KFRI Research Report No.

Standardization for enhanced production of antagonistic principle by *Bacillus subtilis* and *Streptomyces* for the control of sapstain on rubber wood

(Final report of KFRI 602/2010)

E. J. Maria Florence
Forest Protection Division

Kerala Forest Research Institute

Peechi – 680 653, Peechi, Thrissur, Kerala

October 2014

ABSTRACT OF PROJECT PROPOSAL

Project No:	KFRI 602/2010
Title of the project:	Standardization for enhanced production of antagonistic principle by *Bacillus subtilis* and *Streptomyces* for the control of sapstain on rubber wood
Objectives:	1. Standardization of physical parameters for the maximum production of chitinase from *Streptomyces* SA18 2. Standardization of physical parameters for the maximum production of antibiotics from *Bacillus subtilis* B2
Date of commencement:	August 2010
Date of completion:	December 2012
Funding agency:	KFRI Plan grant
Principal Investigator:	Dr. E. J. Maria Florence
Research Fellow:	K.L Sajitha (11/3/2011- 19/10/2011)
Technical Assistant:	Sneha Purushothaman (23/1/2012-31/12/2012)

ABSTRACT

One of the major problems in the wood industry is the wood stain and deterioration caused by various fungi. Bluish black sapstain infection by the dominant fungus *Lasiodiplodia theobromae* is a serious issue in wood utilisation. Bacteria, especially the *Streptomyces* sp. and *Bacillus* sp. are known biocontrol agents against various agricultural plant pathogens. In the previous research studies we have identified a *Streptomyces* sp. SA18 and a *B. subtilis* B2 as effective antagonist against the particular stain fungus *Lasiodiplodia theobromae.* The production of chitinase by *Streptomyces* sp. SA18 and antibiotic iturin A secreted by *B. subtilis* B2 for the fungal biocontrol was proved in the previous experiment. The standardization of chitinase and antibiotic production by the *Streptomyces* sp. SA18 and *B. subtilis* B2 respectively were conducted in the present study. The suitable medium and optimum conditions for the chitinase production by the *Streptomyces* sp. SA18 were standardized as chitin yeast extract salt medium at pH 8 and 35°C. The highest chitinase production was noted in the fourth day of incubation. The conditions standardized for antibiotic production by *B. subtilis* B2 were the sorbitol medium at pH 7 and 30°C. The highest antibiotic production was noted in the sixth day of incubation.

CONTENTS

1. Introduction

Wood has been used by man from time immemorial for construction purpose. Forest was the main source of wood. As the population increased, the demand for wood also increased. With the industrial revolution the demand went further up. The indiscriminate deforestation is showing signs of global warming, melting of polar ice, rising of sea water level, drought and consequent destruction. As science and technology developed, several alternate materials like metals, plastics and composites with improved strength and working properties were developed. But none of them had the feel of wood and it was concluded that there was no true substitute for wood. The continued search for wood led to the utilization of secondary species of wood like rubberwood, a plantation timber.

Hevea brasiliensis, native of Brazil, is the most important commercial source of natural rubber and it is grown in about 9 million hectares in the tropical regions of Asia, Africa and America. After the extraction of latex, at the age of 22 to 29 years, the trees are cut and replanted. Thus the rubber plantation is a sustainable source of rubber as well as timber, contributing positively to the environment. Rubberwood is one of the most important resources that are currently in great demand as raw material for many wood-based industries in Kerala. The current average production of rubber wood per ha is 150 and 180 m^3 in rubber smallholdings and estates respectively. Kerala State accounts for 78% of the area and 90% of total rubber produced in India (George and Chandrasekhar, 2014). Rubberwood represents a relatively sustainable alternative to tropical woods extracted

from natural forests. Furthermore, rubberwood has proven to be very versatile in its use in furniture manufacturing and the wood-based panel industry.

The rubberwood properties are one of the prime rationales for its popularity. Its natural colour, strength and mechanical properties are comparable to traditional timbers. It stains well and has good wood working and machining properties. Rubberwood can substitute for several timber species (ramin, meranti, sersaya, merbau, kapur, tangile and teak) that are essential for the primary and secondary industries in tropical and sub-tropical countries.

One of the most important drawbacks of the rubberwood is its non-durability or the susceptibility to insects and fungal attack. This is caused by the high deposition of carbohydrates which attracts the insects and the fungi. Wood inhabiting fungi are classified into three categories depending on the type of damage such as moulds, 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 were worldwide in distribution and sapstain became a serious problem when wood with high sapwood content was harvested in humid conditions, which was favourable for fungal growth. Sapstain fungus, the primary colonizers of the freshly felled lumber was a great concern in paper and timber industry due to the economic loss [Uzunovic *et al.*, 1999; Bruce *et al.*, 2003]. In tropical countries, the

dominant sapstain fungus (*Lasiodiplodia theobromae* Pat- syn. *Botryodiplodia theobromae*), which restricts the use of rubberwood and reduce its economic value by its bluish black staining and cellwall degradation (Florence *et al.*, 2002). Protection of wood and wood products from fungal deterioration is achieved mainly through chemical wood preservatives. Conventional chemical control has been a successful method of preventing staining fungal growth, but the effects of these chemicals are of concern because they create problems for the environment and public health. Thus an alternative approach, biological control has been recognized as a solution to the problem. Biological control, a process of managing pests (including insects, mites, weeds and microbial plant diseases) using other living organisms is an alternative to chemical pesticides and results in disease diminution or decrease in inoculum potential of a pathogen. The first flourished biocontrol agent used against insects and pathogens was members of the genus *Bacillus* (Powell and Jutsum, 1993) and commercial strains of *B. subtilis* have been marketed as biocontrol agents for fungal diseases in crops (Warrior *et al*, 2002). In agriculture, biological control is widely practised to prevent pests (Bale *et al.*, 2008) and plant pathogens (Prashar *et al.*, 2013).

Actinomycetes are diverse group of heterotrophic prokaryotes forming hyphae at some stages of their growth. Actinomycetes, in particular the genus *Streptomyces*, which are gram positive mycelial bacteria, ubiquitous in soil, are well known as producers of many extracellular enzymes, including chitinases which degrade the fungal cell wall directly (Gupta *et al.*,

1995). The genus *Streptomyces* have many characteristics which make them useful as biocontrol agents (Crawford *et al.*, 1993).

Chitin, a polymer of N-acetyl D-glucosamine is the second most abundant carbohydrate in nature and is a major structural polysaccharide of arthropods, coelenterates and fungi. Chitinases are glycosyl hydrolases which catalyse the degradation of chitin. It is an enzyme complex which comprises an exochitinase, endochitinase and chitobiase components. The first one liberates soluble low molecular weight dimers, the second releases multimers of N-acetyl-glucosamine (NAG) and third hydrolyses chitobiose to NAG (Vyas and Deshpande, 1989). The combined effect of these enzymes results in the complete degradation of entire chitin. Lorito *et al.*, (1996) made a comparative study of the three chitinolytic enzymes and found that endochitinase (EC 3.2.1.14) is most effective for antifungal and lytic activities.

Various studies on the effect of chitinase on plant pathogenic fungi had been conducted (Quecine *et al.*, 2008; Anitha and Rabeeth, 2010). Mendonsa *et al.* (1996) reported the effect of chitinase on moulting process of insects and mosquito control. Not only as biocontrol agents, chitinases had received renewed interest due to its wide range of biotechnological applications like, generation of fungal protoplasts (Vyas and Deshpande, 1989), crustacean chitin waste management (Suresh and Chandrasekaran, 1998), production of single cell protein (Vyas and Deshpande, 1991) and treatment of human fungal diseases. Some of the chito oligosaccharides

including chitohexaose and chitoheptaose have proved antitumour activity. These chito oligosaccharides could be obtained by chitinolytic enzymes.

Bacillus subtilis, also known as hay bacillus or grass bacillus is a gram positive rod shaped non pathogenic bacteria commonly found in soil. It has proven highly amenable to genetic manipulation, and has become widely adopted as a model organism for laboratory studies. *Bacillus subtilis* is well known for the production of enzymes and antibiotics. It has a natural fungicidal activity and is employed as a biocontrol agent in agriculture and horticulture. Most of the antibiotics produced by *Bacillus* are active against gram positive bacteria but a small number have been found to be effective against gram negative bacteria or fungi. All the reported antifungal antibiotics produced by *Bacillus subtilis* strains belong to one of the three groups such as intracellular cyclic peptidolipids, extracellular cyclic peptidolipids or extracellular cyclic peptides, based on the location of the antibiotics and the structure of the molecule.

As a part of the previous project in KFRI (Florence and Balasundaran, 2009), funded by Department of Science and Technology, microorganisms (actinomycetes, bacteria and fungi) antagonistic to the sapstain fungus, *Lasiodiplodia theobromae* was isolated and screened from soil, compost and rubberwood. Most effective actinomycete from soil and bacteria from compost were identified as *Streptomyces* SA18 and *Bacillus subtilis* B2 respectively and deposited in KFRI culture collection. *Streptomyces* SA18 was found to produce the cell wall degrading enzyme chitinase which plays a major role in fungal cell wall degradation and in turn fungal growth

inhibition. *Bacillus subtilis* B2 was proved to produce the antifungal antibiotics which in turn inhibits the fungal growth. In order to find out maximum production of chitinase enzyme from *Streptomyces* SA18 and antibiotics from *Bacillus subtilis* B2 for controlling growth of *L. theobromae* on rubberwood, various physical parameters like medium, pH, temperature, etc. for the higher production of both have to be standardised.

Based on the above facts, the objectives of the present project are: 1. Standardization of physical parameters for the maximum production of chitinase from *Streptomyces* SA18 and 2. Standardization of physical parameters for the maximum production of antibiotics from *Bacillus subtilis* B2.

2. Review of literature

Studies on chitinases have been started since 1960s as several chitinolytic enzymes were reported from different *Streptomyces* sp. like *S. antibioticus* (Jeuniaux, 1966), *S. griseus* (Tarentino *et al.*, 1974), *S. plicatus* (Robbins *et al.*, 1988), *S. lividans* (Miyashita *et al.*, 1991) and *S. venezuelae* P10 (Mukherjee and Sen, 2004), *S. plicatus* strain 101 (Baharlouei *et al.*, 2010). Chitinase enzyme has antifungal property and thus chitinase producing microbes are used as biocontrol agents (Baharlouei *et al.*, 2010) against fungi. A reliable, sensitive, rapid and user friendly method for detection of chitinase activity using calcoflour white, a chitin binding dye on agar plate after polyacrylamide gel electrophoresis was explained by Gohel *et al.* (2005). Anil *et al.* (2007) explained the use of less expensive Ranipal instead of calcoflour white for the detection of chitinase enzyme.

Faramarzi *et al.* (2009) explained the effect of cultural conditions and media composition on the chitinase production of the bacteria *Massilia timonae.* Chitinase production of *Streptomyces hygroscopicus* VMCH2, isolated from the soil samples of different crop fields in Tamil Nadu was optimized by Priya *et al.* (2011).

Various *Bacillus subtilis* strains are known antibiotic producers. Studies on the bacterial antibiotics had been started from decades back. Kaur *et al.* (2011) and Joshi *et al.* (2012) optimised the parameters for maximum production of antibiotics such as subtilin and bacitracin respectively from *Bacillus subtilis.* The immobilization of microbial cells on several matrices for the production and optimization of various metabolites was studied by Awais *et al.* (2010). The production optimization of the bioactive compounds from *Bacillus subtilis* effective against *Salmonella* was studied in detail by Bhatta and Kapadnis (2010). The standardization of physical factors for the enhanced production of bioactive compounds from *Bacillus subtilis* against the wood surface fungal contaminants were discussed by Moita *et al.* (2005).

3. Materials and methods

The microorganisms involved in this study, *Streptomyces* SA18, *Bacillus subtilis* B2 and *Lasiodiplodia theobromae*, isolated from stained rubber wood were taken from the KFRI microbial culture collection.

3.1. Standardization of physical parameters for the maximum production of chitinase from *Streptomyces* SA18

Chitinase activity assay

Extracellular chitinolytic activity in *Streptomyces* SA18 inoculated broth was calculated using chitin as the substrate. Chitin was prepared by the method explained by Hsu and Lockwood (1975). The crude chitin was treated with concentrated HCl for fifty minutes under constant stirring. To this, double the volume of cold distilled water was added a colloidal form of chitin was obtained. It was then washed several times, dried and used in preparing medium. Chitinase activity was determined colorimetrically by detecting the amount of N-acetylglucosamine (NAG) released from a colloidal chitin substrate (Monreal and Reese, 1969). Two millilitre of the culture supernatant was mixed with 1ml distilled water and 0.5 ml of 1.25% chitin in 200 mM phosphate buffer pH 6. The assay mixture was incubated at 25°C for 2 hours, boiled for five minutes and centrifuged to remove insoluble chitin. The resulting adduct of reducing sugars were measured by the dinitrosalicylic acid (DNS) method (Miller, 1959). 8 ml of 5.3 M sodium poatassium tartarate solution was added to 20 ml 96 mM 3,5-Dinitrosalicylic acid solution and made up to 40ml with deionized water to prepare the colour reagent. The centrifuged test supernatant (1ml), deionized water (2 ml) and colour reagent (1.5 ml) along with the blank was placed in the boiling water bath for five minutes, cooled to room temperature. 0.1% w/v of NAG (Sigma) was used as the standard solution. The absorbance and concentration of NAG liberated during the reaction was noted at 540 nm in a spectrophotometer. One unit of chitinase activity was

defined as the amount of enzyme that released 1 µmol NAG from colloidal chitin per minute under the specified conditions.

3.1.1. Optimization of culture media

Fourteen different media numbered 1 to 14; with pH 7 were used to determine the chitinase production by *Streptomyces* SA18. One ml of 24 h *Streptomyces* SA18 broth was inoculated to 100 ml of each medium and incubated at room temperature in a rotary shaker at 120 rpm. From the next day itself the culture was harvested, centrifuged at 10,000 rpm for 15 minutes and the supernatant was used for the chitinase assay. Different media used in the current experiment is shown in Table 1.

Table 1. Composition of media used for testing the chitinase production ability of *Streptomyces* SA18.

Sl.No:	Media (pH-7)	Composition g/l
1.	CYS (Chitin Yeast extract Salt)	Chitin -5, Yeast extract -0.5, K_2HPO_4 - 2, $MgSO_47H_2O$ – 1, $FeSO_47\ H_2O$ - 0.1
2.	LB (Luria Bertani)	Tryptone – 10, Yeast extract – 5, NaCl – 10
3.	LB with 0.5% chitin	Tryptone – 10, Yeast extract – 5, NaCl – 10, Chitin – 5
4.	Colloidal Chitin Broth, No. 4	Colloidal chitin – 10, Na_2HPO_4 – 0.65, KH_2PO_4 – 1.5, NaCl – 0.25, NH_4Cl – 0.5, $MgSO_4$ – 0.12, $CaCl_2$ – 0.005
5.	Colloidal Chitin Broth, No. 5	Colloidal chitin – 15, Yeast extract – 0.5, $(NH_4)_2SO_4$- 1, $MgSO_4$ – 3, KH_2PO_4 – 1.36
6.	Colloidal Chitin Broth, No. 6	Colloidal chitin – 7.5, $(NH_4)_2SO_4$- 1, $MgCl_2$ – 7.5mM, Triton X 100 - 2

7.	Colloidal Chitin Broth, No.7	Colloidal chitin – 5, Yeast extract – 0.2, KH_2PO_4 – 0.7, K_2HPO_4 – 0.3, NaCl – 4, $MgSO_4$ – 0.5, $FeSO_4$ – 0.01, $ZnSO_4$ – trace, $MnSO_4$ – trace
8.	Yeast Dextrose Peptone (YDP)	Yeast extract – 4, Peptone – 4, Dextrose – 20
9.	YDP with 0.5% chitin	Yeast extract – 4, Peptone – 4, Dextrose – 20, Chitin – 5
10.	Yeast Malt Broth (YMB)	Yeast extract – 4, Dextrose – 4, Malt Extract – 10
11.	YMB with 0.5% chitin	Yeast extract – 4, Dextrose – 4, Malt Extract – 10, Chitin –5
12.	Chitin Maltose Broth	Chitin – 4.94, Maltose – 5.56, Yeast extract – 0.62, KH_2PO_4 – 1.33, $MgSO_4.7H_2O$ – 0.65
13.	Chitin Urea Broth	Chitin – 3.75, Urea – 0.33, KH_2PO_4 – 1.17, $MgSO_4.7H_2O$ – 0.3, Yeast extract – 0.65
14.	Colloidal Chitin Broth, No.14	Chitin – 0.1, $NaNO_3$ – 2, KH_2PO_4 – 1, $MgSO_4.7H_2O$ – 1, CaCO3 – 1, $FeSO_4$ 0.01, KCl – 0.5

3.1.2. Optimization of pH

For the optimization of pH, optimum medium with pH ranging from 4 to 10 was used. Standardized medium with different pH was inoculated with 24 h culture broth and incubated at optimum temperature in a rotary shaker at 120 rpm. Here the chitinase assay was conducted as described in the previous experiments.

3.1.3. Optimization of temperature

The optimized medium with the inoculum was incubated at different temperatures like 20, 25, 30, 35, 40 and 45°C at 120 rpm. Each day the chitinase activity was examined.

The quantity of N- acetyl –D- glucosamine (NAG) liberated during the reaction was determined using the standard curve developed in the DNS method with NAG as the substrate (Miller, 1959). The enzyme units liberated was calculated by the equation.

$$\text{Units/ml enzyme} = \frac{(\text{mg NAG released})\ (2.5 + \text{Vol. of NAGase})}{2 \times 1 \times 0.5}$$

where 2.5 - Initial reaction volume of assay

2 - Conversion factor for converting 2hrs to 1hr as per unit definition

1 - Volume (in ml) of supernatant used in colorimetric determination

0.5 - Volume (in ml) of enzyme used

3.2. Standardization of physical parameters for the maximum production of antibiotics from *Bacillus subtilis*

Various physical parameters like the medium, temperature and pH were standardized for the maximum production of antibiotics from *Bacillus subtilis*.

Well method of antibiotic assay

The bacterium was grown in the antibiotic production medium. The antagonistic activity against *L. theobromae* was tested using cell free culture filtrate after removing the potential antibiotic producing organism. The

crude culture filtrate was filtered using Millipore (0.22 µm) filters. To check the antifungal activity of the extract, 1 ml of the filtrate was poured into the well made in Potato dextrose agar (PDA) medium using a cork borer. The test fungus, *L. 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 and inhibition zone formation was measured. Controls adding sterile distilled water in the well were also maintained. Inhibition of the stain fungus in the dual culture (Fig. 1) was assessed by two parameters: the percentage inhibition of radial growth [100 x (r_1-r_2) / r_1] and the width of the zone of inhibition (ZI) measured at the smallest distance between both colonies (Benko, 1988).

r_1 : the distance from the inoculums to the extreme edge of the biggest growth of the test organism – the *L. theobromae*

r_2 : the distance from the inoculums to the edge where inhibitory zone begins

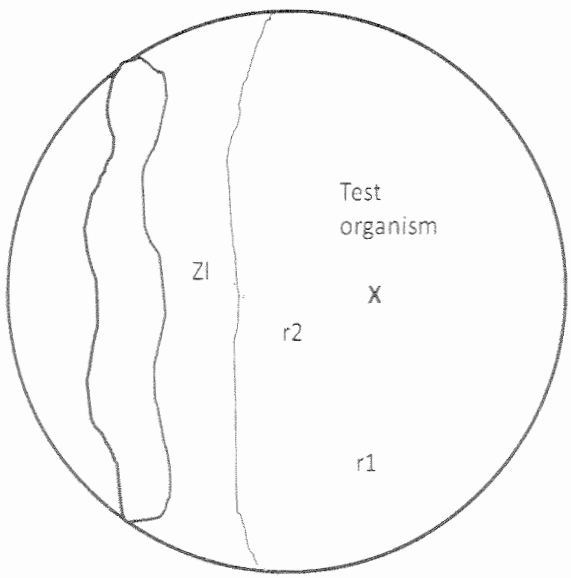

Fig. 1. Diagram showing the inhibition of the test organism by potential antagonist in the dual culture technique.

3.2.1. Optimization of antibiotic production medium

Twelve chemically defined antibiotic production media (Table 2) were used for the standardization of media for the maximum antibiotic production. One ml of 24 h *Bacillus subtilis* broth was inoculated to 100 ml of each medium and incubated at room temperature in a rotary shaker at 120 rpm. Next day onwards, the culture was centrifuged at 10,000 rpm for 15 minutes and the filtrate was filtered through 0.22 µm membrane filter. This filtrate was added to the well bored in the PDA medium which contained inoculated test fungus, *L. theobromae* on the opposite side and incubated at 28°C for one week and examined the activity.

Table 2. Media used for the standardization of maximum antibiotic production ability of *Bacillus subtilis* B2

Sl. No.	Media	Components (g/l)
1.	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
2.	No. 2	Yeast Extract – 4, Dextrose – 2, NaCl – 5, K_2HPO_4 – 2.5, KH_2PO_4 – 0.5
3.	No. 3	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
4.	No. 4	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
5.	No. 5	Trypticase Soya Broth (TSB) Himedia
6.	No. 6	Lactose – 15, Ammonium succinate - 1.5 mg/l

7.	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
8.	No. 8	To medium No.7, 2.5g sucrose was added
9.	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
10	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
11.	No.11	Tryptone – 20, Glycerin – 10, K_2HPO_4 – 15, $MgSO_4$ - 15
12	No. 12	Sucrose – 20, Peptone – 10, Yeast extract – 1, $CaCO_3$ – 0.1, NaCl – 0.1

3.2.2. Optimization of temperature

For the optimization of temperature, the medium No.3 was inoculated with the *B. subtilis* and incubated at different temperatures like 20, 25, 30, 35, 40 and 45°C at 120 rpm. Every day the antibiotic production was examined as indicated in the previous experiment.

3.2.3. Optimization of pH

Medium No.3 with pH ranging from 4 to 10 was used for optimizing the pH. Standardized medium with different pH was inoculated with 24 h *B. subtilis* culture broth and incubated at optimum temperature in a rotary shaker at 120 rpm.

4. Results

4.1. Standardization of physical parameters for the maximum production of chitinase from *Streptomyces* SA18

4.1.1. Optimization of culture media

From the fourteen different culture media tried for the production of chitinase enzyme, chitin yeast extract salt (CYS) medium was found to be effective for the enzyme production at room temperature (Table 3). In CYS medium, *Streptomyces* SA18 was found to produce 0.78125 units of chitinase enzyme. Among all the media tested, the production of enzyme chitinase was found to be more effective in CYS medium.

Table 3. Chitinase enzyme produced by *Streptomyces* SA18 in fourteen different media

Sl.No:	Media (pH-7)	Chitinase enzyme (units/ml) (mean of 3 replicates)
1.	CYS (Chitin Yeast extract Salt)	0.78125
2.	LB (Luria Bertani)	0.625
3.	LB with 0.5% chitin	0.75
4.	Colloidal Chitin Broth, No. 4	0.3125
5.	Colloidal Chitin Broth, No. 5	0.3125
6.	Colloidal Chitin Broth, No. 6	0.547
7.	Colloidal Chitin Broth, No.7	0.156
8.	Yeast Dextrose Peptone (YDP)	0.187
9.	YDP with 0.5% chitin	0.187

10.	Yeast Malt Broth (YMB)	0.469
11.	YMB with 0.5% chitin	0.547
12.	Chitin Maltose Broth	0.625
13.	Chitin Urea Broth	0.46
14.	Colloidal Chitin Broth, No.14	0.312

4.1.2. Optimization of pH

In CYS medium at room temperatue, pH optimization for the increased production of chitinase enzyme was done at pH ranging from 4 to 10. pH 8 was found to be most effective for the production of enzyme chitinase. In CYS medium at pH 8, *Streptomyces* SA18 had a maximum production of 0.9375 units of chitinase enzyme (Table 4).

4.1.3. Optimization of temperature

For testing the optimum temperature for the higher production of chitinase enzyme, temperatures ranging from 20 to 45°C were tried and 35°C was found to be suitable for the maximum enzyme production in CYS medium. CYS medium at a temperature of 35°C with pH 8 found to produce 1.4 units of chitinase enzyme (Table 4). The enzyme production was observed from the second day of incubation and it attained the maximum on the 4th day and after that declined.

Table 4. Chitinase enzyme produced by *Streptomyces* SA18 in CYS media at different pH and temperature

Sl No	pH	Chitinase enzyme (units/ml) (mean of 3 replicates)	Temperature	Chitinase enzyme (units/ml) (mean of 3 replicates)
1	4	No enzyme production	20	0.0523
2	5	0.015	25	0.67
3	6	0.5	30	0.74
4	7	0.782	35	1.4
5	8	0.9375	40	0.542
6	9	0.69	45	0.021
7	10	0.05	50	No enzyme production

4.2. Standardization of physical parameters for the maximum production of antibiotics from *Bacillus subtilis*

4.2.1. Optimization of antibiotic production medium

From the twelve different media used for the antibiotic production, medium No. 3, the sorbitol medium and TSB media were found to be very effective in antibiotic production at room temperature (Fig. 2a & b). Among sorbitol and TSB media, sorbitol medium was selected for the further antibiotic standardization studies.

Fig. 2a

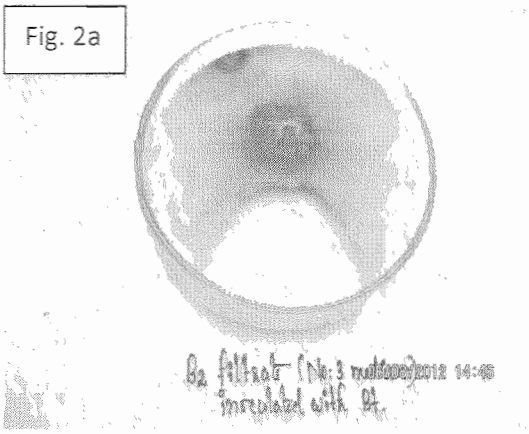

Fig. 2a.Culture filtrate of *B. subtilis* B2 grown in medium No.3 inhibiting the sapstain fungus *L. theobromae*

Fig. 2b

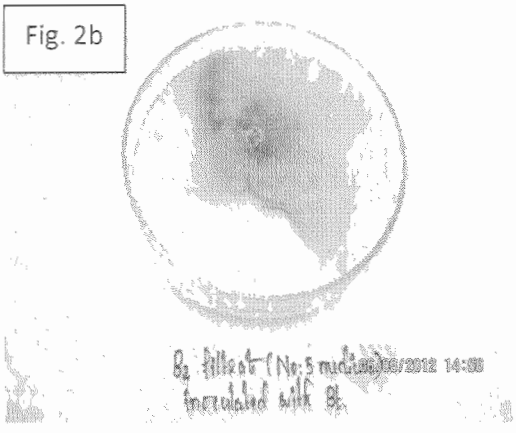

Fig. 2b.Culture filtrate of *B. subtilis* B2 grown in TSB medium inhibiting the sapstain fungus *L. theobromae*

4.2.2. Optimization of temperature and pH

The inhibition percentage was calculated using the formula given in the well assay method. Maximum inhibition of 82.36 % was observed at pH 7. Inhibition of the test fungus was very less at 20, 40 and 45°C (Table 5). At 50°C no inhibition was observed. The percentage inhibition for 25°C, 30°C and 35°C were 60, 81and 50% respectively. No inhibition was noted at pH 4 and pH 10 (Table 6). At pH 6 and 8 the inhibition percentage was 79.4 and 60 respectively. At pH 9, inhibition was only 30% and it reduced to 10% at pH 5. The antibiotic production was found to be maximum in the sixth day and found to decline after the twentieth day.

Table 5. Percentage of inhibition of *L. theobromae* by the culture filtrate of *B. subtilis* B2 obtained after incubating at different temperatures

Sl. No.	Temperature (°C)	% inhibition (mean of three plates)
1	20	Slight inhibition
2	25	60
3	30	81
4	35	50
5	40	20
6	45	Slight inhibition
7	50	No inhibition

Table 6. Percentage of inhibition of *L. theobromae* by the culture filtrate of *B. subtilis* B2 obtained after incubating at different pH

Sl. No.	pH	Inhibition % (mean of three plates)
1	4	No inhibition
2	5	10
3	6	79.4
4	7	82.36
5	8	60
6	9	30
7	10	No inhibition

5. Discussion

Even though the genus *Streptomyces* is reported as good chitinase producers (Hara *et al.*, 1989), the optimized conditions vary from organism to organism. Nawani and Kapadnis (2004) reported that the production of chitinase by *Streptomyces* sp. NK1057 was high after 5 days of incubation while Joo *et al.* (2005) found the maximum yield of chitinase by *Streptomyces halstedii* after 72 h of incubation. Narayana and Vijayalakshmi (2009) explained the production of chitinase just after 24 hours of incubation and maximum production at the 60th hour. In the present experiment, the chitinase enzyme production was noted in the second day and the maximum production in the 4th day which showed the discrepancy in the time for chitinase production even within the genus. Taechowisan *et al.* (2003) reported that the production of chitinase by *S. aureofaciens* was

optimal at pH 6.5-7 and temperature 30-40°C. Maximum chitinase production by the strain *Streptomyces* sp. ANU 6277 (Narayana and Vijayalakshmi, 2009) was observed in the CYS medium incubated at pH 6 and temperature 35°C for 60 h. In the present experiment, the optimum pH observed for maximum chitinase production was 8 and temperature 35°C.

The antibiotic production in *B. subtilis* also varies from strain to strain. The physical parameters play a major role in the metabolite production. Kumar *et al.* (2009) had explained the effect of various physical parameters on the growth and metabolite production by *Bacillus subtilis* strain isolated from garden soil. In that experiment, the maximum inhibition towards the test fungus *Microsporum fulvum* was observed in the second day of incubation in the TSB medim at 37°C and pH 7. But in our experiment, the maximum antifungal effect was observed in the sixth day and continued for some more days. The optimum pH was observed as 7 and temperature as 30°C.

6. Conclusion

Both *Streptomyces* SA18 and *Bacillus subtilis* B2 were found to be effective against the sapstain fungus *Lasiodiplodia theobromae*. The fungal cell wall degrading chitinase enzyme was found to be produced by the *Streptomyces* SA18 which results in the inhibition of the sapstain fungus. *Streptomyces* SA18 when grown in Chitin Yeast extract Salt medium at pH 8 and incubated at 35°C was found to be the optimum condition for the chitinase enzyme production. *Bacillus subtilis* B2 produced the maximum antibiotics in chemically defined sorbitol medium at pH 7, incubated at 30°C. The

maximum antibiotic production against the *L. theobromae* was found in the sixth day.

7. References

Anil, K., Seshagirirao, K. and Podile, A. R. 2007. A simple, rapid and yet less expensive method to detect chitinase in agarose plates. Journal of Biochemical and Biophysical Methods 70 (4): 683–68.

Anitha, A. and Rabeeth, M. 2010. Degradation of fungal cell walls of phytopathogenic fungi by lytic enzyme of *Streptomyces griseus*. African Journal of Plant Science 4 (3): 61-66.

Awais, M., Pervez, A., Asim Yaqub. and Shah, M. M. 2010. Production of antimicrobial metabolites by *Bacillus subtilis* immobilized in Polyacrylamide gel. Pakistan Journal of Zoology 42(3): 267-275.

Baharlouei, A., Sharifi-Sirchi, G. R. and Shahidi Bonjar G. H. 2010. Identification of an antifungal chitinase from a potential biocontrol agent, *Streptomyces plicatus* Strain 101, and its new antagonistic spectrum of activity. The Philippine Agricultural Scientist 93 (4): 439-445.

Bale, J. S., van Lenteren J. C. and Bigler F. 2008. Biological control and sustainable food production, Philosophical Transactions of Royal Society of Biological Sciences, 363:761–776.

Benko, R. 1988. Bacteria as possible organisms for biological control of blue stain. The International Research Group on Wood Preservation, Document No.IRG/WP/1339, Stockholm, Sweden.

Bhatta, D. R. and Kapadnis, B. P. 2010. Production optimization and characterization of bioactive compound against *Salmonella* from *Bacillus subtilis* KBB isolated from Nepal. Scientific World 8(8): 19-29.

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.

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.

Faramarzi, M. A., Fazeli, M., Tabatabaei Yazdi, M., Adrangi, S., Jami Al Ahmadi, K., Tasharrofi, N. and Aziz Mohseni, F. 2009. Optimization

of cultural conditions for production of chitinase by a soil isolate of *Massilia timonae*. Biotechnology 8(1): 93 -99.

Florence, E. J. M and Balasundaran, M. 2009. Use of bioprotectant against fungal deterioration of rubberwood, KFRI Research Report No. 327 pp 43.

Florence, E. J. M., Gnanaharan R., Singh P. A. and Sharma J. K. 2002. Weight loss and cell wall degradation in rubberwood caused by sapstain fungus *Botryodiplodia theobromae*, Holzforschung 56: 225-228.

George, J.G. and Chandrashekar, H.M. 2014. Growth and Trends in Production and Marketing of Natural Rubber in Kerala, India. International Journal of Current Research and Academic Review 2(8): 53-61.

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.

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 Microbiology 78: 378-383.

Hara, S., Yamamura Y., Fugii Y., Mega T. and Ikenaka, T. 1989. Purification and characterization of chitinase produced by *Streptomyces erythraeus*. Journal of Biochemistry 105: 484-489.

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: 422-426.

Jeuniaux, C. 1966. Chitinases. Methods of Enzymology 8: 644–650.

Joo, J. G. 2005. Purification and characterization of an extracellular chitinase from the antifungal biocontrol agent *Streptomyces halstedii*. Biotechnology Letters 27: 1483-1486.

Joshi, R. D., Hamde, V. S., Umrikar, A. M., Kulkarni, S. S. and Bhate, M. A. 2012. Studies on production of peptide antibiotic by thermotolerant Bacillus sp. International Multidisciplinary Research Journal 2(6): 30-33.

Kaur, P., Sharma, P., Ahmed, F., Tembhurkar, V. 2011. Optimization of subtilin production by *Bacillus subtilis*. Indo Global Journal of Pharmaceutical Sciences 1(4): 362-368.

Kumar, A., Saini P. and Shrivastava J. N. 2009. Production of peptide antifungal antibiotic and biocontrol activity of *Bacillus subtilis*.

Indian Journal of Experimental Biology 47: 57-62.

Lorito, M., Woo, S. L, D'ambrosio, M., Harman, G. E., Hayes, C. K., Kubicek, C. P. and Scala, F. 1996. Synergestic interaction between cellwall degrading enzymes and membrane affecting compounds. Molecular Plant-Microbe Interaction 9: 206-213.

Mendonsa, E. S., Vartak, P. H, Rao, J. U., Deshpande, M. V. 1996. An enzyme from *Myrothecium verrucaria* that degrades insect cuticles for biocontrol of *Aedea aegypti* mosquito. Biotechnology Letters 18: 373-376.

Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugars. Analytical Chemistry 31: 426-428.

Miyashita, K., Fujii, T. and Sawada, Y. 1991. Molecular cloning and characterization of chitinase genes from *Streptomyces lividans* 66. Journal of General Microbiology 137: 2065–2072.

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 and Biodegradation 55: 261-269.

Monreal, J. and Reese, E. T. 1969. Canadian Journal of Microbiology 15: 689-696.

Mukherjee, G. and Sen, S. K. 2004. Characterization and identification of chitinase producing *Streptomyces venezuelae* P10. Indian Journal of Experimental Biology 42(5): 541-544.

Narayana, K. J. P. and Vijayalakshmi, M. 2009. Chitinase Production by *Streptomyces* sp. ANU 6277. Brazilian Journal of Microbiology 40(4): 725-733.

Nawani, N. N., Kapadnis, B. P. 2004. Production dynamics and characterization of chitinolytic system of *Streptomyces* sp. NK 1057, a well equipped chitin degrader. World Journal of Microbiology and Biotechnology 20: 487-494.

Powell, K. A. and Jutsum, A. R. 1993. Technical and commercial aspects of biological control products. Pesticide Science 37: 315-321.

Prashar, P., Kapoor N. and Sachdeva S. 2013. Biocontrol of plant pathogens using plant growth promoting bacteria, Sustainable Agriculture Review12: 319-360.

Priya, C. S., Jagannathan, N. and Kalaichelvan, P. T. 2011. Production of chitinase by *Streptomyces hygroscopicus* VMCH2 by optimisation of

cultural conditions. International Journal of Pharmacology and Bio Sciences 2(2): 210 – 219.

Quecine, M. C., Araujo, W. L., Marcon, J., Gai, C. S., Azevedo, J. L. and Pizzirani-Kleiner, A. A. 2008. Chitinolytic activity of endophytic Streptomyces and potential for biocontrol. Letters in Applied Microbiology 47: 486–491.

Robbins, P. W., Albright, C. and Benfield, B. 1988. Cloning and expression of a *Streptomyces plicatus* chitinase (chitinase-63) in *Escherichia coli.* Journal of Biological Chemistry 263: 443–447.

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

Suresh, P. V. and Chandrasekaran, M. 1998. Utilization of prawn waste for chitinase production by marine fungi *Beauveria bassiana* by solid state fermentation. World Journal of Microbiology and Biotechnology 14: 655-660.

Taechowisan, T., Peberdy, J. F. and Lumyong S. 2003. Chitinase production by endophytic *Streptomyces aureofaciens* CMU AC 130 and its antagonism against phytopathogenic fungi. Annals of Microbiology 53: 447-461.

Tarentino, A. L. and Maley, F. 1974. Purification and properties of an endo-b-*N*-acetylglucosaminidase from *Streptomyces griseus*. Journal of Biological Chemistry 249: 811–817.

Uzunovic, A., Yang P., Gagne C., Breuil L., Bernier A., Gignac B. M. and Kim Can S. H. 1999. Fungi that cause sapstain in Canadian softwoods, Canadian Journal of Microbiology 45: 914–922.

Vyas, P. and Deshpande, M. V. 1989. Chitinase production by *Myrothecium verrucaria* and its significance for fungal mycelia degradation. Journal of General and Applied Microbiology 35: 343-350.

Vyas, P. and Deshpande, M. V. 1991. Enzymatic hydrolysis of chitin by *Myrothecium verrucaria* chitinase complex and its utilization to produce SCP. Journal of General and Applied Microbiology 37(3): 267-275.

Warrior, P., Konduru K. and Vasudevan P. 2002. Biological Control of Crop Diseases, Marcel Dekker, New York, 421 pp.

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