

Original Research Paper

Bioactive Endophytic Actinomycetes of *Cinnamomum* sp.; Isolation, Identification, Activity Guided Purification and Process Optimization of Active Metabolite

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Article history

Received: 10-12-2014

Revised: 04-02-2015

Accepted: 26-03-2015

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Abstract: Studying on endophytic actinomycetes of *Cinnamomum* sp. from Indian rainforest, Cherapunji, one of our isolated strains designated as CH1 was found to produce significant antibacterial activity against test pathogens (*Aeromonas caviae* (ATCC 15468), *Vibrio parahaemolyticus* ATCC 1782, *Pseudomonas aeruginosa* (ATCC 9027), *Proteus vulgaris* (ATCC 12453), *Shigella flexnerii* (ATCC 12022), *Escherichia coli* (human sample isolate), *Bacillus subtilis* (ATCC 11774) and *Bacillus cereus* (ATCC 14579). Scanning electron microscopic studies, morphological characterization and 16s ribosomal RNA gene sequence based classification strongly suggest that the isolate is a new strain of *Streptomyces rochei*. The rRNA gene sequenced was submitted to NCBI (GenBank accession number KJ486840.1). An antibacterial metabolite was extracted from fermented culture broth and purified by repeated silica gel column chromatography. TLC and bioautogram study of active fraction showed compound of Rf 0.196 was active metabolite where as HPLC study showed two major peak of RT 2.433 and 2.632 min. Parameters influencing optimal antimicrobial production were also determined and the active compound was found nonmutagenic by Ames test. Based on above experiments it is concluded that this entophytic isolate can be further exploited for industrial and biological applications.

Keywords: Endophyte, *Streptomyces*, Antibacterial, Nonmutagenic, Optimization

Introduction

The emerging field of antimicrobial therapy in recent decades has cultivated much interest in using microbial natural products, such as toxins, proteins, hormones, vitamins and amino acids for diseases control (Fatope *et al.*, 2000). These are virtually biodegradable, specific and generally have low toxicity (Newman *et al.*, 2003). Finding of new antibiotics, which are effective against pathogenic bacteria, are an important area of antibiotic research. Actinobacteria are mainly soil bacteria, play an important role in biotechnological industries as it has the capability to produce a large number of antibiotics and other bioactive secondary metabolites (Roy and Banerjee, 2013). In recent years more than 90% antibiotics have obtained

from actinobacteria and among which about 55% belong to the genus *Streptomyces* (Hamaki *et al.*, 2005). Moreover, the multiplication in the number of drug-resistant pathogens and the throttled success of strategies like combinatorial chemistry in providing new compounds indicate uncertainty for future antimicrobial therapy. Thus finding new groups of microbes from unexplored or less explored habitats became mandatory by pursuing them as sources of novel bioactive compounds. There are a variety of endophytic microorganisms inside the tissue of nearly all healthy plants. Endophytes are synergistic to their host; among them some are thought to be making returns for the nutrition from the plant by producing special substances, such as secondary metabolites. These metabolites protect the host from being attacked successfully by fungi and

pests (Taechowisan *et al.*, 2005). It has been found that some *Streptomyces* have taken up residence in plants which opens the possibility that, this may be an entirely prime source of novel pharmaceuticals (Joseph and Priya, 2011).

Tropical rainforests are vital to the global ecosystem and human existence. They are a world like no other and are unparalleled in terms of their biological diversity. During our study on endophytic bacteria from various plants of Cherapunji rainforest we have found a strain designated as CH1, isolated from *Cinnamomum* sp. (Lauraceae), showed potential antimicrobial property against some pathogenic bacteria. In this study we have identified the endophytic strain, described its antagonism to some selected pathogenic bacteria and purified its non-mutagenic bioactive metabolite. We have also determined various critical parameters that may influence rate of production of active substance.

Materials and Methods

Isolation of Endophytic Actinomycetes

Healthy stems of *Cinnamomum* were collected from the North-East Indian rainforest (Cherapunji, 25.30° N-91.70° E) and brought in the laboratory in sterile zip-lock bags. Stems were washed thoroughly under distilled water, soaked in autoclaved tissue paper and cut at about 0.5 to 1 cm in length. Surface sterilization of samples were made by using sodium hypochlorite and 70% ethanol as surface sterilizing agent and then flame sterilized after dipping in 70% ethanol for 10 to 15 s to remove all epiphytic microorganisms (Zin *et al.*, 2010). Barks were removed with sterile sharp blade and dissected plant stems were placed on various media for isolation of endophytic actinomycetes. Water agar media, actinomycetes isolation agar media, glycerol-asparagine agar media (ISP5) and yeast extract malt extract agar media (ISP2) supplemented with actidione (50 µg mL⁻¹) were used for actinomycetes isolation in this study. Plates were incubated for at least 15 days at 28±2°C (Lee *et al.*, 2008). Actinomycete colonies emerging from stems were immediately brought in pure culture and preserved in 30% (v/v) glycerol at -20°C.

Antimicrobial Spectrum Analysis

Antimicrobial property of isolates was checked against following pathogens on Muller Hinton agar media by agar diffusion method. Individual actinomycetes isolates were grown in liquid media (from where they were isolated) under shaking (250 rpm) at 28 ±2°C for 10 days. Culture filtrate (200 µL) was applied in the wells of MHA plates previously seeded with pathogens. Plates were incubated for 24 h and growth inhibition of pathogens were recorded. In this study *Aeromonas caviae* (ATCC 15468), *Vibrio*

parahemolyticus ATCC 1782, *Pseudomonas aeruginosa* (ATCC 9027), *Proteus vulgaris* (ATCC 12453), *Shigella flexnerii* (ATCC 12022), *Escherichia coli* (human sample isolate from Medical College, Medinipur, WB, India), *Bacillus subtilis* (ATCC 11774), *Bacillus cereus* (ATCC 14579) were selected as test pathogens. Antibacterial potency of isolated bacteria was also compared with few known antibiotics against same pathogens under same condition. Endophytic isolate CH1 was selected due to its broad antibacterial spectrum and was further investigated.

Morphological, Physiological and Biochemical Characterization

CH1 was grown on ISP5 agar media and sterile coverslip was placed at 45° angle at edge of its colony and further allowed to grow. Coverslip was taken out after 7 days and examined under bright field compound microscopy after Gram staining. Colony were further scrapped and studied for scanning electron microscopy (JEOL; JSM 5800, Japan) (Franson *et al.*, 1984). Following standard methods aerial mycelium, spore mass color, substrate mycelium, pigmentation and if any diffusible pigment produced by the strain were recorded for the isolated strain (Shirling and Gottlieb, 1966; Williams *et al.*, 1983). Different sugar utilization pattern was also determined (Gordon *et al.*, 1974). Routine enzyme production by CH1 was checked for amylase, protease, lipase, cellulase and catalase by growing on specific media. Optimum growth temperature and pH was also determined after growing it in various temperature and pH conditions.

DNA Isolation, Amplification and Sequencing of the 16S rRNA Gene

Genomic DNA was isolated of the strain after growing in ISP5 broth for 5 days. Cells were taken in TE buffer (25 mM Tris-HCl [pH 8.0], 25 mM EDTA and treated with lysoszyme, RNase and followed by 10% SDS. DNA was extracted with phenol- chloroform-isoamyl alcohol and finally precipitated with isopropanol. 16S rRNA gene was amplified using the primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') (Santhi and Solomon, 2011). Reactions were performed on a thermocycler (Eppendorf) in 50 µL volumes consisting of 2 µL genomic DNA, 1.0 µM each primer, 21 µL sterile water and 25 µL of REDTaq ReadyMix (HiMedia). Reaction parameters were as follows: Initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 95°C for 60 sec, annealing at 55°C for 60 sec and extension at 72°C for 90 sec. A final extension was done at 72°C for 10 min. PCR products were purified with Hi-PurA™ PCR product purification spin kit (HiMedia). Forward and reverse DNA

sequencing reactions of purified PCR amplicon was carried out with same primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.

Phylogenetic Analysis

Consensus sequence of 1397 bp rRNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out BLAST with the 'nr' database of NCBI genbank. Based on maximum identity score sequences were selected and aligned using multiple alignment software program, Clustal W. Distance matrix was generated using RDP (Ribosomal Database Project) database and the phylogenetic tree was constructed using MEGA 6 (Tamura *et al.*, 2007). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980).

Nucleotide Sequence Accession Numbers

DNA sequences were deposited in GenBank under accession number KJ486840.1

Production and Extraction of Bioactive Metabolite

CH1 grown agar block (surface area 1 cm²) was aseptically transferred to 100 mL Erlenmeyer flask containing 30 mL ISP5 broth and incubated at 28°C for 72 h at 250 rpm for seed production. Seed culture was inoculated (5% v/v) into 250 mL Erlenmeyer flask containing 60 mL production media having composition bacto-tryptone 5 g L⁻¹, dextrose 10 g L⁻¹, yeast extract 2 gm L⁻¹, K₂HPO₄ 2 g L⁻¹, NaCl 2 g L⁻¹ and MgSO₄ · 7H₂O 500 mg L⁻¹ and cultured for 12 days at 28°C in orbital shaking at 250 rpm. After fermentation total 1.8 L culture broth was filtered through Whatman paper no. 1 and then centrifuged at 14,000 x g for 20 min to remove any cell mass. The culture filtrate was then extracted twice with equal volume of ethyl acetate. This organic solvent extract was pooled, made water free by adding anhydrous Na₂SO₄ and then taken to dryness under vacuum in rotary evaporator (HS-2005S, HAHNSHIN, Korea) at 40°C. Residual part was dissolved in 2 ml methanol.

Purification by Flash Column Chromatography

The methanolic extract was soaked in silica gel and loaded onto the silica gel column (230-400 Å mesh size). Extract was eluted first with 50 mL hexane- chloroform (50:0, 4:1, 1:1,1:4, 0:50) of increasing polarity and then with chloroform- methanol (50:0, 48:2, 45:5, 40:10, 25:25, 10:40, 0:50). All collected fractions were concentrated to 2 ml and tested for antimicrobial activity by disk diffusion method (25 µL disk content) against *B*

cereus and *E. coli*. The active fraction was again purified by silica gel column with chloroform- methanol of more finely graded solvent system (chloroform and methanol: 50:0, 49.5:0.5, 49:1, 48.5:1.5, 48:2, 47.5:2.5, 45:5, 0:50). The eluting volume for this time was fixed to 25 mL. Different fractions were concentrated to 1 mL and bioactivity was determined similarly.

HPLC and Activity Guided TLC Study of Active Fraction

Ten micro liters of most the active fraction was analyzed in reverse phase high performance liquid chromatography (Agilnet, 1260, USA) under the following conditions: Flow rate 1.0 mL min⁻¹; stationary phase Delta Pak C18 column (Zorbax high purity porous silica microsphere); 23°C; mobile phase 80% methanol in chloroform; detector wavelength 260 nm. Numbers of peaks with their retention time were recorded.

Five micro liters active fraction was spotted on silica coated alumina TLC plate as thin band and was separated using chloroform-methanol (20:1) followed by UV detection. For bioautogram study, after UV detection, TLC plate was covered with a layer of MHA (40°C) containing *B. cereus* (50 µL of 10⁵ cfu mL⁻¹) and incubated for 24 h at 35°C. Cell viability was checked by spraying methylthiazoletetrazolium (MTT-5 mg mL⁻¹) on pathogen grown media surface. Inhibition zone was observed as clear spots against a purple background.

Mutagenic Potentiality Test of Active Compound

Salmonella typhimurium TA100 was used for this experiment. This bacterium has point mutations in the histidine (*his*) operon, rendering it being incapable of producing histidine. When a mutagenic event occurs, base substitutions or frameshifts within his gene may cause a reversion to histidine prototrophy. Ames mutagenicity assay was performed for purified compound exposing directly and with S9 liver extract activation to the test bacteria. Strain TA 100 was first grown on LB media supplemented with 10 mg mL⁻¹ His and 1 mg mL⁻¹ biotin. Approximately 90 µL aliquot (10⁶ cfu mL⁻¹) was mixed with 10 µL His-Bio solution (0.5 mM) and seeded on Glucose Minimal agar media. Purified sample (15 and 50 µL) was soaked on sterile filter paper disk and with 100 µL S9 liver extract at 37°C separately. Negative control was made with methanol (50 µL) and positive control with NaN₃ (1 µL from stock-1 µg mL⁻¹) soaked paper disk. Paper disks were placed centrally on TA 100 inoculated plates and incubated at 37°C for 24 h. Well developed revertant bacterial colony were counted and analyzed. The compound was judged to be mutagenic if the number of revertants were 2 folds or more relative to the negative control (Rodríguez *et al.*, 2012).

Optimization of Antimicrobial Production by Regulating Various Influencing Parameters

Species-specific variation occurs within *Streptomyces* for cell growth and production of secondary metabolites by the influences of various factors (Vilches *et al.*, 1990; Kojima *et al.*, 1995). In our experiment we have carefully regulated several cultural parameters to determine optimum production of the bioactive compound. Firstly ISP5 broth, TYG broth, mineral salt broth and SCN broth were checked for maximum antimicrobial production. Optimum incubation period was determined by growing CH1 up to 15 days in TYG broth and culture supernatant was verified for antibacterial activity against *B. cereus*. Most desirable carbon and nitrogen source for optimum antimicrobial production were also evaluated. Dextrose, fructose, galactose, maltose, lactose, sucrose (1% w/v) were used as various carbon source and peptone, tryptone, soymeal, casein (0.5% w/v), KNO₃, NaNO₃, NH₄Cl, urea and L-Asn (0.2% w/v) were taken individually as sole nitrogen in TYG broth. Initial pH variation of media (pH-4, 6, 7, 8 and 10) was also checked to determine optimum pH for antimicrobial production.

Results

Endophytic isolate, CH1 was isolated on ISP 5 agar media. The light microscopic characterization of CH1 depicts that it is branched filamentous Gram positive bacteria. SEM study reveals its spore orientation as oval 3-8 spores of about 1 to 1.2 μ m diameter, at tip of the filaments (Fig. 1). CH1

produces elevated chalky, dry, irregular folding white colony on ISP5 agar media. Other morphological and physicochemical properties of strain CH1 was summarized in Table 1. 16s rRNA sequences and phylogeny study showed close relationship between CH1 and various type strains of *Streptomyces rochei* (Fig. 2). It showed 99% 16s rDNA sequence similarity with different type strains of *Streptomyces rochei* like SCSIOZ-SH06, SCSIOZ-SH07, cfcc3115, SCSIOZ-SH12 etc. We propose our isolated strain CH1 as *Streptomyces rochei* CH1 according to its rDNA sequences and other characterization similarities (Acharyabhata *et al.*, 2013; Ma *et al.*, 2008) though it shows similar rRNA sequences homology to few other species of *Streptomyces*.

Antimicrobial assay of CH1 culture filtrate against test pathogens represents its potential antibacterial spectrum though it does not inhibit *Pseudomonas aeruginosa* at all (Table 2). The appearance of evaporated residual was amorphous dark brownish. Active antibacterial compound was eluted with chloroform: Methanol (47:1.5) that showed highest antibacterial activity. Thin layer chromatogram of this fraction indicates two distinct bands X and Y of rf value of 0.196 and 0.410 respectively. Bioautogram study revealed compound X (rf value of 0.196) to be active antibacterial secondary metabolite produced by this strain. The active extract was further analyzed by analytical reverse phase HPLC with a C¹⁸ column based on hydrophobicity. Two major compounds were found with retention time 2.433 and 2.632 min respectively (Fig. 3) among which compound with RT-2.632 was most abundant which might be the active substance.

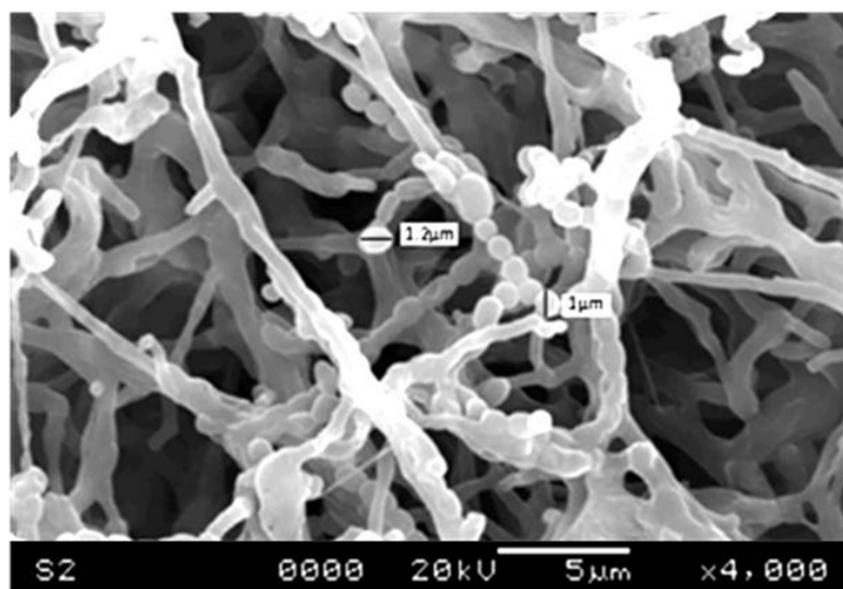


Fig. 1. Highly branched mycellial structure with spore of *Streptomyces rochei* CH1

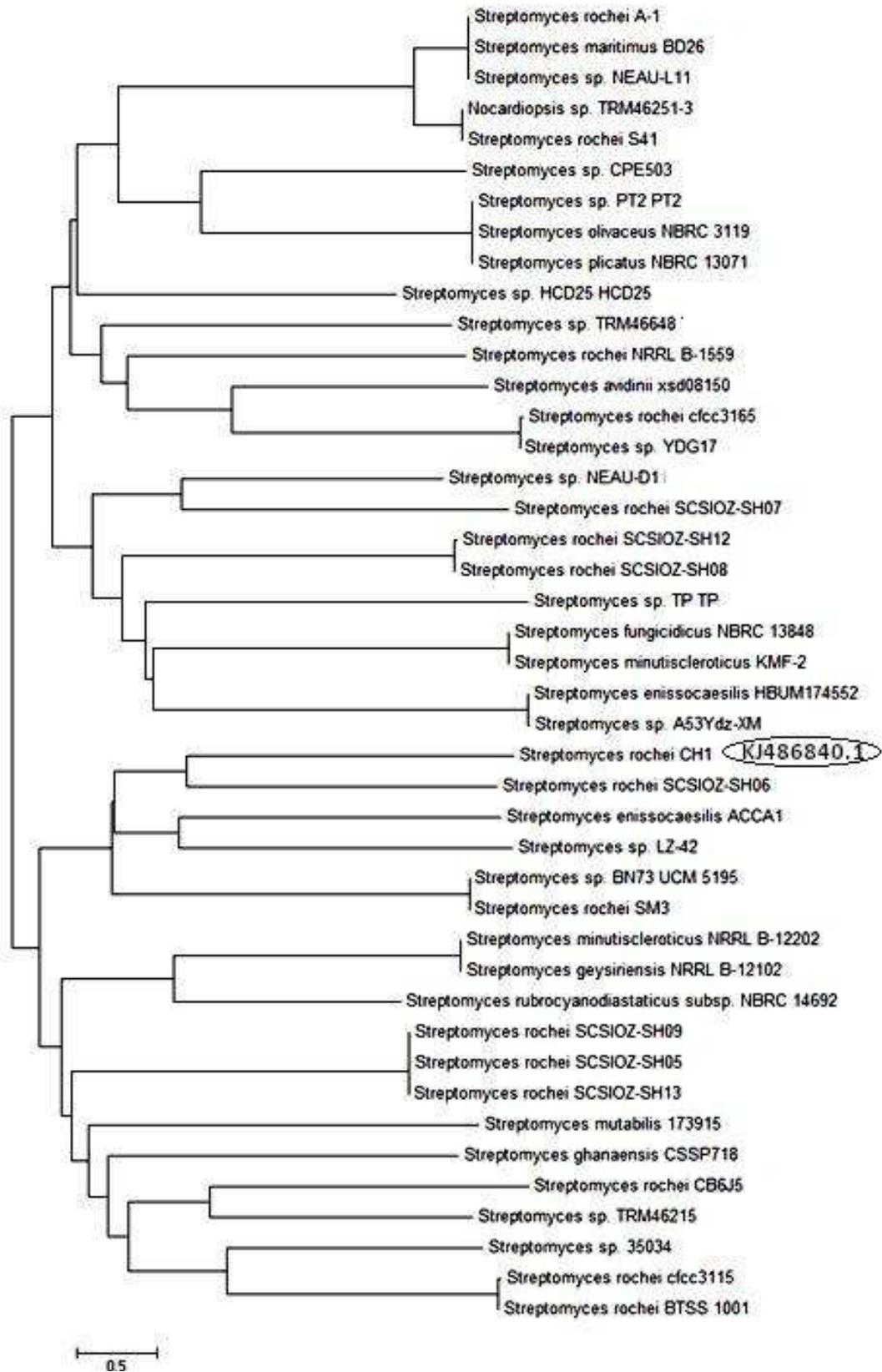


Fig. 2. Phylogenetic position of *Streptomyces rochei* CH1 using 16S rRNA partial sequences

Table 1. Characterization of *Streptomyces rochei* CH1

Colony morphology (ISP5)	Elevated, wrinkled surface, dry, irregular folding white colony
Cultural morphology	
Growth on ISP2	Very poor
Substrate mycelia	White
Aerial mycelia	-
Growth on ISP4	Good
Substrate mycelia	White to violet
Aerial mycelia	White to ash
Growth of ISP5	Good
Substrate mycelia	White to reddish violet
Aerial mycelia	White to bluish grey
Growth on ISP7	Good
Substrate mycelia	White to reddish violet
Aerial mycelia	Bluish Ash
Starch Casein Nitrate agar (SCN)	Good
Substrate mycelia	White to ash
Aerial mycelia	Ash
Pigmentation	
Diffusible pigment	-
Melanoid pigment (ISP1, 6, 7)	-
Cellular and spore morphology	
Vegetative cells	Highly branched filamentous
Spore per chain	3-8 spores at tip of the filaments
Spore shape	Round to oval
Spore surface	Smooth and hairy surface
Spore size	About 1 to 1.2 µm diameter
Gram characetr	Positive
Extra cellular enzymes	
Cellulase	+
Amylase	+
Lipase	+
Protease	-
Catalase	+
Carbon source utilization	
Dextrose	+
Fructose	+
Galactose	+
Lactose	+
Maltose	+
Sucrose	+
Starch	+
Mannitol	+
Growth parameters	
Temperature	20-40°C (28°C optimum)
pH	6-10 (optimum pH 8)

(*Optimum has been determined by measuring dry biomass of culture filtrate; - no growth, + growth)

Table 2. Antibacterial activity of *S. rochei* CH1 fermented crude liquor

Test pathogens	Mean zone diameter (mm)*	Penicillin-G (1 UI)	Ampicillin (25 µg)	Streptomycin (25 µg)	Co-trimoxazole (25 µg)
<i>Aeromonas caviae</i>	16.33	12.00	15.50	34.83	19.50
<i>Aeromonas hydrophila</i>	16.33	14.83	18.66	31.00	18.50
<i>Vibrio parahaemolyticus</i>	15.83	10.50	17.50	34.66	15.33
<i>Pseudomonas aeruginosa</i>	-	-	-	25.50	13.83
<i>Proteus vulgaris</i>	14.66	15.66	17.66	33.66	16.00
<i>Shigella flexnerii</i>	13.83	12.16	16.00	34.83	24.00
<i>Escherichia coli</i>	16.50	11.33	18.00	26.16	18.50
<i>Bacillus subtilis</i>	23.16	35.83	46.50	36.16	24.66
<i>Bacillus cereus</i>	24.00	37.50	45.00	32.50	26.83

(* Results are represented as means of three replicates)

Many of such antimicrobial secondary metabolites possess various toxic effects to human health. A basic study for evaluation of mutagenic potentiality of compound is Ames test. After 48 h no significant increases in colony numbers were found for the sample of interest when compared to spontaneously reverted plate. NaN_3 added plate result more than double colony number than spontaneous reversion as usual (Fig. 4).

Present study was carried out for optimum production of antimicrobial compound from strain CH1. It was found that TYG broth as most suitable media for antimicrobial production from *Streptomyces rochei* CH1. Antimicrobial production in salt media was very poor that may indicate organic nitrogen and carbon is essential for antimicrobial production. Most

effective nitrogen source for antimicrobial production was found to be tryptone and dextrose as effective carbon source, though considerable antimicrobial production was observed with lactose. Less activity was found when peptone, used as organic nitrogen source but surprisingly no other organic or inorganic salt played role in antimicrobial production. It was found that antimicrobial production had started at 6th day of incubation as in early hours actinomycete growth remains in lag phase and secondary metabolite production usually starts at late log phase. Antimicrobial production was found to be highest at 12th day. Strain CH1 was found to produce highest antibacterial activity at initial media pH 7. All the optimization data are depicted in Fig. 5.

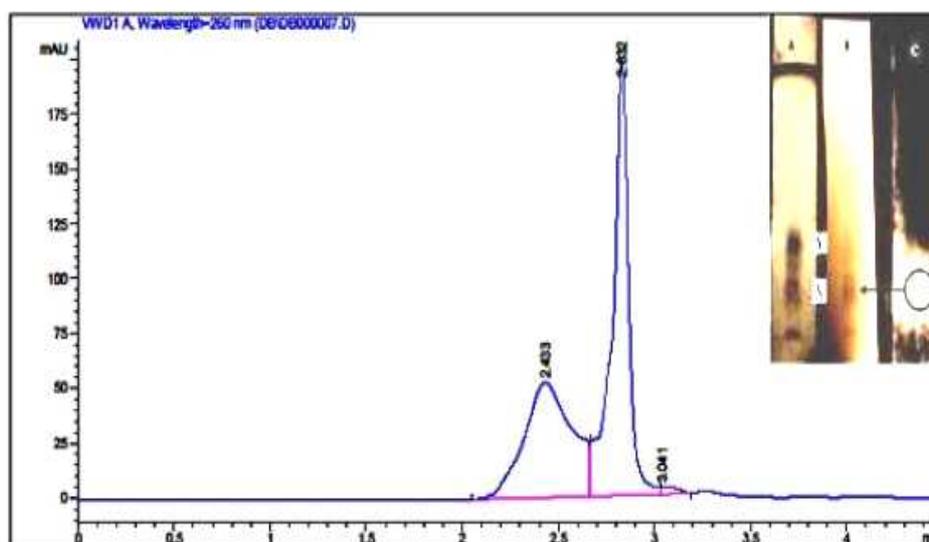


Fig. 3. HPLC analysis of column purified active secondary metabolite. (Inset; TLC and bioautogram of active fraction, A-UV exposed, B-iodine vapour, C-autobiogram (circle indicates dead cells and arrow, corresponding band))

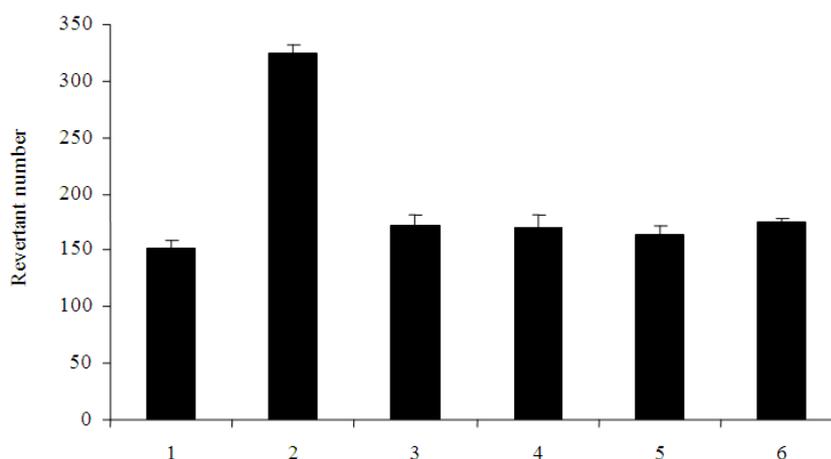


Fig. 4. Ames mutagenicity test of purified bioactive compound; 1-spontaneous reversion, 2- NaN_3 control, 3-purified extract 15 μL , 4-purified extract 50 μL , 5-purified extract 15 μL + S9, 6-purified extract 50 μL + S9 (values represent mean of three replicates and error bar as sd from mean)

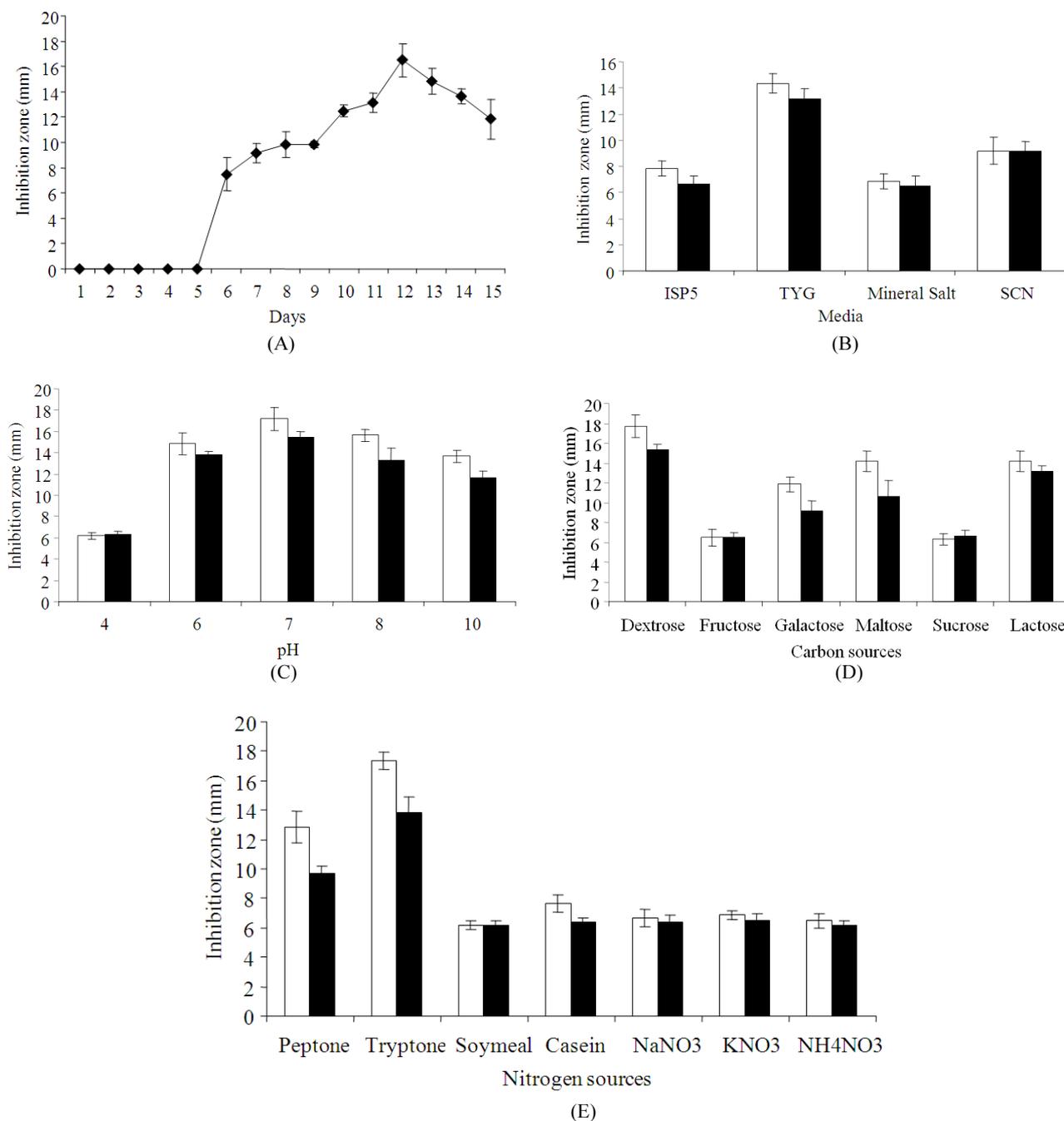


Fig. 5. Characterization of parameters for optimum production of bioactive compound against *B. cereus*. (A) Incubation period (B) Media type (C) Initial pH of media (D) Carbon source type (E) Nitrogen source type. (values represent mean of 3 replicates and error bars used as sd from mean)

Discussion

Most of the endophytic *Streptomyces* so far isolated are good anti fungal producers but less report are found where they are lethal to bacteria (Zin *et al.*, 2007). *Streptomyces rochei* BTSS 1001 a marine actinomycetes with alkaline amylolytic activity (Acharyabhatta *et al.*,

2013) was found to be similar cultural properties with strain CH1 but exceptionally it does not grow well at ISP2 and no pigmentation was found in any media. Ma *et al.* (2008) reported endophytic actinomycetes Lj20 producing butylated hydroxytoluene and 3, 5-di-tert-butyl-4-hydroxybenzyl methyl ether as its secondary metabolite with significant antifungal activity.

Streptomyces rochei is known to produce Lankacidin and Lankamycin group of antibiotic and γ -Butyrolactone, a diffusible signaling molecule, often regulate antibiotic production and/or morphogenesis in this bacterium (Yamamoto *et al.*, 2008). *Streptomyces rochei* F20, non-endophytic actinomycetes is also reported for production of streptothricin class antibiotics (Anukool *et al.*, 2004). However this is the first report of endophytic *Streptomyces rochei* with potential antibacterial activity isolated from *Cinnamomum* of Indian rain forest.

In our experiment we have extensively characterized this bacterium from physical, biochemical and molecular aspect. This isolated strain produced antimicrobial substance which is comparable with penicillin, ampicillin, streptomycin and co-trimoxazole to some extent. The active compound was purified by flash column chromatography which was confirmed by TLC analysis and bioautogram studies. Genetic diversity very often is responsible for production of new class antibiotics. Chance of some genetic diversity is higher in co-evolutionary endophytic relationship lead to the ability for biosynthesizing some unusual compound different from its other strains of different ecological niche by genetic variation; including uptake of some plant DNA in their self genome (Germaine *et al.*, 2004; Stierle *et al.*, 1993). Moreover no mutagenic property of purified bioactive metabolite was found in any concentration and combination tested in this study which indicates its potential applicability as drug. So, further structural characterization of active compound is necessary for this endophytic strain.

Conclusion

The isolated endophytic strain was identified as new strain of *Streptomyces rochei* CH1 (GenBank KJ486840.1). This specific strain exhibits broad-spectrum of non-mutagenic antimicrobial activity which is active against both Gram positive and Gram negative pathogens. Optimizations for antimicrobial production conditions and other significant results may help to exploit this strain in future drug.

Acknowledgement

Authors are thankful to University Grants Commission (UGC), New Delhi for funding, SR is thankful to Oriental Institute of Science and Technology (OIST) for their help during this work.

Author's Contributions

All authors equally contributed in this work.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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