

Fig. 23a

Fig. 23b

Fig. 23. (a) Surface growth on open stacked control rubberwood blocks in the Southwest monsoon after one month of incubation (b) Inhibition of fungal growth on open stacked rubberwood blocks treated with *Bacillus subtilis* B1culture

Fig. 24a

Fig. 24b

Fig. 24. (a) Internal stain in open stacked control rubberwood blocks in the Southwest season after one month of incubation (b) Slight fungal stain in open stacked rubberwood blocks treated with *Bacillus subtilis* B1culture

Statistical analysis

Significant difference at p = 0.05 was observed in analysis of variance followed by Duncan's Multiple Range Test between the means of control and treated wood blocks in all the seasons, in both open and close stacking conditions.

6. DISCUSSION

Among the seven antagonistic bacteria isolated from various aerobic composts in this study, six were different strains of *Bacillus subtilis* and the other *Paenibacillus polymyxa.* Occurrence of *Lactobacillus* and *Bacillus* genera in the mesophilic stages of compost processing was reported (Ryckeboer *et al*., 2003). In the thermophilic stages the bacterial community becomes more thermophilic and thermo-tolerant bacteria such as *Actinobacteria* (Fergus, 1964), *Bacillus* (Blanc *et al*., 1997) and *Thermus* (Beffa *et al*., 1996) become abundant. The presence of various *Bacilli* and other *Actinobacteria* in the commercial and homemade composts were also reported (Vaz-Moreira *et al*., 2008). The incidence of common bacterial members like *Pseudomonas*, *Bacillus*, *Aeromonas* and *Vibrio* in vermicompost is due to their presence in the intestine of *Eisenia foetida* (Toyota and Kimura, 2000).

Paenibacillus polymyxa or *Bacillus polymyxa* is a Gram positive, non-pathogenic nitrogen fixing bacterium usually found in soil or marine sediments. This is the first report on its occurrence in vermicompost. The reasons for the presence of *P. polymyxa* in the vermicompost may be due the worm associated microflora or due to the soil bedding/ plant debris used in the different stages of vermi composting (Munnoli and Bhosle, 2009). It has high economic importance in the agricultural and horticultural field as efficient plant growth promoting rhizobacteria, biofertilizer and as antagonists of various root pathogens (Bloemberg and Lugtenberg, 2001). *Paenibacillus polymyxa* has specific properties like the fermentation of mannitol, lactose and galactose and the production of hydrogen sulphide gas during the TSI test (Zengguo *et al*., 2007). Similar results were observed in the present investigation. This study showed the utility of an integrated approach employing morphological, biochemical and molecular tools for the conclusive identification of the bacterial community present in the aerobic composts of diverse sources. The present work demonstrated the efficacy of the antagonistic property of the *B. subtilis* strains against rubberwood sapstain fungus and supported the previous findings (Feio *et al*., 2004; Wang *et al*., 2012) on the significance of *B. subtilis* as a biocontrol agent.

The *in vitro* antagonistic ability of the bacterium, *B. subtilis* strain B1 against *L. theobromae* was very efficiently demonstrated adopting agar well diffusion assay in the present

study. The same method had been adopted previously for the *in vitro* screening of *B. subtilis* against *Fusarium graminearum* (Chen, 2008). The agar well diffusion assay performed with the different concentrations of *B. subtilis* culture filtrate on *L. theobromae* also proved the biocontrol ability of *B. subtilis* due to the presence of antifungal metabolites. The fungal inhibition displayed by the crude filtrate also supported the reports of Zhang *et al*. (2008). The biocontrol effect of *B. subtilis* and *B. amyloliquefaciens* on *L. theobromae* had also been previously reported (Sajitha *et al*., 2008; Swain and Ray, 2009). Among the antifungal antibiotics, iturins have been shown to exhibit strong antagonistic action against a wide range of yeast and fungi such as, *Mucor miehei*, *Neurospora crassa, Trichoderma viridae, Microbotrytum violaceum, Penicillium chrysogenum*, *Candida albicans, Aspergillus flavus, A. niger, Bipolaris maydis, Gibberellazeae, P. digitatum, Pyricularia oryzae, Rhizoctonia solani, Gloeosporium gleosporioides, Podosphaera fusca*, among others (Besson *et al*., 1976; Winkelmann *et al*., 1983; Latoud *et al*., 1988; Moyne *et al*., 2001; Yu *et al*., 2002; Cho *et al*., 2003; Gong *et al*., 2006; Romero *et al*., 2007). Amongst all iturins, iturin A has been found to be predominantly secreted by most strains of *B. subtilis* and *B. amyloliquefaciens (*Gong *et al*., 2006; Romero *et al*., 2007). This study also proved the efficiency of iturin A in inhibiting the sapstain fungus *L. theobromae*. Iturin A passes through the cell wall and interacts with the lipid components of cell membrane by forming ion conducting pores. Formation of pores in the membrane results in the release of electrolytes and other cytoplasmic components accompanied with the death of the cell. It has also been found to interact with nuclear membrane and membranes of other subcellular organelles (Maget Dana and Peypoux, 1994; Thimon *et al*., 1995). The aggregates of iturin A have been found to interact with phospholipids of cytoplasmic membrane to form a complex (Maget Dana and Peypoux, 1994). Furthermore, iturin A has been shown to induce formation of pores in artificial lipid bilayer at low concentrations probably through activation phospholipases (Maget Dana *et al*., 1985; Latoud *et al*., 1988). The activation of phospholipases may result in degradation of phospholipids resulting in formation of pores in membranes.

Mass production of biocontrol agents and their scaling up with an economically viable culture medium is an essential step for their commercial use as bioproducts (Teixido *et al*.,

2011). The present study reports the use of molasses as the major constituent for mass production of *B. subtilis* ($1.13x10^{11}$ CFU/ ml, 10.5 g/ l) in batch fermentation for biocontrol of rubberwood sapstain fungus. A few other investigations also support the use of molasses in the bacterial growth medium along with other nitrogen sources like ammonium phosphate (Younis *et al*., 2010; Yu-Peng *et al*., 2008). Along with bacterial mass production, antifungal metabolite production in MPB growth medium was also reported. The viable cells and biomass obtained in the present batch cultivation after 24 hours was high when compared to the mass production of *B. subtilis* in another batch reaction with low cost medium containing soyflour and molasses (Mendizabal *et al*., 2012). The result shows the efficiency of molasses broth as the major growth medium since a very small concentration of peptone (0.25 %) is used for the *B. subtilis* B1 mass production. More feasible method of mass production could be achieved in a fed batch bioreactor with regular supply of medium periodically (Matar *et al*., 2009).

The culture initially set at higher values of dissolved oxygen was found to diminish over time, which indicates cell growth and nutrient consumption by bacteria using dissolved oxygen. The oxygen supply was kept above 20 % by slightly adjusting the agitation rate (Matar *et al*., 2009) and this was found to be adequate. The moderate temperature of 30°C which was suggested by Thakaew and Niamsup (2013) was found to be sufficient in this study, for the growth and metabolite production of *B. subtilis* B1. The effect of antifungal metabolite production by bacteria like *Bacillus* to reduce the growth of mould and stain fungi on agar media and on different wood types was previously reported (Melentev *et al*., 2006). The antifungal metabolite production of *B. subtilis* was reported in the late log and stationary phases (Dunlap *et al*., 2011). In the present study, the stationary phase is attained in the second day and it continues upto the sixth day. The effect of secondary metabolites in the culture filtrate on sixth day was tested using well diffusion assay against the sapstain fungus, *L. theobromae* and proved that antibiosis plays an important role in the fungal biocontrol. In the bioreactor, using MPB medium, highest *B. subtilis* B1 growth was observed in the first day but the culture was maintained in the bioreactor till the completion of the stationary phase for the highest antifungal metabolite production (Dunlap *et al*., 2011). These antifungal metabolites along with the culture helped in the initial inhibition of the fungal attack on the rubberwood.

The antifungal metabolite production after the exponential phase, which is observed in this experiment, agrees with the reports on the synthesis of antifungal antibiotics, such as mycobacillin and bacilysin in *B. subtilis* (Katz and Demain, 1977).

The differences in the media composition of landy broth (LB) and molasses peptone broth (MPB) medium resulted in the quantitative differences of metabolite production (Katz and Demain, 1977). MPB is rather a nutrient rich growth medium than an antibiotic production medium, it supports the growth of *B. subtilis*. The high bacterial inoculum (2.7 x 10^9 CFU/ ml) along with the metabolites help preventing fungal growth on rubberwood. Similar reports on the metabolite production of B. subtilis in yeast extract glucose broth from the first day to the fourteenth day and their effect on *Aspergillus* sp. and *Trichoderma* sp. is available (Feio *et al*., 2004). Effective inhibition in the seventh and fourteenth day culture filtrate was also observed, which support the present findings on the highest metabolite production in the sixth day of incubation.

The biocontrol potential of *B. subtilis* against various wood staining and surface contaminant fungi were previously demonstrated (Silva *et al*., 1998; Feio *et al*., 2004; Moita *et al*., 2005). The biocontrol effect of two strains of *Trichoderma* against *L. theobromae* on rubberwood was carried out by Veenin *et al*. (1999) and detected some differences between the treated and control wood planks. The biocontrol effect of *Streptomyces* sp. tested in our laboratory was ineffective against *L. theobromae* on rubberwood due to the uneven growth of *Streptomyces* sp. (Sajitha and Florence, 2013). The present study under aseptic conditions was hundred per cent successful and could prove the biocontrol potential of the bacterium *B. subtilis* B1 and its metabolites. *B. subtilis* B1 attained inhibition of the sapstain fungus *L. theobromae* on rubberwood by competing for space, nutrients and by the antifungal metabolite production (Swain and Ray, 2009). In spite of the favourable temperature and moisture conditions provided under asceptic conditions, total fungal control was observed in the *B. subtilis* B1 treated rubberwood blocks, which again substantiate the biocontrol efficiency of *B. subtilis* B1 against the rubberwood sapstain fungus *L. theobromae*.

Beyond doubt, the efficiency of the biocontrol agent *B. subtilis* B1 against rubberwood sapstain fungus could be proved in the field test conducted across the three seasons. Even in

rainy seasons (Southwest and Northeast), which generally promote the growth of sapstain fungus, the biocontrol treatments were significantly effective. Accordingly, the field efficacy test in summer season was more effective in controlling sapstain infections. As explained previously by Florence and Balasundaran (2009), open stacking was more effective than close stacking for controlling the wood fungal infection. Open stacking provides much aeration between the blocks and dries them. The slightly dried wood blocks along with the bacterial culture containing antifungal antibiotics resulted in fungal growth inhibition on rubberwood blocks in all the seasons. The biocontrol agent, *B. subtilis* B1 and the antifungal antibiotics produced by it through cost effective scale up culture in the bioreactor could thus successfully demonstrate its efficiency to inhibit rubberwood sapstain fungus.

7. SUMMARY AND CONCLUSIONS

Seventeen visually different bacteria isolated from the three aerobic composts *viz.* weed compost (KFRI), vermi compost (KAU) and market available compost were screened by dual culture method to test their efficiency against the sapstain fungus *Lasiodiplodia theobromae*. Seven out of seventeen showed antagonism towards *L. theobromae* and six were identified as various strains of *Bacillus subtilis* and one as *B. polymyxa* by various morphological, staining, biochemical techniques and confirmed by polymerase chain reaction and sequencing of 16S rDNA gene. Among the seven, the most effective *B. subtilis* B1 was selected for the further studies. Twelve different antibiotic production media were used to test the antifungal antibiotic production of *B. subtilis* B1. Culture filtrate of *B. subtilis* B1 grown in Landy broth at pH 7 and temperature 30°C had enough antifungal antibiotics to inhibit the growth of *L. theobromae* to a maximum of 75.7 % in the agar plate. The antibiotics were extracted and qualitative and quantitative analysis of antifungal antibiotic iturin A was conducted in HPLC. Five homologues of iturin A with a concentration of 178.499 mg/ l of iturin A was determined in the sample by the regression equation obtained from the chromatogram of peak areas and concentrations of standard solutions.

Six different media were experimented in the growth study of *B. subtilis* B1. Coconut water glucose peptone broth at 30°C and pH 7 was effective for the growth of *B. subtilis* B1. But due to the non availability of the coconut water in all continents, molasses peptone broth was chosen for mass production of *B. subtilis* B1 in the bioreactor. The sixth day culture (2.7×10^9 CFU/ ml) from the bioreactor was tested in the laboratory under aseptic and non aseptic conditions on the treated and control rubberwood blocks inoculated with the sapstain fungus *L. theobromae*. Hundred percent fungal inhibitions were observed in the aseptic treatment while significant difference in fungal inhibition was noted in the treatments in non-aseptic conditions. The biocontrol potential of mass produced *B. subtilis* B1 was tested in three different seasons (Northeast monsoon, summer season and Southwest monsoon) in the timber saw mill at Thrissur, Kerala, India. After one month of incubation, significant difference in fungal growth was observed between the means of treated and control wood planks. All the wood planks after incubation were plained to check the internal stain. In the Northeast monsoon and

summer season, there was no internal stain in the openly stacked treated wood blocks. Slight infection with a rating of 1 was observed in the treated wood blocks in the close stacked condition in all the three seasons. In all the seasons, there was heavy surface growth and internal stain in all the control wood blocks.

The present study demonstrate the utility of the integrated approach, employing morphological, biochemical and molecular tools, for the conclusive identification of the bacterial community present in the aerobic composts of diverse sources. This is the first report on mass production of the biocontrol agent, *B. subtilis* strain B1 against sapstain fungus on rubberwood using an economically viable medium, which offers scope for further commercialization. This study also emphazises the role of antifungal antibiotics like iturin in controlling the fungal growth. The biocontrol efficiency of *Bacillus subtilis* against wood infecting fungus has been reinforced. Field studies carried out in the timber sawmill demonstrated the ability of the mass produced *B. subtilis* B1 against other stain, decay and mould fungi in addition to the sapstain fungus on rubberwood. Further experiments are in progress for the formulation of the biocontrol agent towards developing a holistic biocontrol approach against fungal infections on rubberwood timber logs.

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