Chapter 3:

Production, extraction and characterization of bioactive metabolite (antibiotic S2) produced by *Bacillus altitudinis* S2

3.1. Introduction

One of effective mode of control of BB demonstrated is the spraying of certain chemicals/antibiotic compounds (Singh et al., 1980). Sprays of Agrimycin (250 ppm) was demonstrated to effectively check the spread of the disease. Five sprays of Agrimycin along with Fytolan (50:500 ppm) or combination of streptomycin sulphate (1.75 %), terramycin (0.17 %) and tribasic copper sulphate (42.4 %) at concentration of 665 ppm could reduce the BB to satisfactory level (Singh et al., 1980). Antibiotic like streptomycin have been used since many decades in China to control BB (Xue et al., 1973,). There is a constant need to search for new potential candidates having these properties as many of these compounds become less effective due to development of resistance in the target organism. Streptomycin resistance gene have been recently reported from *Erwinia amylovora*, *Pseudomonas syringae*, *Xanthomonas campestris* and *Xanthomonas oryzae* pv. oryzae (Sundin & Wang, 2018; Xu et al., 2013).

The member of *Bacillus* genera are commonly applied in the biocontrol of many plant diseases. The *Bacillus* species used in biocontrol of phytopathogens have demonstrated different modes of action. Antibiosis has been shown to be one of the most important mechanism involved in the inhibition of the pathogens (Thomashow & Weller, 1996). Also, many members of *Bacillus* produces agents which are antimicrobial in nature. They have been categorized into different classes depending on their structure, composition, mode of action etc. Recent advances in whole genome sequencing have revealed that members of *Bacillus* genus produce diverse kind of antibiotics (Ficker et al., 2012; Stein, 2005). Some of these members like *Bacillus subtilis* have been shown to harbour gene clusters for antibiotic, which cover almost 4 % of their whole genome content (Chen et al., 2009b; Kunst et al., 1997). They synthesize various class of antibiotics like Polyketides (PKs) (e.g., difficidin, bacillaene), Non-ribosomal Peptides (NRPs) (e.g., kurstakin, gramicidin), Bacteriocins (e.g., epidermin, subtilin) as well as some unusual antibiotics (e.g., rhizocticins, bacilysin) (Arguelles-Arias et al., 2009; Chen et al., 2009a; Kunst et al., 1997).

For optimum production of antibiotics from the producing organisms, development of rapid screening techniques for potent antibiotic producing microorganisms and easy extraction protocols are needed (Omura et al., 1986).

Factors like nutrient, oxygen etc. are depleted in the stationary phase where the secondary metabolites are produced by the microorganisms. These factors elicit stress response in the bacterial cells which resultantly leads to increased production of secondary metabolites. Their production is also dependent on temperature and pH of the medium used. Hence, optimizing these factors and conditions will lead to optimum production of the metabolites of interest. There are three main methods used for extraction of secondary metabolites from micro-organisms namely solvent extraction which is liquid –liquid extraction, reverse phase micelles and solid phase extraction (Gu, 2000;Yarbrough et al, 1993).

An approach utilising simultaneous purification and characterization are commonly used in rapid identification of the metabolite. One such approach is Direct Bioautography (DB) which is one of the most useful method of separation and characterization of the bioactive metabolite using test microbe. In this test, microbial suspension is applied to the TLC plate after the sample is resolved and tetrazolium salts like p-Iodine tetrazolium violet is used for visualization. These salts undergo a conversion by the dehydrogenases of living cells present in the suspension to form intensely coloured formazan. Other techniques HPLC combined with mass spectrometric technique have helped in detail characterization of many such compounds (Schymanski et al., 2015,2014; Moritz et al 2015).

This chapter includes screening media for antibiotic production by the selected isolate *B. altitudinis* S2 termed antibiotic S2 from here onwards. Extraction, purification and characterization by TLC, TLC-DB, HPLC, mass spectrometry for identification of the class of this antibiotic has been attempted. The efficacy of the partially purified antibiotic was tested on the Kresek disease model, an infection model of rice seedling with pathogen Xoo BXO43. The amplification of genes for polyketide antibiotic synthesis by PCR and mechanism of the antibiotic action of antibiotic S2 has been carried out.

3.2. Materials & Methods

3.2.1. Chemicals

All the organic solvents: hexane, ethyl acetate, n-butanol, chloroform, methanol, isopropanol and ethanol were procured from Merck, India.

3.2.2. Media

Following media and their components were procured from Himedia, India.

Luria Bertani broth: Details given in section 2.2.

1/4th strength Peptone sucrose (PS) broth: PS broth as described in section 2.2 was diluted four times with distilled water and sterilized by autoclaving at 10 psi for 20 min.

Tryptic soy broth (TSB): composition (g/l): Pancreatic digest of casein, 17.0; Papaic digest of soyabean meal, 3.0; NaCl, 5.0; Dextrose, 2.5; KH_2PO_4, 2.5; Final pH 7.3 ±0 at 25 °C. (Himedia, India)

Sucrose Bushnell Haas (SBH) broth: composition (g/l): Sucrose, 20.0; Bushnell Haas, 3.27; Final pH 7.3 ±0.

Potato Dextrose agar (PDA): composition (g/l): Potato infusion, 4.0; Dextrose, 20.0; agar, 20.0.

3.2.3. Screening of media for antibiotic production

Luria-Bertani (LB) broth, Tryptic soya broth (TSB), Peptone sucrose (PS) broth, 1/4th strength PS broth, Sucrose Bushnell Haas (SBH) broth were used for assessing the production of the bioactive secondary metabolite from *B. altitudinis* S2. Overnight grown culture was inoculated in 50 ml of individually prepared abovementioned media in 250 ml Erlenmeyer flask to attain 0.1 OD and was incubated on a rotary shaker at 120 rpm and 30 °C for 3 days. In one experiment, PS broth was co-inoculated with Xoo BXO43 along with *B. altitudinis* S2 to evaluate the production of bioactive compound in the presence of pathogen. 1% v/v of 0.5 OD_{600} of Xoo BXO43 and overnight grown culture of *B. altitudinis* S2 was inoculated in 50 ml different media in 250 ml Erlenmeyer flask to attain 0.1 OD and was incubated on a rotary shaker at 120 rpm and 30 °C for 3 days. The culture broth obtained from above media was filter sterilized by passing through 0.2 µm pore size nylon membrane filter (PALL, India) and the antibacterial activity of CFS was checked after every 24 hrs using the test strain Xoo BXO43. For antibacterial activity, 100 µl of 0.5 OD_{600} Xoo BXO43 was spread on PS Agar plates and then a 13 mm well was bored in which the filter sterilised extract was dispensed and the

plates were incubated at 30 °C for 48 hrs. The activity was noted in term of zone of clearance due to inhibition of growth.

To test production of bioactive metabolites on SBH agar plates, the agar pieces were cut and added in 5 ml of methanol and homogenized using a homogenizer (Remi) at 10,000 rpm. The homogenized agar was centrifuged and the supernatant methanol was taken in fresh sugar tube and was evaporated at 30 °C. The residue was dissolved in distilled water, the solution was filter sterilised and antibacterial activity was checked using test strain Xoo BXO43 on PS agar plates.

3.2.4. Stability test of the crude antibiotic S2 in CFS of *B. altitudinis* S2

Different parameters like pH, temperature were used to analyze the stability of crude antibiotic in CFS produced by *B. altitudinis* S2. For assessing the thermal stability, the CFS was exposed to temperatures 40, 45, 50, 60, 65 °C for 2 hrs and 121 °C for 15 minutes. The treated samples were cooled to room temperature before testing against the target organism of Xoo BXO43 by agar well diffusion method.

For the pH stability test, the CFS was adjusted to a pH value ranging from 2 to 10 by using buffers of respective pH. This was incubated for 6 hrs at 30 °C before determining the antimicrobial activity by agar well diffusion method as above.

Different enzymes namely proteinase K, trypsin, lysozyme, and amylase in 0.1 mg/ml final concentration were tested on CFS by incubating these enzymes with the extract at 37 °C for 1 hour. A control without treatment was kept for comparing the results. The enzymes were heat inactivated before testing for antimicrobial activity as above.

3.2.5. Effect of different carbon sources and other physical parameters on production of antibiotic S2 production

Different carbon sources like sucrose, glucose, mannitol, xylose, citrate, fructose, trehalose in percent carbon equivalent were used in the medium to study their effect on antibiotic production. The antibiotic production was checked at different temperature values (25, 30, 35 and 42 °C) and pH values (5.0, 5.8, 7.0, 8.0

and 9.0). The amount of antibiotic was quantified by micro-dilution broth assay wherein two-fold serial dilution of crude extract contained antibiotic S2 was carried out with sterile distilled water. Equal volume of all dilutions containing varying concentration of antibiotic and media were added in individual wells. Xoo BXO43 was added to attain 0.2 OD in all the wells and plate was incubated at 30 ^{o}C for 20 hrs. Thus, the antibiotic titre was defined in terms of arbitrary unit (AU) of antibacterial activity per millilitre (AU/ml) as follows:

$$\mathrm{AU} = \frac{(2^{n} \times 1000\ \mu l)}{V\ \mu l}$$

where n is the reciprocal of the highest dilution that resulted in no increase in turbidity in the wells as observed by unaided eye and V is the volume of CFS containing bioactive metabolite used in the test (Mayr-Harting, 1972).

3.2.6. Time-course production of antibiotic S2 from *B. altitudinis* S2

For studying the course of antibiotic production, overnight grown culture of *B. altitudinis* S2 was inoculated in 100 ml SBH broth in 250 ml Erlenmeyer flask to attain 0.1 OD and kept for incubation on a rotary shaker at 120 rpm. The antibacterial activity and culture growth in terms of OD_{600} was examined from CFS at every 24 hrs till 10 days. Production was checked by microdilution broth assay in terms of Arbitrary Units (AU) using Xoo BXO43 test strain as mention in above section 3.2.5.

3.2.7. Extraction of antibiotic S2 from CFS

Extraction was carried out using organic solvents having different strength of polarity: hexane, ethyl acetate, n-butanol, chloroform isopropanol, ethanol and methanol. The CFS was obtained from the culture grown in SBH medium as mentioned in section 3.2.3. For miscible organic solvents the lyophilised CFS was dissolved in the solvent. For immiscible organic solvents approximately equal volume of broth and organic solvent were taken and the mixture was vortexed for 15 min and the two layers were separated by centrifugation at 7,000 rpm for 5 min at 25 °C. The process of extraction was carried out thrice for each organic solvent

and the 3 volumes were pooled. The organic solvent was evaporated completely and the residue was dissolved in distilled water. This aqueous solution of solvent extracted antibiotic S2 was filter sterilized and the antibacterial activity was checked by agar diffusion well assay as mentioned above section 3.2.3.

3.2.8. Thin Layer Chromatography

The constituents of solvent extracted antibiotic S2 were separated on an aluminium-backed Thin Layer Chromatography (TLC) plates (Silica gel $60F_{254}$, 10 x 3.5 cm, layer thickness 0.25 mm; Merck, Germany). The TLC plates were developed under saturated conditions with optimized mobile phase using acetonitrile/water/glacial acetic acid (6:3:0.5) and separated components were detected using saturated chamber of iodine vapours and ultraviolet light.

Preparative TLC was performed multiple times and the activity band from developed TLC plate was scraped. The scrapings from TLC were further suspended in methanol, vortexed to separate silica gel and methanol was separated from the silica gel. The process was done thrice and methanol extracts were pooled, evaporated and the residue was weighed and dissolved in sterile distilled water. A solution of 6.26 mg/ml partially purified antimicrobial metabolite or antibiotic S2 so obtained was used further.

3.2.9. Direct Bioautography using Thin Layer chromatography (TLC)

Ten µl of *B. altitudinis* S2 solvent extracted antibiotic S2 was loaded on TLC plate as a band. TLC was performed as above and the organic solvent from TLC plate was evaporated completely in laminar airflow till the smell of acetic acid could no longer be detected. Xoo BXO43 and *S. aureus* in their early log phase were used for detection of active antimicrobial metabolite on TLC plates. For this, 0.5 ml of the respective culture was seeded in molten soft agar at approximately 35 ^{o}C temperature which was immediately poured on TLC plate and allowed to form a uniform film. The TLC plates were transferred in a sterile jar after the agar containing the test organism solidified on TLC and incubated at 30 ^{o}C. After incubation for 12 hrs in case of *S. aureus* and 24 hrs in case of Xoo BXO43, the TLC plates were soaked in 0.1 mg/ml concentration tetrazolium dye solution for

colour development. Pink colour so developed indicated bacterial growth and no colour indicated no growth due to presence of antibacterial compound demonstrating activity band (Choma & Grzelak, 2011; Tyihák et al., 2004).

3.2.10. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of partially purified antibiotic S2 produced by *B. altitudinis* S2

Antimicrobial titre was quantified by serial dilution method in sterile, disposable 96-well microdilution plates as described by Park et al., (2011) and Dung et al., (2008). Overnight grown Xoo BXO43 was adjusted to value of 0.5 OD_{600} measured using UV-Vis Spectrophotometer (Shimadzu) and subsequently diluted to 1:20 (v/v) in PS broth. To determine the minimal inhibitory concentration (MIC) of the bioactive compound, was diluted as recommended by the National Committee for Clinical Laboratory Standards (NCCLS) from 6.26 mg/ml stock of partially purified antibiotic S2 obtained in preparative TLC (section 3.2.8) to attain concentrations from 0.5 to 128 µg/ml in 150 µl system. All the wells contained equal amount of media. The plates were incubated at 28 ^{o}C for 18 hrs. The MIC was determined as the lowest concentration that shows an increase in bacterial growth of not more than 2-5 % as measured by the OD at 600 nm taken by a Synergy-4 multimode Microplate Reader (Tecan). This value was calculated using the following formula:

$$\%\ increase\ in\ bacterial\ growth =$$

$$[1 - \frac{(control\ 24 - control\ 0) - (treated\ 24 - treated\ 0)}{(control\ 24 - control\ 0)}] * 100$$

Where, control 24- well without antibiotic S2 incubated for 24 hrs; control 0- well without antibiotic S2 at 0 hr, treated 24- well incubated with antibiotic S2 for 24 hrs; treated 0- well with antibiotic S2 at 0 hr. The experiment was done twice in triplicates.

The MBC was determined from the same experiment by plating the content of the wells before and after well corresponding to MIC on the PS agar plates and incubated for 48 hrs. MBC was considered as the lowest concentration which showed no growth on agar plate.

3.2.11. Time-kill assay of partially purified antibiotic S2

Xoo BXO43 was grown in PS broth at 28 °C and incubated for 16 -20 hrs to achieve mid-log phase (OD_{600} ~0.5). For a time-kill assay, Xoo BXO43 cultures were treated with two different concentrations of the partially purified antibiotic i.e. 8 µg/ml and 16 µg/ml. Aliquots were drawn at different time points 0, 1, 2, 4, 10 and 20 min, serially diluted and 10 µl of different dilutions were spotted on PS agar plates and incubated at 28 °C for 2-3 days. The growth was measured in terms of CFU/ml (Tripathi et al., 2015).

3.2.12. Semi-preparative HPLC

Semi-preparative HPLC was carried out for separating the components of partially purified antibiotic S2, detected by PDA detector in HPLC (Waters). The LC column used was symmetry C-18 with pore size 3.5 µm. The column dimensions were 4.6 mm x 75 mm. The mobile phase was composed of water (A) and methanol (B). HPLC was conducted in gradient mode with 95 % A for 1 min; then the concentration of B was increased linearly from 5 % to 95 % over 19 min, and was held at 95 % B for 5 min. The fractions collected at the interval of every 30 units of retention time were concentrated by evaporating organic solvent and the antimicrobial activity was checked by agar well diffusion assay against the sensitive bacteria Xoo BXO43 and *S. aureus*.

3.2.13. HRLC-MS/MS analysis

Samples were outsourced and it was analyzed on Q-ToF HRLC-MS/MS system (Agilent Technologies, USA) from SAIF, IIT Bombay with ESI in both positive and negative modes. LC column Luna (r) 5µm C18, 150 x 2 mm (Phenomenex) was used and the flow rate was kept at 0.3 ml/min. The mobile phase for Liquid chromatography was composed of (A) water and (B) acetonitrile. Gradient mode used was with 95 % A for 2 min; the concentration of B was then increased linearly from 5 % to 95 % over 26 min, and further at 95 % B for 4 min. Mass spectra were obtained by electrospray ionization both in negative and positive ionization modes at the range of 60 -1,000 m/z with the scan rate 1 spectra/min.

3.2.14. Scanning Electron Microscopy (SEM)

SEM analysis was carried out to determine the effect of antibiotic S2 present in extract of *B. altitudinis* S2 on Xoo BXO43 cells at the ultra-structure level. Xoo cells at its mid-log phase were treated with 16 µg/ml of partially purified antibiotic S2 and incubated for 1 hr at 28 ^{o}C. Cells were centrifuged and primary fixation was carried out with 2.5 % glutaraldehyde solution. Fixed cells were rinsed three times for 15 minutes each with 100 mM phosphate buffer, post-fixed with 1 % osmium tetroxide for 3 hrs. Dehydration was done through an acetone gradient from 30 % to 100 %. For SEM analysis, samples were coated with gold and analyzed on JEOL JSM-7600F FEG-SEM (Field Emission Gun-Scanning Electron Microscopes) at the facility of SAIF (Sophisticated Analytical Instrument Facility), IIT Bombay.

3.2.15. Live-dead staining of antibiotic treated Xoo BXO43 and *S. aureus*

To assess the viability of the Xoo cells, a live-dead staining method was performed using LIVE/DEAD BacLight bacterial viability staining kit L7012 (Invitrogen, Molecular Probes, USA). The kit consists of two coloured fluorescence stains: a green-fluorescent SYTO9 and a red-fluorescent propidium iodide (PI). When used in an appropriate mixture, live bacteria with intact membranes fluoresce green, while dead bacteria fluoresce red. Concentration of the dyes were prepared as mentioned in user's manual. Xoo BXO43 cells were treated with 16 µg/ml of partially purified antibiotic S2 for 5 hrs and then centrifuged at 2000 x g for 7 min, and resuspended in 10 mM sodium phosphate buffer (pH 7.4) to give OD_{600} ~0.5 (10^{12}–10^{14} cells/ml). Control included cells without antibiotic S2 treatment. Then 100 µl of the dye cocktail prepared as recommended by the manufacturer was added, and the cell suspension was incubated for 15 min in the dark. A direct microscopy using an epifluorescence microscope (Olympus model BX43) was used to view live cells (green emission at 530 nm) and dead cells (red emission at 630 nm) with excitation at 485 nm. Minimums of 30 fields or 300 cells were counted in all samples. Thus, the total cell count was live plus dead cells by direct microscopic count. Viable and dead counts were determined based on a microscopic LIVE/DEAD BacLight assay (Smith & Oliver, 2006; Adams et al., 2003).

3.2.16. Determination of protein concentration

Bradford assay was used to determine the protein concentration (Bradford, 1976) using Bovine serum albumin as a standard.

3.2.17. SDS-PAGE analysis

Xoo BXO43 culture was grown in PS medium at 28°C for 16 -20 hrs to achieve an inoculum of approximate mid-log phase (OD_{600} ~0.5). Then, antibiotic S2 at final concentrations of 8 and 4 µg/ml and streptomycin at 25 and 15 µg/ml concentration were added in different samples and incubated in a rotary shaker (220 rpm) at 28°C. After 5 hrs, cells were collected and OD_{600} was measured to determine the growth of cells. 0.2 OD_{600} equivalent cells were taken and an SDS-PAGE analysis was performed using 12 % polyacrylamide gels. Gels were stained with Coomassie brilliant blue R-250. Control consisted of cells not treated with antibiotic S2 (Sambrook & Russel, 2001; Shi et al., 2016).

Tricine-SDS-PAGE was carried out for detection of small molecular weight proteins in the extracted antibiotic S2 scrapped from TLC plate Rf 0.92 (Schagger, 2006).

3.2.18. Kresek Model of rice plant to study the effect of solvent extracted antibiotic S2 on prevention of BB

Seeds of susceptible rice variety TN-1 were surface sterilized by thoroughly washing them 3 -4 times with sterile distilled water, followed by immersing the seeds in 0.05 % $HgCl_2$ solution with constant stirring for 3 min and then with 70 % ethanol for 2 min. Finally the remaining $HgCl_2$ and ethanol were removed from the seeds by washing them for 3 -4 times with sterile distilled water. The sterilized seeds were transferred aseptically in sterile 0.8 % water-agar plates and incubated at 30 ^{o}C in dark for germination. After 2 -3 days, most of the seeds showed germination. The root hairs of germinated rice seedlings were trimmed in aseptic conditions for facilitating the entry of pathogen without disturbing the primary root and then the seedlings were transferred to 2 ml Xoo BXO43 saline suspension contained in sterile test tubes, for the development of seedling wilt or Kresek. For

preparation of saline suspension, Xoo BXO43 was grown in PS broth for 48 hrs was centrifuged at 10,000 rpm for 10 min, the pellet obtained was washed and suspended in sterile saline. 60 AU/ml of solvent extracted antibiotic S2 was used in the experiment. After the incubation, rice seedlings were transferred to the sterile MS agar tubes and incubated in the green house which was maintained at 28 -30 ^{o}C with 60 % humidity for 10 days. Controls without antibiotic and Xoo BXO43 and with only Xoo BXO43 were kept. After growth period the plant health was measured in terms of shoot and root heights, wet and dried shoot-root biomass. The efficacy of antibiotic to control the disease on rice plant was assessed by comparing the parameters of plant treated with antibiotic, without antibiotics and with pathogen. The experiment was done independently twice with triplicates.

3.2.19. Design of primers for genes for type III polyketide from *B. altitudinis* database using bioinformatics and their validation

Representative type III polyketide genes pks (polyketide synthase) and pmt (phospholipid methyltransferase) from database of different strains of *B. altitudinis* available online was used for designing the primers. The whole Genome data of the type strain *B. altitudinis* 41KF2b and other strains namely, *B. altitudinis* P-10, *B. altitudinis* SGAir0031, *B. altitudinis* FD48, *B. altitudinis* G8 and *B. pumilus* MTCC B6033 were used for primer designing. Primers were designed using Primer design tool of NCBI and are listed in Table 3.1. Genomic DNA isolation of the isolates was carried out according to the protocol of Sambrook & Russel (2001). 10 µL PCR mixture contained 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 200 µM each of dNTP, 100 ng primer, 1 U of Taq polymerase (Invitrogen) and 10 -30 ng of genomic DNA template. Negative control contained all the components of PCR mixture except the DNA template. 16S rRNA was taken as positive control for PCR using primers mentioned in section 2.2.7. of chapter 2. PCR amplification was carried out in Dice thermocycler (Taqara) using the following conditions: 1 cycle of 4 min at 94 °C; 35 cycles of 1 min at 94 °C, 1 min at 61.5 °C for pks, 63 °C for pmt and 58 °C for 16S rRNA, 1.5 min at 72 °C; and one cycle of a final extension for 10 min at 72 °C. Amplified PCR products separated on a 1% agarose gel in 1× TAE buffer, containing ethidium bromide (1 µg/ml) were visualized and photographed using transmitted UV light at 295 nm.

Table 3.1: Primers designed for genes from polyketide synthesis pathway

Name of the gene	Primer sequence (5' -3')	Amplicon size (bps)
pks	CGGCGGTAAAATCCCATC TGGCGAAATAGAGAGCAGGC	593
pmt	CAATCGCCACCACCACATAG GCGATTGAATTTGGTGCAAGC	283

3.3. RESULTS

3.3.1. Screening of production media and extraction solvents for antibiotic S2 from *B. altitudinis* S2

Production of antibiotic S2 by *B. altitudinis* S2 was carried out in different media and its activity was checked qualitatively by agar diffusion well bioassay using the test organism Xoo BXO43 (Table 3.1). Antibiotic production was observed in synthetic medium Sucrose-Bushnell Haas (SBH) broth which contains sucrose and minimal salts; while no production was observed in rich media containing complex media components viz. peptone, tryptone. No antibiotic activity was observed even after reducing the strength of the rich media like PS broth. Nevertheless, the antibiotic activity was observed when target strain i.e. Xoo BXO43 was co-inoculated in PS broth along with the antibiotic producing strain, i.e. *B. altitudinis* S2.

Extraction of antibiotic S2 was carried out using organic solvents having different strength of polarity viz. Hexane, Ethyl acetate, n-butanol, Chloroform, Isopropanol, Ethanol and Methanol. The antibiotic activity was observed only in polar solvents like Isopropanol, Ethanol and Methanol extract. Isopropanol was chosen as solvent for extraction as it does not extract sucrose of the media. Methanol and ethanol though were good solvents for antibiotic extraction also extracted sucrose from the medium. Table 3.2 depicts the results of the activity obtained in the extracted antibiotic S2 prepared from dissolving the residue in distilled water.

Table 3.2: Screening of media and organic solvents for production and extraction of antibiotic S2 produced by *B. altitudinis* S2

Media		Extraction	
Type	**Activity in CFS**	**Organic solvent**	**Activity of the extracted antibiotic**
LB, TSB, PS broth	-	ND	-
1/4th strength PS broth	-	ND	-
PS broth with Xoo BXO43	+	Methanol	+
SBH agar	+	Methanol	+
SBH broth	+	Hexane	-
		1-butanol	-
		Ethyl Acetate	-
		Chloroform	-
		Isopropanol	+
		Ethanol	+
		Methanol	+

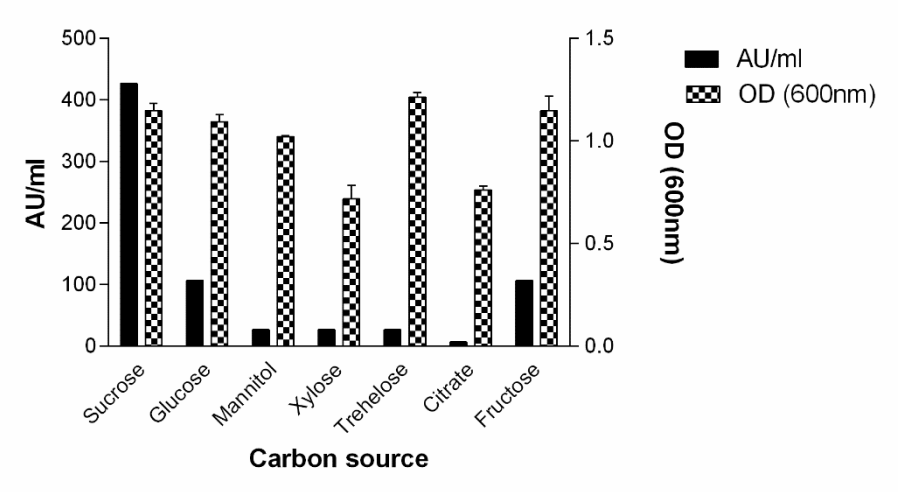
ND – Not Determined, LB- Luria Bertani broth, PSA- Peptone Sucrose Agar, SBH – Sucrose Bushnell Hass medium, TSB-Tryptic Soy broth, CFS is cell free supernatant of *B. altitudinis* S2

3.3.2. Effect of different carbon sources, temperature and pH on antibiotic S2 production by *B. altitudinis* S2

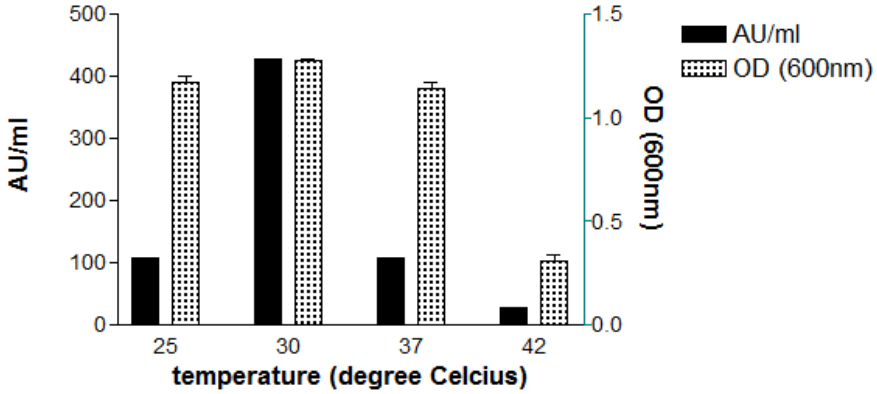
When different sugars were used to study the effect on the production of antibiotic S2 by *B. altutidinis* S2 the growth of the bacteria was supported in all the sugars. Comparatively higher growth of 1.21 ±0.02 OD were achieved with trehalose, relatively lower growth of 1.15 ±0.07 OD when sucrose and fructose were used as carbon source individually and growth of 1.02 OD was observed in case of mannitol. Xylose and citrate supported relatively less growth of OD < 0.8. Noticeably, no correlation of the antibiotic S2 production with the growth in individual carbon source was observed. Highest production of antibiotic S2 of about 426.67 AU/ml was observed in medium with sucrose, while antibiotic production in media containing other sugars was < 110 AU/ml (Fig. 3.1a).

The growth and antibiotic production by *B. altutidinis* S2 was obtained in the temperature range from 25 -42 ^{o}C studied; however, the optimum temperature for the production of bioactive metabolite was found to be 30 ^{o}C as about 426.67 AU/ml (Fig. 3.1b). When different pH 5.8 -8 were tested, higher growth of the organism was observed at 7 and 7.5, while optimum pH for the production of antibiotic S2 of about 426.67 AU/ml, found was pH 7 (Fig. 3.1c).

(a)



(b)



(c)

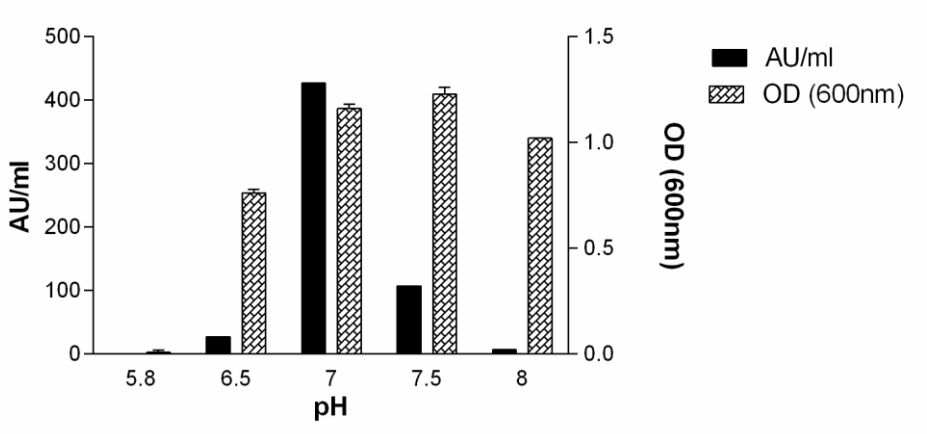


Fig. 3.1:Effect of physical parameters on antibiotic S2 production.
Effect of a) different carbon source, b) temperature and c) pH on growth of *B. altitudinis* S2 and production of antibiotic S2.

3.3.3. Time-course production of antibiotic S2 and growth of *B. altitudinis* S2

The growth and time course production of antibiotic S2 by *B. altitudinis* S2 was monitored in Sucrose Bushnell Haas (SBH) broth where the production was analyzed using agar diffusion well assay and quantitated in terms of AU using 96 as per section 3.2.5. The antibiotic S2 production was initiated at 48 hrs in the early stationary phase and quantified as 7 AU/ml. Further in the stationary phase which was observed to extend till 192 hrs, the production of antibiotic S2 was found to increase linearly and reach the maximum of 6823 AU/ml at 192 and after 192 hrs, the production was found to be constant (Fig. 3.2).

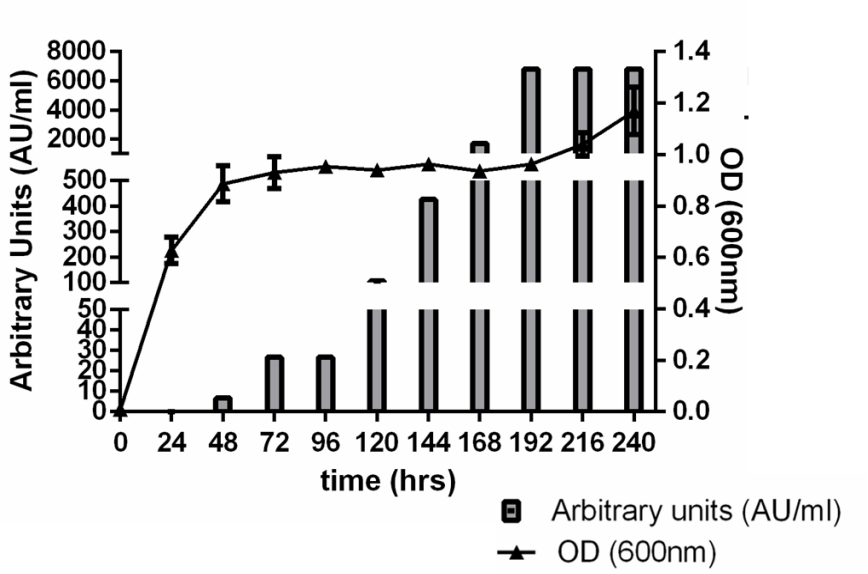


Fig. 3.2: Time-course Production of antbiotic S2
Time-course production of antibiotic S2 measured as Arbitrary Units and growth (OD_{600}) of *B. altitudinis* S2 in SBH medium

3.3.4. Determination of efficacy of solvent extracted antibiotic S2 in controlling the Kresek disease

Kresek model was adopted to study the efficacy of the antibiotic S2 to protect the plant from disease when infected with Xoo BXO43 in green house conditions. The susceptible rice variety TN-1 and Xoo BXO43 culture of different optical density i.e. 0.2, 0.4, 0.7 and 1.0 OD_{600} was used to optimise the infectious dose the 3 days old germinated rice seedlings as described in section 3.2.18 for development of Kresek disease model. 1.0 OD was found to be optimum in showing lesions on leaves and overall growth retardation of the rice plant as was observed after 10 days of incubation in the green house. Seedlings treated with 60 AU/ml of

the antibiotic S2 did not show any diseased symptoms like yellowing of leaves or growth retardation of the plant and the growth was found as healthy as untreated control after 10 days of incubation as shown in Fig. 3.3. The effect of the antibiotic S2 on rice plant was evaluated using different parameters like length of root and height of shoot, wet and dry weight of the shoot and root of the rice plants infected with pathogen Xoo BXO43, the results of which are shown in Table 3.3. The growth parameters of the plants treated with antibiotic S2 before infection with Xoo BXO43, was almost near to the healthy untreated rice plants, while that of the plants treated with pathogen only showed reduced growth parameters of the plant.

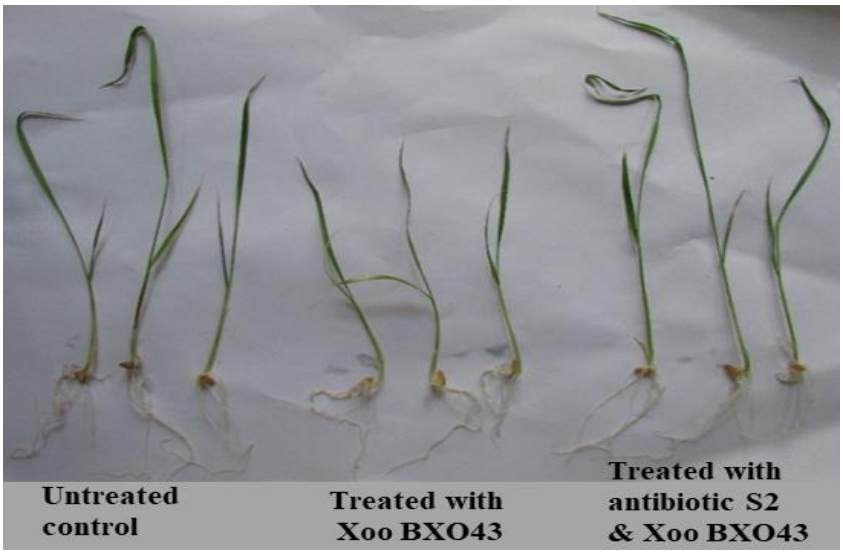


Fig. 3.3: Kresek disease model showing potential of antibiotic S2
Kresek model of rice plant showing yellow lesions on leaves and retarded growth of the plant when treated with Xoo BXO43 and no disease symptoms on the plant infected with Xoo BXO43 and treated with antibiotic S2

Table 3.3: Plant parameters to evaluate efficacy of antibiotic S2 in protecting rice plant

Experimental set	Shoot Length (cm)	Root Length (cm)	wet weight (mg)		Dry weight (mg)	
			Shoot	Root	Shoot	Root
control	14.03 (1.27)	5.90 (0.72)	46.27 (2.63)	43.96 (10.23)	8.23 (0.39)	8.58 (0.48)
Xoo BXO43	7.63 (1.44)*	4.03 (2.78)	16.36 (4.28)*	26.88 (4.54)	3.69 (1.40)*	6.05 (0.37)
Xoo BXO43 +antibiotic S2	15.63 (2.57)	5.50 (1.00)	47.51 (11.51)	24.63 (2.73)	8.30 (1.48)	7.63 (0.38)

Results are mean of three independent experiments. Data were analyzed using one-way ANOVA measures followed by Bonferroni's multiple comparison tests. *, $p < 0.01$ level of significance.

3.3.5. Effect of different physicochemical parameters on solvent extracted antibiotic S2 activity

The stability of solvent extracted antibiotic S2 from *B. altitudinis* S2 was examined at different temperatures and pH. The antibiotic S2 aqueous solution was incubated at different temperatures 40, 45, 50, 55, 60, 65 for 2 hrs and 121 °C for 15 min and after cooling it at room temperature the activity was assessed by agar diffusion well assay. It was found to tolerate the temperatures from 40 –65 °C; while 28 % activity loss was observed when autoclaved at 121 °C for 15 minutes. It was also found to tolerate acidic and alkaline pH when tested at pH 2, 5, 8 and 9. The solvent extracted antibiotic S2 retained its antimicrobial activity even after treatment with enzymes Proteinase K, Trypsin and β-amylase. The results of the temperature, pH and various enzymes stability tests are summarized in table 3.4.

Table 3. 4: Stability of antibiotic S2 in different treatments.

Experimental set	Zone of inhibition (mm)	Residual activity (%)[#]
Untreated control	27 ±2	100
Temperature(°C)		
40	27 ±0	100
45	27 ±0	100
50	27 ±2	100
60	27 ±2	100
65	27 ±2	100
121	19 ±4	72
pH		
2	27 ±2	100
5	27 ±0	100
8	27 ±6	100
9	27 ±2	100
Enzymes		
Proteinase K	27 ±2	100
Trypsin	27 ±1	100
Amylase	27 ±2	100

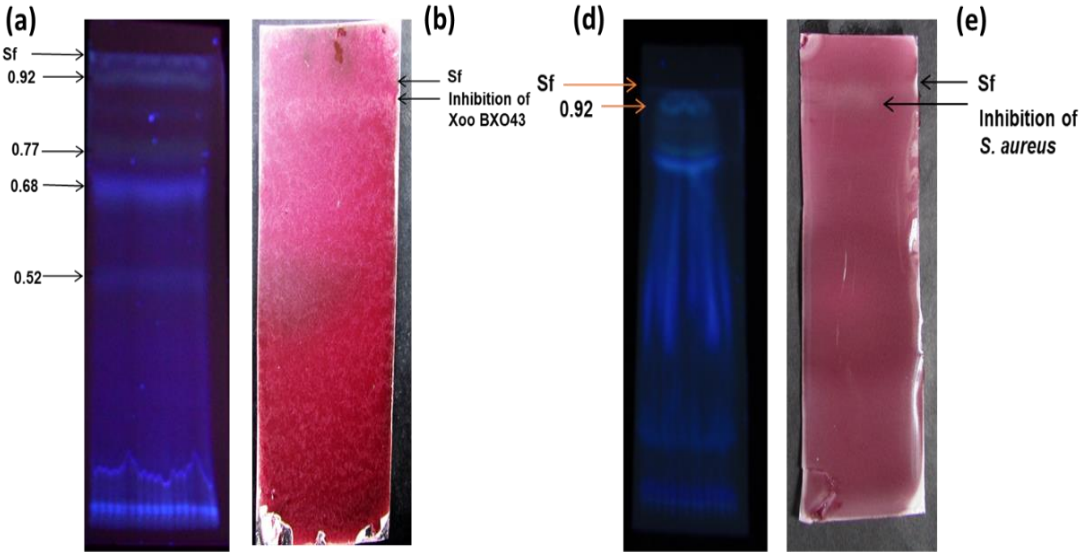
Effect of temperature, pH and enzymes on antibiotic S2 produced by the isolate *B. altitudinis* S2 tested against Xoo BXO43 in agar well diffusion assay
[#]Residual activity = (inhibition zone/ inhibition zone of untreated) x 100

3.3.6. Separation and detection of bioactive component from solvent extracted antibiotic S2

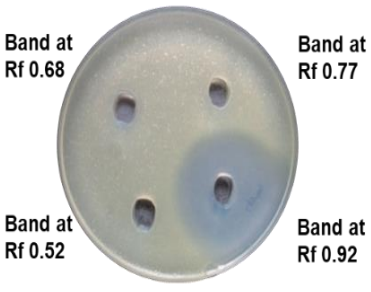
Different bands at Rf values 0.92, 0.77, 0.68 and 0.52 were observed under UV radiation when the solvent extracted antibiotic S2 was run on the TLC plate

(Fig 3.4a). Notably, only the band corresponding to Rf 0.92 gave the desired activity (Fig 3.4 b and c) detected by tretrazolium dye (method described in section 3.2.9). The band with antibiotic activity was detected by direct bioautography using Xoo BXO43 (Fig 4.3b). The four bands obtained were scrapped and antibiotic activity against Xoo BXO43 was checked by agar well diffusion method (Fig 3.4c). Fig 3.4d and e depicts the TLC-DB using *S. aureus*. Similar band showed activity against *S. aureus*.

The active band was eluted from TLC with Rf 0.92 and dissolved in methanol was analysed for absorption spectra in 190 -700 nm range of wavelength. As shown in the Fig. 3.5, it showed absorption maxima in UV region at 225 -227 nm. The absorption spectra of methanol did not show any prominent peak over the scanned region



(c)



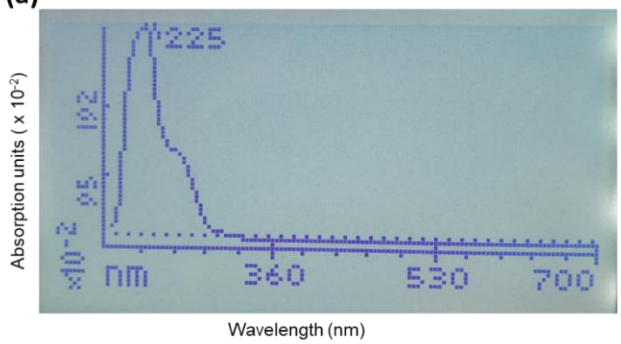
(f)

Rf of bands	Antibiotic activity
0.92	+
0.77	ND
0.68	ND
0.52	ND
+ inhibitory activity detected; ND – Not Detected	

Fig. 3.4: TLC-DB of crude extract of *B. altitudinis* S2

a) TLC showing resolved components of antibiotic S2 as observed under UV, b) direct bioautography using the test organism Xoo BXO43, c) Agar diffusion well assay depicting antibiotic activity of the individual bands from TLC, and d) & e) TLC-DB performed with *S. aureus*. (f) bands on TLC at Rf values evaluated for inhibition activity.

(a)



(b)

Wavelength (nm)	Absorbtion units (AU)
225	3.188
227	3.188
418	0.006
653	0.006

Fig. 3.5: Absorption spectrum of extract containing antibiotic S2
Absorption spectrum of the eluted band Rf 0.92 from TLC carried out with solvent extracted antibiotic S2 that showed inhibition of Xoo BXO43 and *S. aureus*.

3.3.7. Purification and characterization of the antibiotic S2

The band in TLC corresponding to the one which showed inhibition of the test organisms, i.e. Xoo BXO43 and *S. aureus* was scraped and eluted with methanol. The presence of antibiotic activity in the methanol extract was confirmed using both the test organisms Xoo BXO43 and *S. aureus*. The partially purified antibiotic S2 was examined for the presence of protein/peptide by tricine-SDS-PAGE for low molecular weight proteins. No bands were detected by silver staining, indicating absence of proteins of major size in the extracted sample containing bioactive metabolite. Hence, ESI-MS mass spectrometry was chosen for further analysis.

Semi-preparative HPLC was carried out as described in section 3.2.12. with partially purified antibiotic S2 to separate the bioactive metabolite, specifically which showed inhibition of the test organisms. The fractions at RT 9.8 -10.2 showed activity when tested against Xoo BXO43 and *S. aureus* for their activity. Further, HRLC-MS and MS/MS was carried out of the active fraction. The LC-ESI-MS spectral analysis of the fraction showed two prominent peaks (Fig.3.6 a). One at RT 1.081 min with m/z 365 [peak I] and another, at RT 10.018 min $[M + H]^+$ ion with m/z 573.2131 [peak II]. Further, ESI-MS/MS of the two peaks was carried out. The fragmentation pattern corresponding to MS2 fragmentation of the peak II gave 113.948, 119.0129, 136.9132, 147.0054, 156.9892, 174.9986 along with 573.2167 when collision energy used was 43.39 % (Fig 3.6b). Amongst these peaks, peak with mass value 113.948 was the most abundant. Notably, mass value of 573.2167

is obtained when 5 units of 113.948 summed up; indicating the parent ion is probably composed of repeated units of 113.948.

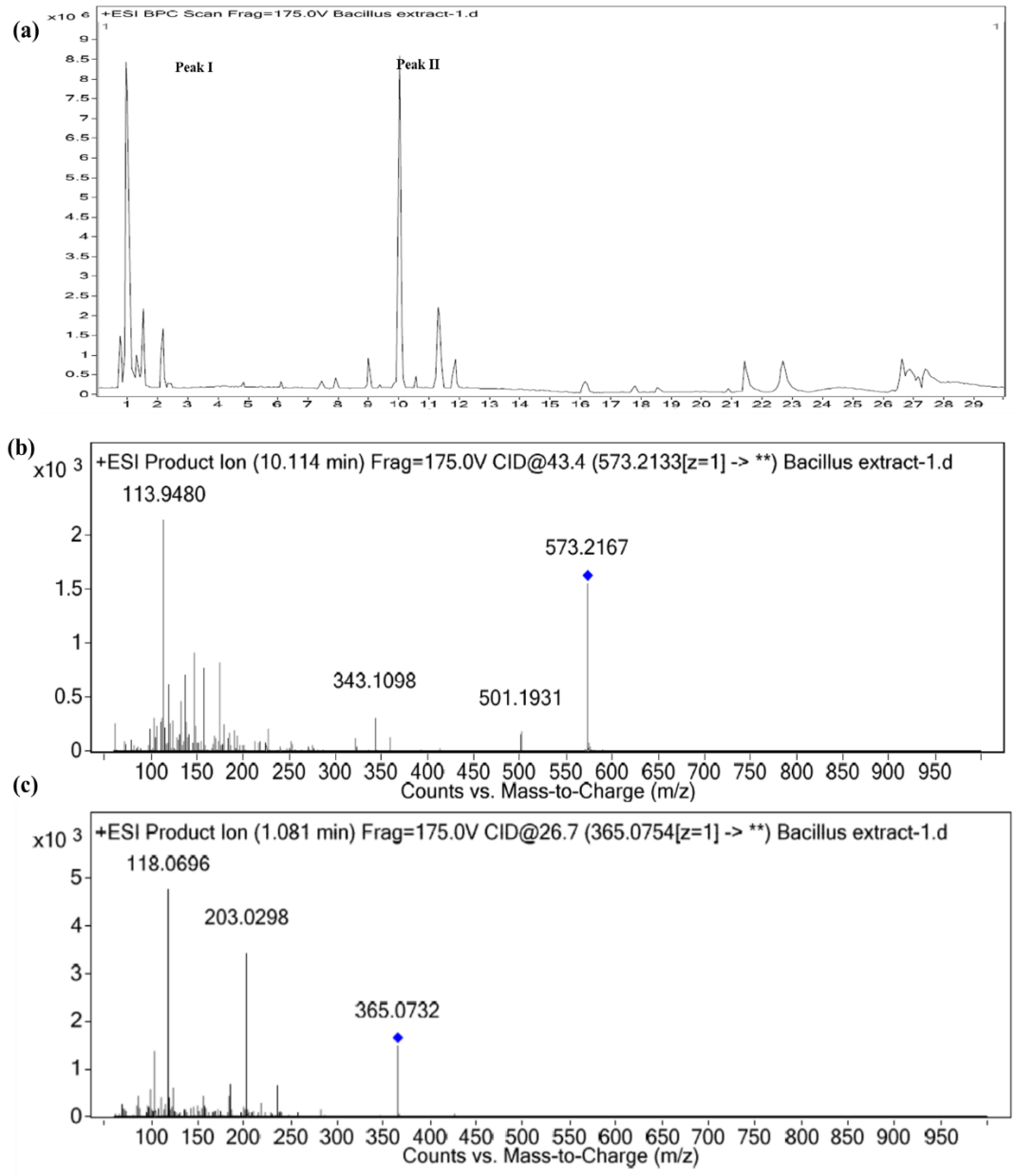


Fig. 3.6: chromatographs obtained by HPLC and peaks by mass spectrometry of antibiotic S2

HPLC profile of band containing antibiotic S2 purified from semi-preparative HPLC, **b)** ESI-MS/MS of m/z 573.2147 when 43.4 % collision energy was applied **c)** ESI-MS/MS of the peak with m/z 365.07 when 26.7 % collision energy was applied.

3.3.8. Detection of type III polyketide genes from *B. altitudinis* S2

Presence of type III polyketide genes in the genome of *B. altitudinis* S2 was determined to validate the possibility of synthesis of polyketide antibiotic. Primers were designed and further PCR was carried out for the genes pks and pmt as

described in section 3.2.19. The amplicon size of pks was 593 bps and that of pmt was 283 bps, obtained matched the expected band size. Similar results were obtained with a type strain *B. altitudinis* 41KF2b by (Shivaji et al., 2006) (Fig. 3.7).

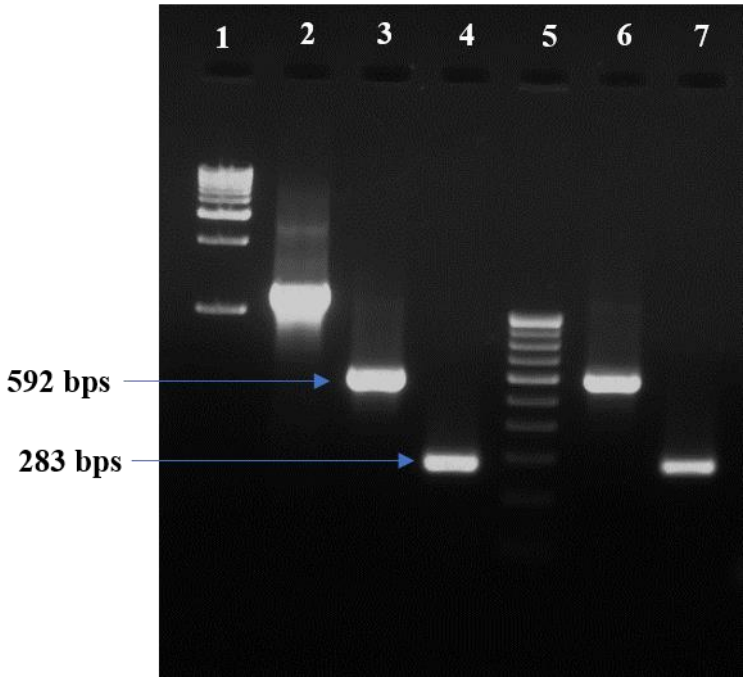


Fig. 3.7: Gel image showing amplification of pks and pmt genes from *B. altitudinis* S2 and type strain *B. altitudinis* 41KF2b
Lane 1- 1 kb StepUp ladder, Lane 2- 16S rRNA amplicon from *B. altitudinis* S2, Lane 3- pks amplicon of approx. size 600 bps (expected 592 bps) from *B. altitudinis* S2, Lane 4- pmt amplicon of approx. size 300 bps (expected 283 bps) from *B. altitudinis* S2, Lane 5- 100 bp StepUp ladder, Lane 6- pks amplicon from *B. altitudinis* 41KF2b, Lane 7- pmt amplicon from *B. altitudinis* 41KF2b.

3.3.8. Determination of MIC and MBC of partially purified antibiotic produced by *B. altitudinis* S2 against Xoo BXO43

Determination of MIC and MBC values of the antibiotic showing inhibitory activity against Xoo BXO43 was done by microdilution plate assay as described in the section 3.2.10. Different concentrations of antibiotic were prepared by two fold serial dilutions and their effect evaluated in terms of optical density determined the MIC obtained to be 8 µg/ml. MBC of the antibiotic was evaluated by sub-culturing from the broth of the well with MIC of the antibiotic as well as three wells preceding and succeeding it on PSA plates and it was determined to be 16 µg/ml. This indicates that the bioactive compound is bactericidal in nature.

The antibacterial activity was further confirmed by the viability cells assay using LIVE/DEAD backlight assay (Fig. 3.8) and the time-kill assay (Fig. 3.9). For evaluation of effect of antibiotic on viability of sensitive target strains Xoo BXO43 and *S. aureus* phase contrast/fluorescence microscopy in combination with LIVE/DEAD BacLight bacterial viability staining was used as described in section 3.2.15. As observed in the untreated control of Xoo BXO43 i.e. the cells with no exposure to antibiotic gave intense green colour (Fig 3.8 a ii) and faint intensity of red colour (Fig. 3.8 aiii). Whereas, the cells exposed to antibiotic gave very intense red colour (Fig 3.8 avi) while the images in Fig. 3.8 a iv and v are phase contrast and fluorescence image respectively. The images in Fig 3.8 bi-vi show identical results with *S. aureus*.

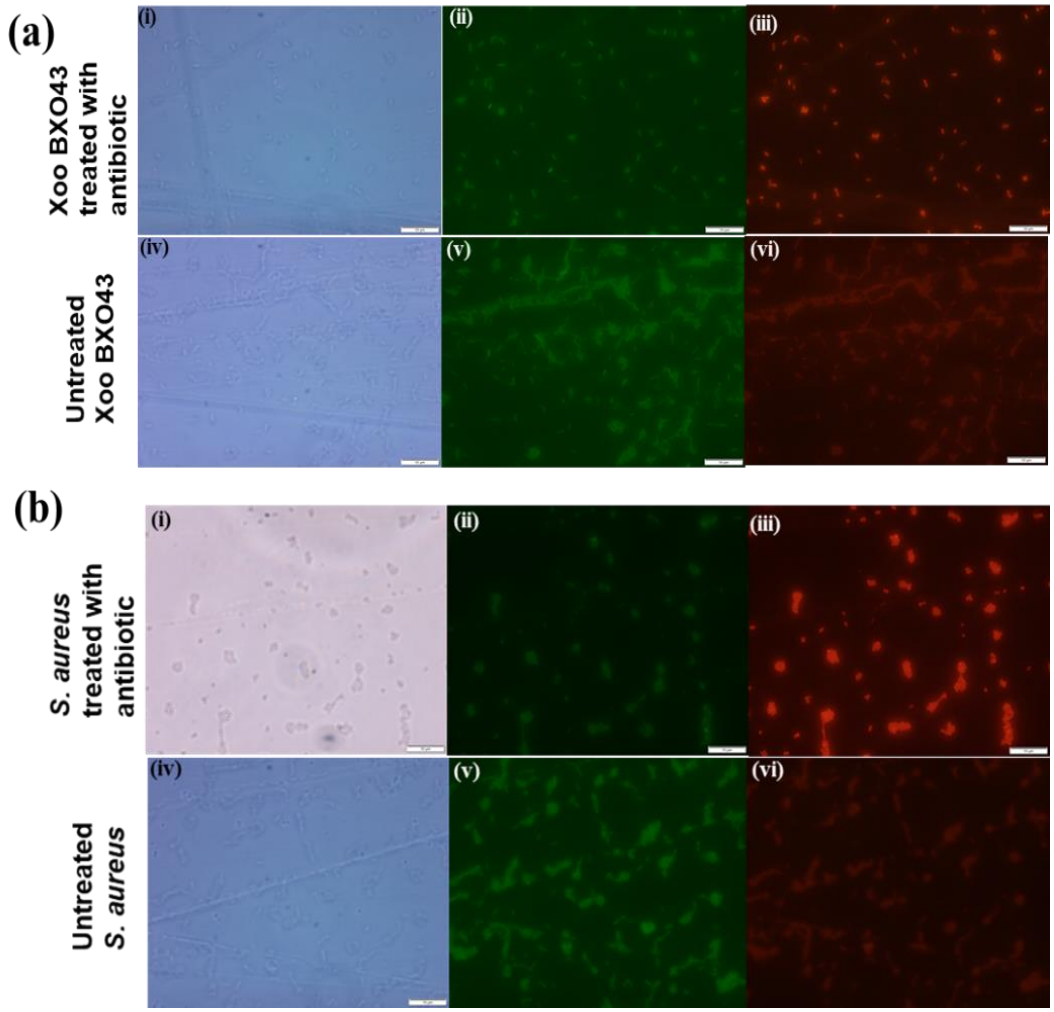


Fig. 3.8: Micrographs showing effect of exposure to antibiotic S2 using Live-Dead staining kit

SYTO9 stains all cells as indicated by green colour, while dead cells show higher intensity of red colour as propidium iodide stains penetrates only dead cells. (a) Untreated Xoo BXO43 (i-iii) and cells treated with MBC of antibiotic (iv-vi). (b) Untreated *S. aureus* (i-

iii) and cells treated MBC of antibiotic S2 (iv-vi).

Further, for time-kill assay, the aliquots from samples treated with antibiotic S2 were taken at different time points as described in section 3.2.11 and plated on PS agar plates for viable cell count. At 8 µg/ml concentration, the antibiotic inhibited the growth of Xoo BXO43 from 10^{11} to 10^{4} CFU/ml as observed over period of 20 min. When the concentration of the antibiotic S2 increased to 16 µg/ml, the number of viable cells decreased almost to undetectable level and no viable cells were detected from the sample taken beyond 4 min of incubation time (Fig 3.9).

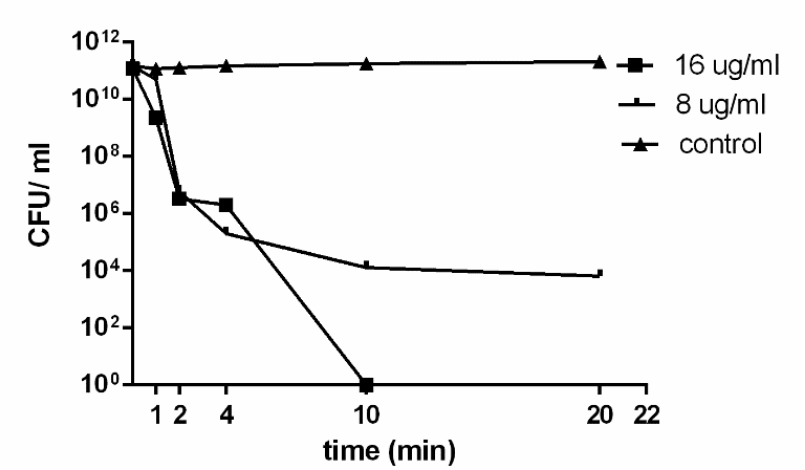


Fig. 3.9: Time-kill assay of antibiotic S2 using Xoo BXO43.
Xoo BXO43 treated with MIC (8 µg/ml) and MBC (16 µg/ml).of antibiotic S2. The inhibitory effect was evaluated in terms of CFU/ml survival of the pathogen after treatment with antibiotic at various time points. No treatment of the pathogen was taken as control

3.3.9. Effect of partially purified antibiotic S2 on morphology of Xoo BXO43 and *S. aureus*

Scanning Electron microscopy was carried out for visualizing the effect of antibiotic on the sensitive target bacterial cells. Here the untreated Xoo BXO43 cells appeared intact and normal smooth rod-shaped cells. On exposure to antibiotic no noticeable change was observed (Fig. 3.10 a). Similar observation were noted for *S. aureus* when treated with antibiotic (3.10 b). This indicates that probably the antibiotic does not rupture the cells of the target bacterial cells

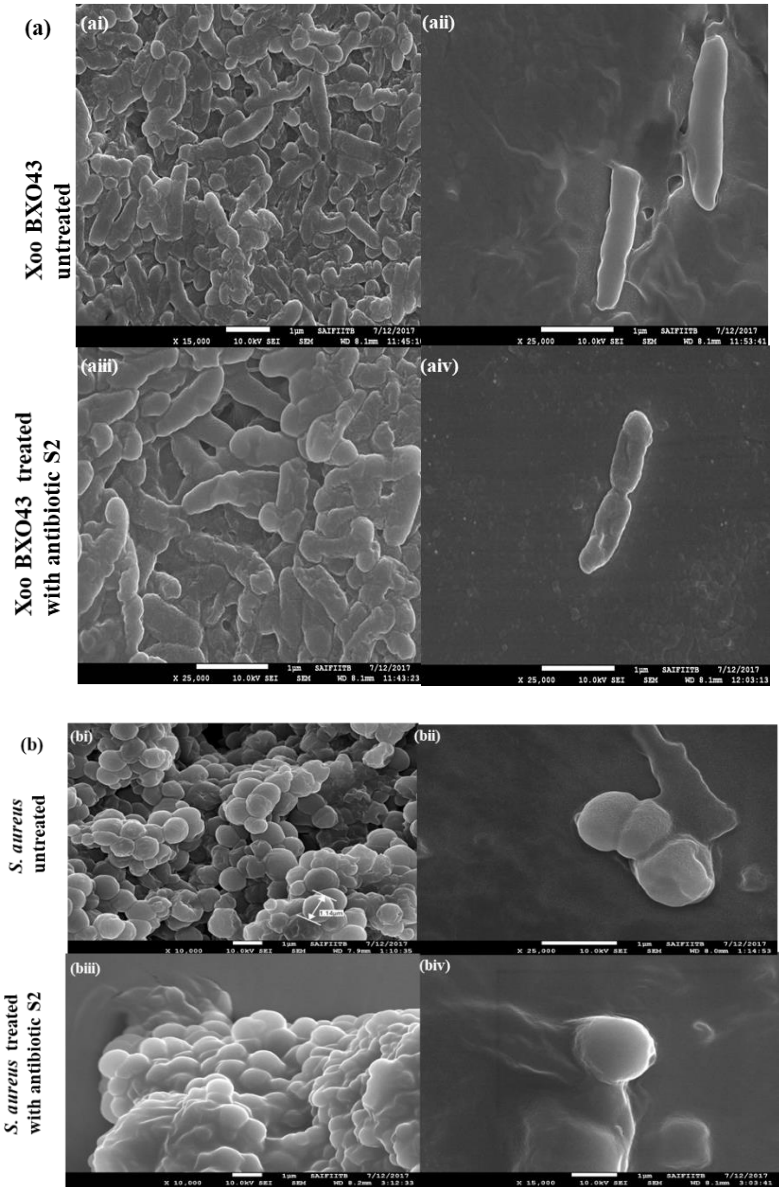


Fig. 3.10: Scanning electron micrograph depicting surface morphology of Xoo BXO43 and *S. aureus* when exposed to antibiotic S2

ai) untreated Xoo BXO43 cells, 10,000x (aii) untreated Xoo BXO43 cells, 25,000x, (aiii) Xoo BXO43 cells treated with antibiotic S2, 10,000x, (aiv) Xoo BXO43 cells treated with antibiotic S2, 25,000x; bi) untreated *S. aureus* cells, 10,000x, (bii) untreated *S. aureus* cells, 25,000x, (biii) *S. aureus* cells treated with antibiotic S2, 10,000x and (biv) *S. aureus* cells treated with antibiotic S2, 25,000x

3.3.10. Effect on total protein of Xoo BXO43 when treated with antibiotic S2

To evaluate the mechanism of action of the antibiotic on the Xoo cells, total protein content of the XooBXO43 cells treated with antibiotic S2 were analysed. After treatment with 8 μg/ml and 16 μg/ml antibiotic for 5 hrs, total protein was extracted and measured by SDS-PAGE. Protein equivalent to 0.16 OD_{600} of cell mass of all the samples were used for normalization. The total protein profile of

Xoo BXO43 was decreased with increase in concentration of antibiotic treatment from 8 to 16 µg/ml (Fig. 3.11) as indicated by decrease in intensity of certain bands or complete loss of certain bands as observed in PAGE. Streptomycin treatment was taken as control as its mode of action is inhibition of protein synthesis in sensitive strains.

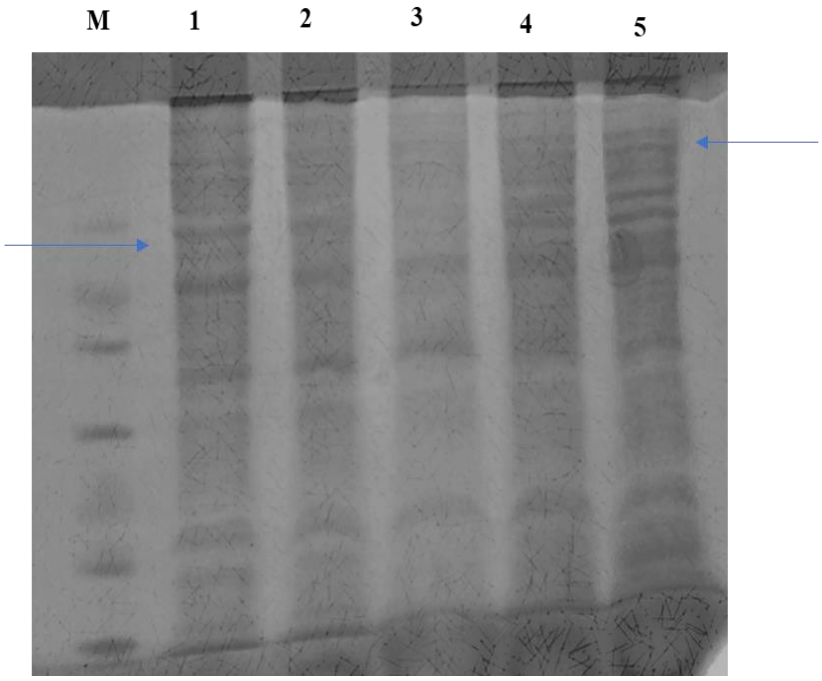


Fig. 3.11: SDS-PAGE showing effect of antibiotic S2 on total protein of Xoo BXO43

Effect of antibiotic S2 and Streptomycin on total protein of Xoo BXO43. Lane M - Protein Marker (70-14 kDa), Lane 1 - PBS treatment control; Lane 2 - 4 µg/ml of antibiotic treatment for 5 hrs; Lane 3 - 8 µg/ml of antibiotic treatment for 5 hrs; Lane 4 - 25 µg/ml of Streptomycin for 5 hrs; Lane 5 - 15 µg/ml of Streptomycin for 5 hrs. The arrows indicate decreased intensity or lost band(s)

3.4. Discussion

The selected isolate identified as *B. altitudinis* S2 obtained from plant root after screening of antagonist against Xoo BXO43 showed strong inhibitory activity in plate bioassay and detached leaf assay. Two genera *Bacillus* and *Pseudomonas* are the most predominant inhabitants of the rhizosphere of plants (Yusuf et al., 2011). However, members of *Bacillus* group have been reported to produce diverse secondary metabolites which can be potent biocontrol agents (Fickers, 2012). The aim of studies in this chapter was the production and characterization of the bioactive metabolite produced by *B. altitudinis* S2, the XooBXO43 antagonist.

Production media composition is foremost important factor in antibiotic production. Additionally, production of antibiotic by the bacteria is profoundly influenced by various environmental factors such as temperature, pH and incubation (Iwai et al., 1973). Therefore, different media were screened for production of antibiotic by *B. altitudinis* S2. No antibacterial activity was detected when it was grown in LB or NB, the common media used for growth of the *Bacillus* strains or even in TS broth, which is commonly used for antibiotic production (Vijayakumari et al., 2013; Slininger et al., 1995). When medium like PS broth on which screening of *B. altitudinis* S2 was performed or even 1/4th strength PS medium was used, no antibacterial activity was observed. Only in the minimal media SBH broth out of all the media screened, the bioactivity was detected, which was also observed during screening of the isolates. SBH broth is a synthetic medium which contains Sucrose as carbon source and minimal salts present in synthetic Bushnell Hass medium. This indicated that abiotic or biotic stress could act as trigger for production of the antibiotic in *B. altitudinis* S2. The minimal media exerted abiotic stress which must have induced the bacterium to produce the antibiotic. In complex medium there was no antibiotic production due to absence of stress. While the production of antibiotic in presence of the target bacterium Xoo BXO43 can be attributed to biotic stress. This also indicated that production of antibiotic is inducible in nature since it is triggered in stress condition. For selection of extraction solvent the extraction of antibiotic was performed in solvents with different polarity where it was obtained in polar solvents like methanol, ethanol and isopropanol which indicated that the antibiotic is probably hydrophilic in nature. It has been demonstrated that different organic solvents are used for extraction of secondary metabolites and solubility of these compounds is favoured in solvents with similar polarity (Raynie et al., 2006).

Sucrose was found to be the best carbon source for the production of antibiotic S2. The optimum temperature and pH for antibiotic production were 30 ^{o}C and 7 pH, respectively. For maximum extraction of the antibiotic, it is important to know the phase at which the optimum production of antibiotic is achieved. Hence, time-course production of the antibiotic was carried out in SBH medium. The CFS exhibited antibacterial activity at 24 hrs of growth and maximum production was found to be in late stationary phase which was achieved at 96th hrs.

In one of the studies similar results were obtained where *B. altitudinis* ZJ186 grown in Laddy medium, a synthetic medium, composed of Glucose and minimal salts for antifungal metabolite production showed activity from 20 hrs which attained maximum at 96^{th} hrs (Jin et al., 2012).

Kresek model of the rice plant was developed to study the pathogen control ability of the solvent extracted antibiotic S2 in the green house. The treatment of seedling with the solvent extracted antibiotic S2 prior to infection with Xoo BXO43 showed significant protection against the bacterial blight symptoms caused by the pathogen Xoo. This suggests that crude extract containing antibiotic can be used to control the disease symptoms on rice plant caused by Xoo.

Physicochemical characteristics of the bioactive metabolites can help in understanding the nature of the bioactive metabolites produced by the microorganisms. Hence, temperature and pH stability studies were carried out. The antibiotic activity was relatively stable upto 65 ^{o}C; however it lost some activity at high temperature. The antibiotic S2 was stable over wide pH range of acidic to alkaline. It did not lose the activity after treatment with various enzymes like Proteinase K and trypsin indicating that the antibiotic S2 probably might not be proteinaceous in nature. Since certain small peptidic antibiotics are resistant to these enzymatic treatments owing to different types of amino acids as their components (Guder et al., 2000; Benz et al., 1991). Resistance to β-amylase indicates it might not have any oligosaccharide moiety; however, proteinaceous nature of the bioactive metabolite could not be completely ruled out as many peptidic/proteinaceous antibiotic are made of different types of amino acid and have been reported to be resistant to Proteinase K and trypsin (Katz & Demain, 1977).

Bioautography using Thin Layer chromatography technique has distinct advantage as the separation and bioactivity detection are performed simultaneously on the TLC plate. This helps in identification of the bioactive metabolites against the test organism in short time and minimal labour. The antibiotic S2 produced by the isolate did not show antifungal activity when most common phytopathogenic fungi were tested. However it also showed activity against the Gram positive bacterium *S. aureus*. The band at Rf 0.92 was identified as active band containing the antibiotic by TLC-DB which showed activity against both Xoo BXO43 and *S. aureus*. This indicated that probably the same antibiotic S2 is active against both

the strains. Hence, it could be inferred that either same metabolite is active against both the test organisms or the metabolites responsible for their inhibition are coeluted in TLC.

Further, partial purification by preparative HPLC and ESI-MS/MS provided two peaks of m/z value 365 and 573.214 at RT 1.081 and 0.018.min, respectively. When MS/MS of peak of 573.214 value was carried out, it was noticed that after around 43.39 % collision energy, the most predominant product ion obtained was of m/z value 113.948. A close observation of the MS/MS spectra of 573.214 m/z reveals that the parent ion is made of multiple repeat unit of 113.948. This gives a clue that the metabolite produced by *B. altitudinis* S2 possibly was comprised of repetitive units.

Bacillus strains possess huge array of compounds which possess anti-bacterial and anti-fungal activities which are attributed to either ribosomal synthesis bacteriocins or to lipopeptides and polyketides that are made by non-ribosomal synthesis (Zuber et al., 1993; Fickers, 2012). From available literature, it was found that antibiotic compounds like bacteriocins and bacteriocine-like inhibitory substances (BLIS) are proteinaceous and their size varies from 0.77-210 kDa (Stein et al., 2005; Begley et al., 2009; Barboza-Corona et al., 2007). The cyclic peptides and lipopeptides of the mass range of 800–5000 Da are typically used as biomarkers for *Bacillus,* and they possess distinct antimicrobial properties as well (Vater et al., 2002; Price et al., 2007). Although, polyketides are widespread secondary metabolites from bacteria, only a few have been isolated and characterized from *Bacillus* (Schneider et al., 2007).

Widespread studies are cited in literature where *Bacillus* genus has been shown to produce antimicrobial metabolites. *Bacillus amyloliquefaciens* FZB42 has been shown to harbour an array of giant gene clusters that produce several secondary metabolites with antimicrobial activity, both by genome sequence and mutational studies (Chen et al., 2009; Koumoutsi et al., 2004). Non-ribosomally synthesized cyclic lipopeptides; surfactins, Bacillomycin D and Fengycin have been shown to possess antimicrobial mainly antifungal activity, while its antibacterial activity is mainly due to non-ribosomal synthesis of polyketides. Other antibiotics with nematocidal and algicidal activities have also been studied. Two polyketides namely Difficidin and Bacilysin from *B. amyloliquefaciens* FZB42

have demonstrated to have antibacterial activity against *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* (Wu et al., 2015). Two antibacterial polyketides have also been shown to be produced by *B. subtilis*. Macrolactin has been isolated from *Bacillus sp*. AH159-1, *Actinomadura* and uncharacterized species (Zheng et al., 2007). Difficidin and Oxydifficidin have been isolated from *B. subtilis* (Wilson et al., 1987).

Genome mining experiments were carried out amongst the members of *Bacillales* by Aleti & group (2015). They concluded that several strains harboured gene cluster well described for Difficidin, Bacillaene and Macrolactin synthesis, along with new variants which have not been anticipated earlier in *B. amyloliquefaciens* and *B. subtilis*. Two species *B. amyloliquefaciens* and *B. subtilis* are noted as prolific producers of Polyketides. Their work included other *Bacillus* species, amongst which *Brevibacillus brevis, B. atrophaeus* and *B. mojavensis* were reported to possess polyketide genes. The genome mining carried out on *Bacillales* indicated that bacterial strains associated with plant, harboured polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS) while bacteria not associated with plant do not harbour these genes (Aleti et al., 2015).

The antibacterial polyketides isolated and characterized from various *Bacillus* species have been detected with m/z values below 1000. Chakraborty et al. (2017a) isolated various polyketides and characterized them by NMR and spectrometry techniques. They found that all the antibacterial polyketides were in the range of 200-500 m/z value as measured by ESI-MS. An antibacterial polyketide isolated from *B. subtilis* showed m/z of 445 (Chakraborty et al., 2014). Difficidin and Oxydifficidin purified from different *B. subtilis* strains and *B. amyloliquefaciens* are highly unsaturated 22-membered macrocyclic polyene polyketides of m/z values 543.4 [M-H] and 559.3 [M-H], respectively (Chen et al., 2006; Wilson et al., 1987). Bacillaene have been characterized to have m/z value of 581 [M+H] by HRMS (Chen et al., 2006; Patel et al., 1995). The antibiotic S2 isolated from *B. altitudinis* S2 also has m/z value in the range of the general value of other reported polyketides and does not match with those reported for other antibiotics like Bacteriocins and Lipopeptides. The data obtained does not give any conclusive support towards identification of antibiotic S2. However, to the best of our knowledge, till date no polyketide antibiotic has been isolated from *B.*

altitudinis strains. However, many *B. altitudinis* strains have been demonstrated to have biocontrol activities against various phytopathogens (Budiharjo et al., 2017; Lu et al., 2017; Sunar et al., 2015).

The whole genome sequence of few *B. altitudinis* strains have been recently studied. *B. altitudinis* Lc5 has been predicted to contain 26 gene cluster types comprising a terpene-siderophore, type III polyketide synthase, bacteriocin, nonribosomal peptide synthetase, sactipeptide, and biosynthetic gene clusters of bacilysin, pseudomonine, fengycin, surfactin, and lichenysin (Potshangbam et al., 2018). The whole genome sequence of *B. altitudinis* P-10 which demonstrated to protect rice plants against bacterial blight caused by Xoo, was predicted to contain variuos genes of lichenysin or bacilysin like non-ribosomal peptides, terpenes bacteriocin and type III polyketide (Budiharjo et al., 2017). Type III polyketide antibiotic is synthesized by two enzymes namely, type III polyketide synthases (PKS) also known as Chalcon-like PKS (also known as CHS-like PKSs) and Phospholipid methyl transferase (PMT) (Nakano et al., 2009; Funa et al., 2006; Capuano et al., 1996). PKS enzyme which is made up of homodimer wherein activation of starter unit, condensation of extender unit and termination cycle is iteratively catalysed by both the monomer units (Katsuyama & Ohnishi, 2012; Yu et al., 2012). It exhibits broad starter unit specificity (Sankaranarayanan et al., 2004; Austin & Neol, 2003**)**. PMT, the gene which is present as a part of operon, carries out methylation giving rise to methylated polyketide (Nakano et al., 2009). Both the genes for type III polyketide synthesis have been found in whole genome of various *B. altitudinis* strains (Kumaravel et al., 2018; Potshangbam et al., 2018; Budiharjo et al., 2017). Primers were designed using type strain *B. altitudinis* 41KF2b and other strains whose whole genome sequence is available online. Results of Polymerase Chain Reaction exhibited the presence of the operon in the isolate *B. altitudinis* S2 indicating a high probability of the synthesis of type III polyketide antibiotic by the isolate.

The MIC and MBC results indicate that the antibiotic S2 was bactericidal in nature and at MBC concentrations, it kills the bacteria at very short duration of time. The antibiotic did not show any lytic or morphological deformity as analysed by SEM. While, SDS-PAGE analysis showed the decrease in total protein of Xoo BXO43 with increasing concentration of the antibiotic treatment. This indicated

that the antibiotic was not lytic but definitely affect protein synthesis of the pathogen. Various polyketides reported to be produced by different *Bacillus* strains like Bacillaene, Difficidin and Oxydifficidin and Enacyloxin have been demonstrated to inhibit protein synthesis of the sensitive bacterial strains (Krokidis et al., 2014; Wilson, 2014; Canu et al., 2002; Patel et al., 1995).

In conclusion, the mass spectrometry of the purified antibiotic S2 isolated from the isolate *B. altitudinis* S2 revealed its mass value of 573.217 which is a multiple of 113.94 while MS^2 spectra indicated the presence of a peak of mass value 113.94 as the most abundant peak. Also, the SDS-PAGE of the solvent extracted antibiotic S2 did not reveal any high molecular protein band, indicating that it is not a peptidic antibiotic. This could further be supported from the fact that the bioactivity was retained even after exposure of the antibiotic S2 to proteolytic enzymes like proteinase K and trypsin. The presence of pks and pmt genes in the genome of *B. altitudinis* S2 implied that it probably produces class type III polyketide antibiotic. Further experiments with antibiotic S2 from *B. altitudinis* S2, could aid in revealing the structural details of this potent antibiotic.