Purification and biological evaluation of the metabolites produced by *Streptomyces* sp. TK-VL_333

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Abstract

An Actinobacterium strain isolated from laterite soils of the Guntur region was identified as *Streptomyces* sp. TK-VL_333 by 16S rRNA analysis. Cultural, morphological and physiological characteristics of the strain were recorded. The secondary metabolites produced by the strain cultured on galactose--tyrosine broth were extracted and concentrated followed by defatting of the crude extract with cyclohexane to afford polar and non-polar residues. Purification of the two residues by column chromatography led to isolation of five polar and one non-polar fraction. Bioactivity- guided fractions were rechromatographed on a silica gel column to obtain four compounds, namely 1H-indole-3-carboxylic acid, 2,3-dihydroxy-5-(hydroxymethyl) benzaldehyde, 4-(4-hydroxyphenoxy) butan-2-one and acetic acid-2-hydroxy-6-(3-oxo-butyl)-phenyl ester from three active polar fractions and 8-methyl decanoic acid from one non-polar fraction. The structure of the compounds was elucidated on the basis of FT-IR, mass and NMR spectroscopy. The antimicrobial activity of the bioactive compounds produced by the strain was tested against the bacteria and fungi and expressed in terms of minimum inhibitory concentration. Antifungal activity of indole-3-carboxylic acid was further evaluated under *in vitro* and *in vivo* conditions. This is the first report of 2,3-dihydroxy-5-(hydroxymethyl) benzaldehyde, 4-(4-hydroxyphenoxy) butan-2-one, acetic acid-2-hydroxy-6-(3-oxo-butyl)-phenyl ester and 8-methyl decanoic acid from the genus *Streptomyces*.

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Keywords: Actinobacteria; *Streptomyces* sp. TK-VL_333; Bioactive metabolites; 1H-indole-3-carboxylic acid; 2,3-Dihydroxy-5-(hydroxymethyl) benzaldehyde; Acetic acid-2-hydroxy-6-(3-oxo-butyl)-phenyl ester

1. Introduction

Nature acts as a prominent reservoir for new and novel therapeutics. By employing sophisticated techniques in various screening programs, the rate of discovery of natural compounds exceeds 1 million. Out of 22, 500 biologically active compounds that have been extracted thus far from microbes, 45% are produced by Actinobacteria, 38% by fungi and 17% by unicellular bacteria (Demain and Sanchez, 2009). Members of the class Actinobacteria, and especially *Streptomyces* spp. have long been recognized as prolific sources of useful bioactive metabolites, providing more than half of the naturally occurring antibiotics discovered to date and continuing to be a source of new bioactive metabolites (Berdy, 2005). Unfortunately, the emergence of drug-resistant pathogens and the increase in diseases affecting the immune system have greatly intensified the need to investigate new bioactive metabolites for potential pharmaceutical and industrial applications (Demain and Sanchez, 2009; Wise, 2008). The search for new antimicrobials has not been limited to the medicinal field, but also extends to crop protection. Development of fungicide-resistant plant pathogens as well as excessive and indiscriminate use of synthetic agrochemicals has led to ecological imbalances in soil and human health (Thind, 2008). Therefore, the search for alternatives to chemical control of plant pathogens, such as biological control, has gained momentum in recent years.

Several approaches, including cultural, genomic and metagenomic analysis, have been employed for the isolation of
novel bioactive compounds. In cultural methods, potent bioactive metabolite-producing Actinobacteria strains were isolated by pretreatment of soil samples collected from deserts (Hozzein et al., 2008), caves (Nakaew et al., 2009), marine (Fenical and Jensen, 2006) and other unexplored terrestrial areas (Sait et al., 2002; Wu et al., 2009) with calcium carbonate (El-Nakeeb and Lechevalier, 1963), dry heat (Nonomura and Ohara, 1969), phenol (Hayakawa et al., 2004), irradiation with microwaves (Bulina et al., 1997), sodium dodecyl sulfate and yeast extract (Yamamura et al., 2007), etc. followed by plating them on various selective culture media like Gauze I agar (Zakharova et al., 2003), Kuster agar (Vargas et al., 2009) and himic acid vitamin agar incorporated with antibiotics, viz., trimethoprim, nalidixic acid (Hayakawa, 2008) etc. On the other hand, ‘genome mining’ aims at the genes that encode tailoring enzymes from natural product biosynthesis pathways and serve as indicator genes for identification of strains that have the genetic potential to produce novel natural products (Hornung et al., 2007). Metagenomics is a new powerful tool for identification of uncultured microbial genes encoding for bioactive compounds (Gillespie et al., 2002), new polyketide synthases (Courtois et al., 2003) and even new functions like a membrane-associated proteolytic system (Beja et al., 2000). In our continuous quest for new bioactive metabolites, Actinobacterium strain TK-VL_333 having good antimicrobial potential was selected amongst fifty isolated strains from the soils of the Guntur region unexplored for Actinobacteria using a CaCO3-based approach. The present study reveals the fermentation, isolation, characterization and biological evaluation of the metabolites produced by strain TK-VL_333 along with its taxonomic study.

2. Materials and methods

2.1. Isolation

Actinobacterium strain TK-VL_333 found to be predominant in laterite soils of the Guntur region was isolated on asparagine-glucose agar medium incubated for 7 days at 37 °C using a soil dilution technique. The strain was maintained on yeast extract-malt extract-dextrose (YMD) agar medium at 4 °C for further study (Williams and Cross, 1971).

2.2. Taxonomic studies

Cultural characters of strain TK-VL_333 were studied on International Streptomyces Project (ISP) and non-ISP media (Dietz and Thayer, 1980). The micromorphology of the strain cultured on ISP medium 2 at 37 °C for 4 days was examined under scanning electron microscopy (model JOEL-JSM 5600) at various magnifications (×7500 and ×18,000) (Bozzola and Russell, 1999). Utilization of carbon sources by the strain was carried out according to the method of Gottlieb (1961). Tolerance of the strain to lysozyme, phenol, NaCl and the ability of the strain to produce different enzymes, H2S, indole and acid were tested in accordance with standard protocols (Holding and Collee, 1971). In addition, the sensitivity of the strain to different antibiotics was determined by the paper disc method (Cappuccino and Sherman, 2004).

Extraction of genomic DNA of the strain was performed according to the method described by Rainey et al. (1996). 16S rRNA gene was amplified with primers forward (5’-TGCCTCGCTCCTCAAGGTC-3’) and reverse (5’-TGCACTGTTTAGGGCCTGA-3’). The amplified DNA fragment was separated on 1% agarose gel, eluted and purified using the QIAquick gel extraction kit (Qiagen, Germany). The purified PCR product was sequenced using the Big-Dye terminator kit ABI 310 Genetic Analyzer (Applied Biosystems, USA). The phylogenetic position of the isolated strain (TK-VL_333) was assessed by performing a nucleotide sequence database search using the BLAST program from NCBI GenBank. Sequence data of related species were retrieved from NCBI GenBank. Nucleotide substitution rates (Kmt values) were calculated (Kimura, 1980) and the phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987). The statistical significance of the tree topology was evaluated by bootstrap analysis of sequence data using CLUSTAL W software (Thompson et al., 1997).

2.3. Fermentation, extraction, purification and structural elucidation of bioactive metabolites

For isolation and identification of bioactive metabolites, a loopful culture of Streptomyces sp. TK-VL_333 was cultivated in YMD broth (seed broth) and incubated on a rotary shaker (250 rpm) at 30 °C. After 48 h of incubation, the seed culture (10% v/v) was transferred to the optimized fermentation medium consisting of 2% galactose, 1% tyrosine, 0.05% K2HPO4, 0.05% NaCl and 0.001% FeSO4·7H2O with pH adjusted to 7.0 (Kavitha and Vijayalakshmi, 2009).

The culture filtrates (25 L) obtained after cultivation of the strain in 2.5 L conical flasks for 96 h were extracted twice with ethyl acetate and concentrated to dryness under a vacuum at 35 °C. The crude extract (1.8 g) thus obtained was defatted with cyclohexane to give polar and non-polar residues. The resulting red-colored polar residue was chromatographed on a silica gel column (25 × 5 cm, Silica gel 60, Merck, Mumbai, India) using the gradient solvent system of CHCl3:MeOH (100–30:70 v/v). Three fractions collected at different eluent (CHCl3:MeOH) conditions (first at 80:20 v/v; second at 70:30 v/v and third at 60:40 v/v) showed antimicrobial activity against the Firmicute (Bacillus cereus), the Gammaproteobacteria (Escherichia coli) and the yeast (Candida albicans).

On the other hand, the non-polar residue of the crude extract was subjected to silica gel column (25 × 5 cm, silica gel 60, Merck, Mumbai, India) using a gradient solvent system of hexane:ethyl acetate (100–70:30 v/v). Elutions were collected and recl chromatographed on a silica gel column (25 × 5 cm, Silica gel 100, Merck, Mumbai, India) to yield one major fraction (fourth). The structure of the compounds was elucidated and confirmed on the basis of FT-IR (Fourier transform infra-red; model: Thermo Nicolet Nexus 670...
spectrophotometer with NaCl optics), EIMS/ESIMS (Electron ionization mass/electron spray ionization mass; model: micromass VG-7070H, 70 eV spectrophotometer) and nuclear magnetic resonance (1H and 13C NMR; model: Varian Gemini 200 and samples were made in CCl4/CDCl3 + DMSO using tetramethyl silane as internal standard) spectroscopy.

2.4. Biological assays

The antimicrobial spectra of the compounds were tested in terms of minimum inhibitory concentration (MIC) against the test bacteria like B. cereus (MTCC 430), Bacillus megaterium (NCIM 2187), Bacillus subtilis (MTCC 441), Corynebacterium diphtheriae (MTCC 116), E. coli (MTCC 40), Proteus vulgaris (ATCC 6380), Pseudomonas aeruginosa (MTCC 424), Pseudomonas solanacearum (NCIM 5103), Serratia marcescens (MTCC 118), Staphylococcus aureus (MTCC 96), Staphylococcus epidermidis (MTCC 120), Xanthomonas malvacearum (NCIM 2954), Xanthomonas campestris (NCIM 2310) and fungi including Aspergillus flavus, Aspergillus niger, Alternaria alternata, C. albicans (MTCC 183), Curvularia maculans, Curvularia lunata, Epidermophytum floccosum (MTCC 145), Fusarium oxysporum (MTCC 218) and Penicillium citrinum by using the agar well diffusion assay (Hwang et al., 2001). The compounds dissolved in DMSO at different concentrations ranging from 0 to 1000 μg/ml were employed for antimicrobial assay. The lowest concentration of the bioactive metabolite exhibiting antimicrobial activity against the test organisms was taken as the MIC of the compound (Kavitha et al., 2009).

The antagonism of Streptomyces sp. TK-VL_333 against phytopathogenic fungi like F. oxysporum was tested through the agar streak method (Taechowisan et al., 2005a). The strain was streaked radially at one end of the YMD agar plate followed by incubation at 30 ºC. After the growth of the strain for 5 days, the plate was inoculated with the test fungus at a distance of 6 cm away from the strain and incubated for 4 days at 30 ºC. Simultaneously, the test fungus alone grown on YMD plate served as a control. The inhibition zone in between the strain and the test fungus revealed the antagonistic nature of the strain. The efficacy of the strain towards the wilt pathogen F. oxysporum was further confirmed by conducting a greenhouse trial (Singh and Reddy, 1979) in which polyethylene bags containing 500 g of autoclaved soil were employed for five different kinds of treatment: (i) soil inoculated with the pathogen alone; (ii) soil inoculated with the antagonist alone; (iii) simultaneous inoculation of the soil with the pathogen and antagonist; (iv) soil initially incubated with the antagonist for 4 days inoculated with the pathogen; and (v) uninoculated soil that served as control. The pathogen and strain TK-VL_333 grown individually on YMD agar were employed for soil inoculation. The antagonistic nature of the strain was tested by raising surface-sterilized sorghum seeds in the bags and the incidence of the wilted plants was recorded after 15 days. The experiment was done in triplicate and the results are the means of 10 plants per treatment. Data were statistically evaluated with one-way analysis of variance (ANOVA) and standard deviations of the mean values were analyzed.

Among the bioactive metabolites, 1H-indole-3-carboxylic acid produced by the strain showed strong inhibition against the growth of F. oxysporum causing wilt of Sorghum bicolor. Therefore, its antifungal potential was further tested by in vitro and in vivo studies against F. oxysporum, and bioefficacy was compared with that of commercially available systemic fungicides like mancozeb and carbendazim. The conidial suspensions of F. oxysporum grown on Czapek-Dox agar at 30 ºC for 10 days were treated with different concentrations, i.e. 0, 1, 10, 50, 100, 150, 200 and 500 μg/ml of 1H-indole-3-carboxylic acid, mancozeb and carbendazim. The effect of these compounds on conidial germination was recorded. After 48 h of incubation at 28 ºC, conidial germination was examined microscopically in 3 replicates (Hwang et al., 2001). In a greenhouse trial, seeds of sorghum sown in plastic bags containing steam-sterilized soil were drenched with antifungal solution (30 ml) prepared by dissolving the compounds individually in water + methanol (95:5) to give different concentrations such as 0, 50, 100, 200, 400, 500, 700 and 1000 μg/ml. Sixty seedlings of sorghum (three-days-old) were inoculated with conidial suspensions of F. oxysporum (10⁵ spores/ml) by using the soil drench method (Hwang et al., 2001). Disease severity on sorghum plants was rated after 15 days of inoculation based on the percent of wilted plants. The results were statistically analyzed by two-way ANOVA and standard deviations of the mean values were evaluated.

3. Results and discussion

3.1. Taxonomy of the strain

Cultural characteristics of strain TK-VL_333 are presented in Table 1. It exhibited good growth on ISP-1, ISP-2, ISP-3, ISP-4, ISP-5, ISP-7, malt extract and maltose tryptone agar media. Growth was moderate on nutrient agar, while it was poor on Sabouraud agar media. The color of the aerial mycelium appeared white, while that of substrate mycelium went from light to dark brown. The strain produced reddish brown pigment on ISP-2, ISP-4 and ISP-5 and exhibited

### Table 1

<table>
<thead>
<tr>
<th>Culture media</th>
<th>Growth</th>
<th>Pigment production</th>
</tr>
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<tbody>
<tr>
<td>ISP-1</td>
<td>Good</td>
<td>~</td>
</tr>
<tr>
<td>ISP-2</td>
<td>Good</td>
<td>Reddish brown</td>
</tr>
<tr>
<td>ISP-3</td>
<td>Good</td>
<td>~</td>
</tr>
<tr>
<td>ISP-4</td>
<td>Good</td>
<td>Reddish brown</td>
</tr>
<tr>
<td>ISP-5</td>
<td>Good</td>
<td>Reddish brown</td>
</tr>
<tr>
<td>ISP-7</td>
<td>Good</td>
<td>Melanin</td>
</tr>
<tr>
<td>Malt extract agar</td>
<td>Good</td>
<td>~</td>
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<tr>
<td>Maltose tryptone agar</td>
<td>Good</td>
<td>~</td>
</tr>
<tr>
<td>Nutrient agar</td>
<td>Moderate</td>
<td>~</td>
</tr>
<tr>
<td>Sabouraud’s agar</td>
<td>Poor</td>
<td>~</td>
</tr>
</tbody>
</table>

a International Streptomyces Project media.

b No production.
melanin pigmentation on ISP-7. As depicted in Fig. 1, sporophore morphology of the strain was of a spiral type, which may be placed in the spira group of the family Streptomycetaceae and the genus *Streptomyces*. These results are in close agreement with the findings of Pridham et al. (1958) and Williams et al. (1983).

Table 2 depicts physiological and biochemical characteristics of strain TK-VL_333. The strain utilized d-fructose, d-galactose, d-glucose, d-xylose, glycerol, mannitol, starch and sucrose as carbon sources indicating its wide pattern of carbon assimilation. It exhibited salt tolerance up to 7% that may be placed in the intermediate group of salt tolerance, as suggested by Tresner et al. (1968). Production of melanoid pigments on ISP-7 medium and enzymes such as amylase, L-asparaginase, catalase, cellulase, chitinase, nitrate reductase, protease and urease by the strain was noticed. It was found positive for biochemical tests like H_2S, indole and citrate utilization. Kampfer et al. (1991) suggested that all these tests are indispensable tools for classification of Actinobacteria. It showed sensitivity to a variety of antibiotics, but was resistant to ampicillin, cephalxin, cloxacillin, colistin, co-trimazine, deoxycycline hydrochloride, methicillin, nalidixic acid, nitrofurantoxin, rifampicin and trimethoprim, suggesting that bioactive compounds produced by the strain may be responsible for the resistance of the strain to these antibiotics.

The phylogenetic position of *Streptomyces* sp. TK-VL_333 was established by amplifying the 16S rRNA region and the sequence of the strain (705 bases) was examined by BLAST analysis. The 16S rRNA genome sequence of the strain showed 100% identity with that of *Streptomyces tendae* (Fig. 2); hence, strain TK-VL_333 was assigned to the S. *tendae* cluster and the 16S rRNA sequence was submitted to the NCBI GenBank with accession number FJ877150.

### 3.2. Extraction, purification, identification and structural elucidation of bioactive metabolites produced by *Streptomyces* sp. TK-VL_333

The fermented broth obtained after culturing the strain in the optimized culture medium composed of 2% galactose, 1% tyrosine, 0.05% K_2HPO_4, 0.05% NaCl and 0.001% FeSO_4*7H_2O* with initial pH adjusted to 7.0 for 96 h was extracted with ethyl acetate and concentrated under a vacuum to yield a red-colored crude extract. It was later defatted with cyclohexane to separate polar and non-polar residues which in turn subjected to a silica gel column using CHCl_3:MeOH (70:30 v/v) and hexane:ethyl acetate (80:20 v/v) as the eluting solvent system, respectively. Three different fractions collected from polar residue and one from a non-polar fraction of the crude extract were active against *B. cereus, E. coli* and *C. albicans*. Further purification of these fractions through a silica gel column
column yielded one compound \((T_1)\) from the first fraction, two \((T_2, T_3)\) from the second and one \((T_4)\) from the third of the polar residue. On the other hand, one compound \((T_5)\) was extracted from the non-polar component (fourth) by employing silica gel column chromatography. The entire flow chart showing extraction and purification of bioactive metabolites from the culture filtrate of \textit{Streptomyces} sp. is presented in Fig. 3.

Compound \(T_1\) was obtained as light-brown amorphous powder. It was completely soluble in DMSO, MeOH and partially in CHCl\(_3\). The IR spectrum exhibited absorption bands at \(\nu_{\text{max}} 3388.4 \text{ cm}^{-1}/\nu_{\text{C-O}}\) and \(1700.7 \text{ cm}^{-1}/\nu_{\text{C-O}}\) indicating NH and C=O groups in the structure respectively (Supplementary Fig. 1A). EIMS analysis of the compound gave a molecular ion at \(m/z \ 184.0 [\text{M} + \text{Na}]^+\) (Supplementary Fig. 1B). The \(^1\text{H} \text{NMR (CDCl}_3 + \text{DMSO, 200 MHz)}\) spectrum of compound \(T_1\) showed 4 signals at 10.2 (1H, br, s), 7.64 (1H, d, \(J = 8.7 \) Hz), 7.40 (1H, d, \(J = 8.5 \) Hz) and 7.14 (4H, m) (Supplementary Fig. 1C), whereas \(^{13}\text{C} \text{NMR (CDCl}_3 + \text{DMSO, 50 MHz)}\) exhibited 7 signals at 174.39 (e COOH), 136.11 (C-9), 127.10 (C-2), 123.58 (C-8), 118.88 (C-6), 118.5 (C-7), 111.42 (C-4) and 107.66 (C-3) (Supplementary Fig. 1D). Based on the above spectral data, bioactive compound \(T_1\) was identified as 1\(H\)-indole-3-carboxylic acid (Fig. 4A) with the molecular formula \(\text{C}_9\text{H}_7\text{O}_2\text{N}\).

Compound \(T_2\) appeared as a pale yellow solid which was completely soluble in DMSO and MeOH and partially in CHCl\(_3\). The IR absorption bands at \(\nu_{\text{max}} 3449.5 \text{ cm}^{-1}\) and 2923.87 cm\(^{-1}\) suggested the presence of OH groups and \(\nu_{\text{max}} 1637.9 \text{ cm}^{-1}\) indicated a CHO group in the structure, respectively (Supplementary Fig. 2A). EIMS analysis of the compound displayed a molecular ion at \(m/z \ 192 [\text{M} + \text{H}]^+\) (Supplementary Fig. 2B). Analysis of the \(^1\text{H} \text{NMR (CDCl}_3 + \text{DMSO, 200 MHz)}\) spectrum of the compound revealed 4 signals at 9.61 (1H, s), 7.16 (1H, d, \(J = 3.39 \) Hz), 6.48 (1H, d, \(J = 3.58 \) Hz) and 4.69 (2H, s) (Supplementary Fig. 2C), whereas \(^{13}\text{C} \text{NMR (CDCl}_3 + \text{DMSO, 50 MHz)}\) exhibited 7 signals at 200.48 (e CHO), 155.96 (C-2), 148.41 (C-3), 146.63 (C-5), 139.90 (C-6), 132.47 (C-4) and 57.73 (e CH2, C-7) (Supplementary Fig. 2D). Compound \(T_2\) was identified and confirmed as 2,3-dihydroxy-5-(hydroxymethyl) benzaldehyde (Fig. 4B) with the molecular formula \(\text{C}_8\text{H}_8\text{O}_4\) by employing the above spectral data.

Compound \(T_3\) is a light yellow solid and was found to be completely soluble in DMSO and MeOH, but partially in CHCl\(_3\). The IR spectrum indicated the presence of C=O and C=O groups in the structure by showing absorption bands at \(\nu_{\text{max}} 1637.9 \text{ cm}^{-1}\) and 1218.9 cm\(^{-1}\) (Supplementary Fig. 3A). In EIMS analysis, the compound exhibited a molecular ion at \(m/z \ 180 [\text{M}]^+\) (Supplementary Fig. 3B). Analysis of the \(^1\text{H} \text{NMR (CDCl}_3 + \text{DMSO, 200 MHz)}\) spectrum of the compound revealed 4 signals at 9.61 (1H, s), 7.16 (1H, d, \(J = 3.39 \) Hz), 6.48 (1H, d, \(J = 3.58 \) Hz) and 4.69 (2H, s) (Supplementary Fig. 2C), whereas \(^{13}\text{C} \text{NMR (CDCl}_3 + \text{DMSO, 50 MHz)}\) exhibited 7 signals at 200.48 (e CHO), 155.96 (C-2), 148.41 (C-3), 146.63 (C-5), 139.90 (C-6), 132.47 (C-4) and 57.73 (e CH2, C-7) (Supplementary Fig. 2D). Compound \(T_2\) was identified and confirmed as 2,3-dihydroxy-5-(hydroxymethyl) benzaldehyde (Fig. 4B) with the molecular formula \(\text{C}_8\text{H}_8\text{O}_4\) by employing the above spectral data.

Compound \(T_2\) appeared as a pale yellow solid which was completely soluble in DMSO and MeOH and partially in CHCl\(_3\). The IR absorption bands at \(\nu_{\text{max}} 3449.5 \text{ cm}^{-1}\) and 2923.87 cm\(^{-1}\) suggested the presence of OH groups and \(\nu_{\text{max}} 1637.9 \text{ cm}^{-1}\) indicated a CHO group in the structure, respectively (Supplementary Fig. 2A). EIMS analysis of the compound displayed a molecular ion at \(m/z \ 192 [\text{M} + \text{H}]^+\) (Supplementary Fig. 2B). Analysis of the \(^1\text{H} \text{NMR (CDCl}_3 + \text{DMSO, 200 MHz)}\) spectrum of the compound revealed 4 signals at 9.61 (1H, s), 7.16 (1H, d, \(J = 3.39 \) Hz), 6.48 (1H, d, \(J = 3.58 \) Hz) and 4.69 (2H, s) (Supplementary Fig. 2C), whereas \(^{13}\text{C} \text{NMR (CDCl}_3 + \text{DMSO, 50 MHz)}\) exhibited 7 signals at 200.48 (e CHO), 155.96 (C-2), 148.41 (C-3), 146.63 (C-5), 139.90 (C-6), 132.47 (C-4) and 57.73 (e CH2, C-7) (Supplementary Fig. 2D). Compound \(T_2\) was identified and confirmed as 2,3-dihydroxy-5-(hydroxymethyl) benzaldehyde (Fig. 4B) with the molecular formula \(\text{C}_8\text{H}_8\text{O}_4\) by employing the above spectral data.
Fig. 3B). The $^1$H NMR (CDCl$_3$+DMSO, 200 MHz) spectrum of the compound depicted 5 signals at 7.04 (2H, d, $J = 8.08$ Hz), 6.71 (2H, d, $J = 8.06$ Hz), 3.78 (2H, t, $J = 5.88$ Hz), 2.76 (2H, t, $J = 5.90$ Hz) and 2.14 (3H, s) (Supplementary Fig. 3C) while $^{13}$C NMR (CDCl$_3$+DMSO, 50 MHz) inferred 8 signals at 187.53 (C-2, 6C=O) 147.3 (C-4), 134.83 (C-1), 130.14 (C-3, C-5), 115.44 (C-2, C-6), 63.82 (C-4'), 38.26 (C-3') and 29.69 (C-1') (Supplementary Fig. 3D). On the basis of the above spectral information, compound $T_3$ was characterized as 4-(4-hydroxyphenoxy) butan-2-one (Fig. 4C) with the molecular formula C$_{10}$H$_{12}$O$_3$.

Compound $T_4$ was a yellow solid found to be completely soluble in DMSO, MeOH and partially in CHCl$_3$. Its IR spectrum showed absorption bands at $V_{max}$ 3474.2 cm$^{-1}$ (C=O) and C–O groups in the structure respectively (Supplementary Fig. 4A). EI-MS analysis of the compound suggested a molecular ion at $m/z$ 186 [M + Na]$^+$ (Supplementary Fig. 4B). Analysis of the $^1$H NMR (CDCl$_3$ + DMSO, 200 MHz) spectrum of the compound informed 4 signals at 7.25 (3H, m), 2.96 (2H, t, $J = 7.55$ Hz), 2.68 (2H, t, $J = 7.55$ Hz) and 2.17 (6H, s) (Supplementary Fig. 4C), whereas $^{13}$C NMR (CDCl$_3$ + DMSO, 50 MHz) displayed 10 signals at 176.64 (C-3'), 168.0 (C-1'), 148.52 (C-2), 140.18 (C-1), 129.34 (C-6), 128.25 (C-3, C-5), 126.35 (C-4), 35.22 (C-2'), 30.90 (C-4') and 30.63 (C-1', C-2') (Supplementary Fig. 4D). From the above spectral information, compound $T_4$ was characterized as acetic acid-2-hydroxy-6-(3-oxo-butyl)-phenyl ester (Fig. 4D) with the molecular formula C$_{12}$H$_{14}$O$_4$.

Compounds $T_5$ is a yellow solid which was completely soluble in CHCl$_3$, DMSO and MeOH. The IR absorption maxima at $V_{max}$ 1711.0 cm$^{-1}$ indicated the presence of a C=O group in the structure (Supplementary Fig. 5A). ESIMS analysis of the compound suggested a molecular ion at $m/z$ 186 [M + Na]$^+$ (Supplementary Fig. 5B). The $^1$H NMR (CDCl$_3$, 200 MHz) spectrum of the compound $T_5$ displayed 5 signals at 2.32 (2H, t, $J = 7.55$ Hz), 1.99 (1H, m), 1.61 (2H, m) and 1.20–1.30 (10H, m) and 0.9–0.82 (6H, m) (Supplementary Fig. 5C). $^{13}$C NMR (CDCl$_3$, 50 MHz) showed 11 signals at 180.20 (C-1), 34.17 (C-2), 32.03 (C-3), 29.79 (C-
Fig. 4. Molecular structures of A: 1H-indole-3-carboxylic acid ($T_1$), B: 2,3-dihydroxy-5-(hydroxymethyl) benzaldehyde ($T_2$), C: 4-(4-hydroxyphenoxy) butan-2-one ($T_3$), D: Acetic acid-2-hydroxy-6-(3-oxo-butyl)-phenyl ester ($T_4$) and E: 8-methyl decanoic acid ($T_5$).

4), 29.55 (C-5), 29.47 (C-6), 29.38 (C-7), 29.19 (C-8), 24.76 (C-9), 22.30 (C-10) and 14.27 (C-8′) (Supplementary Fig. 5D). Using the above spectral data, compound $T_5$ was elucidated as 8-methyl decanoic acid (Fig. 4E) with the molecular formula $C_{11}H_{22}O_2$.

3.3. Biological assays

The antimicrobial profile of the bioactive compounds in terms of MIC is shown in Table 3. Among the five bioactive compounds isolated from *Streptomyces* sp., 1H-indole-3-carboxylic acid ($T_1$) exhibited good antimicrobial activity followed by acetic acid-2-hydroxy-6-(3-oxo-butyl)-phenyl ester ($T_4$) and 2,3-dihydroxy-5-(hydroxymethyl) benzaldehyde ($T_2$). Of the opportunistic and pathogenic Gram-positive bacteria tested, *S. aureus* and *S. epidermidis* were highly sensitive to metabolites $T_1$, $T_2$, $T_4$ and $T_5$. Among the Gram-negative bacteria, *E. coli* and *X. campestris* showed high sensitivity to $T_1$, $T_2$, $T_3$ and $T_4$. As compared to tetracycline (positive control), $T_1$ was more active against several bacteria tested, whereas compounds $T_2$ and $T_4$ exhibited good activity against a few bacteria. In other cases, tetracycline exhibited good antibacterial activity over the metabolites produced by the strain. MIC values of compounds $T_1$, $T_2$, $T_3$, $T_4$, $T_5$ and tetracycline (positive control) ranged from 10 to 45 µg/ml, 15 to 55 µg/ml, 45 to 125 µg/ml, 15 to 55 µg/ml, 30 to 95 µg/ml and 25 to 75 µg/ml respectively.

The bioactive compounds exhibited good antifungal activity against dermatophytes such as *C. albicans* and *E. floccosum*, for which MIC values recorded included 10 and 20 µg/ml ($T_1$), 30 and 45 µg/ml ($T_2$), 65 and 70 µg/ml ($T_3$), 25 and 40 µg/ml ($T_4$), 55 and 75 µg/ml ($T_5$) and 50 and 60 µg/ml (griseofulvin). The compounds $T_1$, $T_2$ and $T_4$ showed superior antifungal activity against the dermatophytes tested when compared to that of griseofulvin (positive control).

Among the filamentous fungi tested, *A. niger* and *F. oxysporum* were sensitive to metabolites of the strain as well as to the commercial fungicide carbendazim (positive control). Carbendazim showed high antifungal activity when compared to $T_1$, $T_4$ and $T_5$. MIC values of the compounds, i.e. $T_1$ (15–150 µg/ml), $T_2$ (20–175 µg/ml), $T_3$ (85–400 µg/ml), $T_4$ (20–160 µg/ml), $T_5$ (50–230 µg/ml) and carbendazim (2–12 µg/ml) were recorded.

*S. tendae* represents a prominent source of production of novel bioactive compounds. It has been reported to produce nucleoside peptide antibiotics such as nikkomycins Z, X, J and I (Dahn et al., 1976). Fiedler et al. (1994) recorded a new naphthoquinone antibiotic, juglomycin Z from *S. tendae* Tu 901/8c, active against Gram-positive and Gram-negative bacteria and fungi. Other bioactive compounds reported from *S. tendae* include echoseline (Blum et al., 1995), dioxolides, anhydroshikimate, para-hydrobenzamide (Blum et al., 1996) and cervimycins A-D (Herold et al., 2005). However, in the present study, production of five bioactive metabolites, namely 1H-indole-3-carboxylic acid ($T_1$), 2,3-dihydroxy-5-(hydroxymethyl) benzaldehyde ($T_2$), 4-(4-hydroxyphenoxy) butan-2-one ($T_3$), acetic acid-2-hydroxy-6-(3-oxo-butyl)-phenyl ester ($T_4$) and 8-methyl decanoic acid ($T_5$) by *Streptomyces* sp. TK-VL-333 closely related to *S. tendae* cluster was recorded.

Aldridge et al. (1971) isolated indole-3-carboxylic acid from culture filtrates of *Lasiodiplodia theobromae*. Indole-3-carboxylic acid has been isolated and characterized from *Streptomyces* sp. Act80115 by Shaaban et al. (2008) through electrospray ionization mass spectra. Indole-3-carboxylic acid-related metabolites, indole-3-carboxylic acid, 2-desoxythymidin and 5-dimethylallylidene-3-carboxylic acid were recorded from *Streptomyces* sp. B8000 (Poumale et al., 2006) and *Streptomyces* sp. MS239 (Motoshishi et al., 2008) respectively. To our knowledge, information regarding the natural occurrence of the compounds 2,3-dihydroxy-5-(hydroxymethyl) benzaldehyde ($T_2$), 4-(4-hydroxyphenoxy) butan-2-one ($T_3$), acetic acid-2-hydroxy-6-(3-oxo-butyl)-phenyl ester ($T_4$) and 8-methyl decanoic acid ($T_5$) from microorganisms is not yet reported. In the present study, five bioactive compounds extracted from strain TK-VL-333 showed antimicrobial activity against opportunistic and pathogenic bacteria and fungi, and ours is the first report of isolation and characterization of compounds $T_2$, $T_3$, $T_4$ and $T_5$ from Actinobacteria.

*Streptomyces* sp. TK-VL-333 as an antagonistic agent against the wilt pathogen *F. oxysporum* was determined by the agar plate method. A clear zone of inhibition (30 mm) was noted between *Streptomyces* sp. TK-VL-333 and *F. oxysporum*, suggesting the antagonistic nature of the strain. The
degree of antagonism of the strain towards the wilt pathogen analyzed through greenhouse trials is shown in Table 4. Simultaneous treatment of the soil with the pathogen and strain reduced the incidence of Fusarium wilt on sorghum plants up to 53.3% when compared to that of plants raised in soil inoculated with the pathogen alone. A nearly 80% drastic reduction in wilt occurrence was noted in sorghum plants pretreated with antagonist followed by pathogen inoculation. This may be due to the elaboration of bioactive metabolites by the strain prior to the establishment of the pathogen. On the other hand, sorghum plants raised in soils inoculated with antagonist alone/untreated soils appeared healthy.

Majority of Actinobacteria found in soils belong to the genus Streptomyces (Goodfellow and Simpson, 1987) and most of them act as potent antagonists against a variety of phytopathogenic fungi by elaborating various bioactive compounds (Prapagdee et al., 2008). In the present study, Streptomyces sp. TK-VL_333 showed inhibitory activity against Fusarium wilt. Likewise, Yuan and Crawford (1995) recorded the antifungal activity of Streptomyces lydicus against Pythium ultimum and Rhizoctonia solani. Anitha and Rebeeth (2009) reported the inhibition of fungal pathogens like F. oxysporum, Fusarium solani, R. solani and A. alternata by Streptomyces griseus using the dual culture method.

### Table 3
Antimicrobial activities of bioactive metabolites produced by Streptomyces sp. TK-VL_333.

<table>
<thead>
<tr>
<th>Test organism</th>
<th>MIC (µg/ml)</th>
<th>1H-indole-3-carboxylic acid</th>
<th>2,3-Dihydroxy-5-(hydroxymethyl) benzaldehyde</th>
<th>4-(4-Hydroxyphenoxo) butan-2-one</th>
<th>Acetic acid-2-hydroxy-6-(3-oxo-butyl)-phenyl ester</th>
<th>8-Methyl decanoic acid</th>
<th>Ant&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>25</td>
<td>35</td>
<td>45</td>
<td>30</td>
<td>45</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>B. megaterium</td>
<td>20</td>
<td>35</td>
<td>60</td>
<td>25</td>
<td>55</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>B. subtilis</td>
<td>30</td>
<td>40</td>
<td>85</td>
<td>35</td>
<td>95</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Corynebacterium diphteriae</td>
<td>40</td>
<td>55</td>
<td>100</td>
<td>50</td>
<td>70</td>
<td>50</td>
<td></td>
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<tr>
<td>Escherichia coli</td>
<td>25</td>
<td>35</td>
<td>60</td>
<td>30</td>
<td>50</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Proteus vulgaris</td>
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<td>50</td>
<td>70</td>
<td>45</td>
<td>65</td>
<td>25</td>
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<tr>
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<td>75</td>
<td>50</td>
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<td></td>
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<tr>
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<td>40</td>
<td>70</td>
<td>40</td>
<td>60</td>
<td>50</td>
<td></td>
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<tr>
<td>Serratia marcescens</td>
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<td>50</td>
<td>125</td>
<td>55</td>
<td>75</td>
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<tr>
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<td>15</td>
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<tr>
<td>S. epidermis</td>
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<td>Xanthomonas campestris</td>
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<td>40</td>
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<tr>
<td><strong>Yeast</strong></td>
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<td><strong>Filamentous fungi</strong></td>
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<td>45</td>
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<td>A. niger</td>
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<td>20</td>
<td>85</td>
<td>25</td>
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<td>5</td>
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<tr>
<td>Alternaria alternata</td>
<td>150</td>
<td>175</td>
<td>400</td>
<td>160</td>
<td>230</td>
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<tr>
<td>Carvularia maculans</td>
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<td>145</td>
<td>350</td>
<td>140</td>
<td>175</td>
<td>10</td>
<td></td>
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<tr>
<td>C. lunata</td>
<td>80</td>
<td>95</td>
<td>210</td>
<td>85</td>
<td>125</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Fusarium oxysporum</td>
<td>15</td>
<td>25</td>
<td>95</td>
<td>20</td>
<td>65</td>
<td>5</td>
<td></td>
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<tr>
<td>Penicillium citrinum</td>
<td>20</td>
<td>30</td>
<td>100</td>
<td>30</td>
<td>50</td>
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</tr>
</tbody>
</table>

<sup>a</sup> Antibiotic: tetracycline against bacteria, griseofulvin against yeast and carbendazim against fungi.

### Table 4
Antifungal spectrum of Streptomyces sp. TK-VL_333 against F. oxysporum under in vivo conditions.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Treatment</th>
<th>Percent of wilted sorghum plants</th>
<th>Percent reduction in wilting</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Soil inoculated with the pathogen alone</td>
<td>100 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>2.</td>
<td>Soil inoculated with the antagonist alone</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>3.</td>
<td>Simultaneous inoculation of the soil with the pathogen and antagonist</td>
<td>46.6 ± 0.92</td>
<td>53.3 ± 0.92</td>
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<tr>
<td>4.</td>
<td>Soil incubated with the antagonist for 4 days followed by pathogen inoculation</td>
<td>20.0 ± 0.81</td>
<td>80.0 ± 0.81</td>
</tr>
<tr>
<td>5.</td>
<td>Uninoculated soil</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

Average of 3 trials with 10 plants per treatment (±SD). Data were statistically analyzed by one-way ANOVA and found to be significant at 5%.
The bioactive metabolite 1H-indole-3-carboxylic acid obtained from *Streptomyces* sp. TK-VL_333 showed good antifungal activity against *F. oxysporum* causing wilt of *S. bicolor*. Therefore, its antifungal potential was further tested *in vitro* and *in vivo* studies against *F. oxysporum*. As shown in Fig. 5, 1H-indole-3-carboxylic acid inhibited conidial germination of *F. oxysporum* under *in vitro* conditions at a concentration of 150 µg/ml, and the fungicides, mancozeb and carbendazim showed inhibitory activity at a level of 500 µg/ml and 50 µg/ml respectively. Under *in vivo* conditions, the bioefficacy of the compound against *Fusarium* wilt of sorghum was checked in a greenhouse trial in comparison with that of systemic fungicides. The compounds 1H-indole-3-carboxylic acid, mancozeb and carbendazim were effective in controlling *Fusarium* wilt at concentrations of 400 µg/ml, 700 µg/ml and 100 µg/ml respectively (Fig. 6).

Matsuda et al. (1998) noted the suppression of bacterial wilt in tomato caused by *Ralstonia solanacearum* with 3-indole propionic acid. The metabolites of different Actinobacteria species have been reported to be active against *Fusarium* spp. and other plant pathogens. Soil-borne phytopathogens such as *F. oxysporum*, *Fusarium moniliforme*, *Fusarium semitectum*, *F. solani* and *R. solani* were highly sensitive to 2-methylheptyl isonicotinate produced by *Streptomyces* sp. 201 (Bordolo et al., 2002). Zhong et al. (2004) reported that wuyiencin isolated from *Streptomyces hygroscopicus* var. *wuyiensis* inhibited conidial germination of *Botrytis cinerea*. Secondary metabolites such as 5,7-dimethoxy-4-p-methoxyphenylcoumarin and 5,7-dimethoxy-4-phenylcoumarin produced by *Streptomyces aureofaciens* CMUAc 130 showed antifungal activity against phytopathogenic fungi (Taechowisan et al., 2005b).

In the present study, the efficacy of the bioactive compound indole-3-carboxylic acid against the wilt pathogen was found to be better than that of mancozeb, but less active than carbendazim. Bioactive compounds, because of their natural origin, are biodegradable and they do not leave toxic residues or byproducts to contaminate the environment (http://www.ias.ac.in/currenSci/jan25/articles22.htm-pesticides), whereas commercial fungicides such as carbendazim pose severe toxicity to humans, plants and animals (Mantovani et al., 1998). In order to provide tools for integrated agriculture, biological control offers an eco-friendly alternative to the use of synthetic fungicides for controlling plant diseases, and the bioactive compound, 1H-indole-3-carboxylic acid produced by the strain may be used to control *Fusarium* wilt. From the present investigation, it is apparent that the five bioactive compounds obtained from *Streptomyces* sp. TK-VL_333 have antimicrobial potential against a wide variety of bacteria, yeast and filamentous fungi.

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**Appendix. Supplementary data**

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.resmic.2010.03.011.

**References**


