

IDENTIFICATION, ISOLATION AND OPTIMIZATION OF ANTIFUNGAL METABOLITES FROM THE *STREPTOMYCES MALACHITOFUSCUS* CTF9

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ABSTRACT

An indigenous *Streptomyces* isolate CTF9, exhibiting promising antifungal activity against *Mucor miehei* and *Candida albicans* in pre-screening studies, was investigated by cultivation in a 50-L fermenter and by subsequent isolation, purification, and structure elucidation of the active metabolites. Based on the morphological, biochemical, and physiological characterization, as well as the 16S rRNA gene sequence, the isolate CTF9 was identified as *Streptomyces malachitofuscus*. Using a series of chromatographic techniques, two pure compounds were isolated from the obtained extracts after the fermentation of the isolate CTF9. The isolated compounds were identified as phenylacetic acid and indolyl-3-lactic acid by mass spectrometry (MS) and NMR analysis. The culture optimization studies revealed that the isolate CTF9 can use a variety of low-cost carbon and nitrogen sources to generate the maximum quantity of industrially important metabolites at an elevated temperature of 35°C and at a pH 7.8.

Key words: Phenylacetic acid, Indolyl-3-lactic acid, indigenous *Streptomyces* sp. CTF9

INTRODUCTION

Actinomycetes are the major producers of pharmaceuticals, agricultural pesticides, and veterinary medicine because of their ability to produce antibiotics. Among the actinomycetes, the genus *Streptomyces* has continued to provide a larger number and wider variety of new antibiotics than any other genus, suggesting that a substantial number of *Streptomyces* species or strains with novel antibiotic productivity exist in nature. These bacteria produce about 75% of commercially and medically useful antibiotics (3, 18). Moreover, approximately 60% of the antibiotics developed for

agricultural use are isolated from *Streptomyces* spp. (26). Fungal pathogens have been the major culprits for significant agricultural losses due to the plant diseases that they cause; they are also the causal agents of many medically important diseases. Therefore, currently, there is an immense need for antifungal agents. Several members of the streptomycetes family have been reported as potential biocontrol agents and as the potent producers of antifungal compounds (16, 24), indicating that these filamentous bacteria possess a vast potential for producing antifungal metabolites. In search for antifungal agents, *Streptomyces* strains have been isolated from various types of soils, including rice paddy, lake mud and

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water, deciduous forest, tropical forest, wasteland, and cave soils (6, 8, 11, 23, 25, 29). Their natural habitat soil is nutritionally, biologically and physically complex and variable, demanding their fast adaptation. As a consequence, they are able to perform a broad range of metabolic processes and to produce an immense diversity of bioactive secondary metabolites.

In our search for bioactive secondary metabolites from indigenous *Streptomyces*, more than 100 active *Streptomyces* strains were isolated from the soil samples collected from saline agricultural farmlands in our area (Punjab, Pakistan) and were screened for antimicrobial activity and the metabolites that they produced (21). The isolate *Streptomyces* sp. CTF9 exhibited promising antifungal activity against *Candida albicans* and *Mucor miehei*, suggesting it as a promising strain to be further evaluated by preparative screening for the isolation, purification and structure elucidation of active metabolites. The microscopic, morphological, biochemical and physiological characterization strongly suggests that the isolate belongs to the genus *Streptomyces*. Its 16S rRNA gene sequence shows a maximum similarity (99%) to *Streptomyces malachitofuscus*; the 1426-bp sequence of the 16S rRNA gene of the isolate *Streptomyces* sp. CTF9 is available at GenBank, with the accession number of EU294138. Here, we reported the fermentation, isolation, purification, and structure elucidation of the active antifungal metabolites produced by the isolate *Streptomyces* sp. CTF9, along with the optimization of the production of these metabolites under different media compositions and culture conditions.

MATERIALS AND METHODS

Cultivation of the Strain *Streptomyces* sp. CTF9

The *Streptomyces* sp. CTF9 was cultivated in a 50-L fermenter (Biostat U) (working volume: 30 L) using M₂ medium (malt extract 10 g, yeast extract 4 g, and glucose 4 g in 1 L of distilled water). The fermenter was filled with 27 L of

water mixed with the corresponding quantities of M₂ medium components. The medium was sterilized by keeping the fermenter in the sterilization mode for 30 minutes at 121°C. After the air supply was turned on, a stirring motor and water circulation pumps were subsequently turned on. The acid (2 N HCl), base (2 N NaOH) and antifoam (1% Niax/70% ethanol) were filled in their respective jars and were connected to the fermenter. The pH electrode was sterilized using 70% ethanol and was adjusted. The preculture was prepared by inoculating the isolate CTF9 from well-grown plates into 12 x 250 mL of M₂ medium in 1-L Erlenmeyer flasks (total volume: 3 L). The flasks were incubated at 28°C on a linear shaker for 4 days; 10% of the preculture was used to inoculate the fermenter. The growth parameters were adjusted as follows: temperature 28°C, pH 6.5 ± 1.5, and aeration 1.8 m³/h. Fermentation was conducted for 5 days.

Extraction and purification of the active compounds

After harvesting the dark brown culture, the broth was filtered over celite using a filter press (Schenk Niro 212 B40) to separate the mycelium from the liquid phase. The mycelial cake was extracted using ethyl acetate (3 times) and acetone (1 time). The water phase was extracted by adsorption on Amberlite XAD-16 resin in a large-size glass column (100 × 5 cm) and was eluted with methanol. The solvents (ethyl acetate, acetone, and methanol) containing bioactive metabolites were dried using a rotary evaporator (*Rotavapor* R152) yielding 5.64 g of crude extracts.

Both mycelia and water-phase extracts were then collected, based on the characterization using TLC. The crude extract (5.64 g) was fractionated on a silica gel column (Kieselgel 60, 70 ~ 230 mesh, Merck, Darmstadt, Germany, 30 g, 1.5 × 50 cm) using a CH₂Cl₂: MeOH gradient (0, 5, 10, 20, 30, 40, and 50%, vol/vol) and was divided into two fractions. Fraction I was characterized as fat (1.85 g) using TLC and spraying reagents. Fraction II was further purified by silica gel column chromatography (1.2 × 30 cm, packed with 15 g of silica gel),

preparative TLC and Sephadex LH-20 (25 ~ 100 mesh, Pharmacia Fine Chemicals, Uppsala, Sweden) in a column (86 × 2.0 cm) and was eluted with CH₂Cl₂: 40% MeOH, yielding colorless solids of phenylacetic acid (1; 25.7 mg) and indolyl-3-lactic acid (3; 12.8 mg).

Structure determination of the purified compounds

Mass spectra: The purified fractions were analyzed by EI MS at 70 eV with Varian MAT 731, Varian 311A, AMD-402, ESI MS using a Quattro Triple Quadrupole mass spectrometer Finigan MAT-Incos 50, ESI-MS LCQ (Finnigan) and ESI-HRMS using perfluorokerosene as standard.

Nuclear Magnetic Resonance (NMR) Spectroscopy: ¹H NMR spectra were measured by Varian Unity 300 (300 MHz), Bruker AMX 300 (300 MHz), and Varian Inova 500 (499.8 MHz). Coupling constants (*J*) in Hz. ¹³C NMR spectra were measured by Varian Unity 300 (75.5 MHz) and Varian Inova 500 (125.7 MHz). Chemical shifts (δ) were measured, relative to the internal control tetramethylsilane.

Production optimization

The six different media A, B, C, D, E and F used in the study were as follows: A: malt extract 10 g, yeast extract 4 g, and glucose 4 g in 1 L of DH₂O, B: soya been fat 20 g and mannitol 20 g in 1 L of DH₂O, C: glucose anhydrous 10 g, peptone 2 g, yeast extract 1 g, and meat extract 1 g in 1 L of DH₂O, D: yeast extract 40 g, glucose 5 g, and CaCl₂ 45 g in 1 L of DH₂O, E: trypton 10 g, yeast extract 5 g, NaCl 10 g, and glucose 5 g in 1 L of DH₂O, F: glucose 21 g, fish flour 5 g, flour 10 g, MgSO₄ 0.5 g, NaCl 1 g, CaCl₂ 0.5 g, and trace elements 10 ml in 1 L of DH₂O, (trace element stock solution: FeSO₄ 0.2 g, COCl₂ 0.04 g, CaCl₂ 0.04 g, manganese(II) chloride 0.04 g, zinc sulphate 0.08 g, and sodium borate 0.08 g in 1 L of DH₂O). In addition to different media compositions, the following four sets of culture conditions were applied: CC1: pH 6.5, incubation temperature of 28°C, shaking at 95 rpm, for 7 days. CC2: pH 6.5, incubation temperature of 35°C,

shaking at 110 rpm, for 7 days. CC3: pH 7.8, incubation temperature of 28°C, shaking at 95 rpm, for 7 days. CC4: pH 7.8, incubation temperature of 35°C, shaking at 110 rpm, for 7 days.

For each of the six media, 3 L were prepared and dispensed into 12 1-L flasks (12 × 250 mL culture broth). The flasks were grouped according to the proposed culture conditions; the pH in each flask was individually adjusted, and the flasks were sterilized by autoclaving and were inoculated with *Streptomyces* sp. CTF9 from well-grown plates. The inoculated flasks were incubated on linear shakers under the proposed culture conditions (CC1, CC2, CC3 and CC4). The strains were harvested after five days, the culture broth in each flask was freeze dried (Christ Alpha 2-4 D) and was individually extracted (3 ×) with ethyl acetate. Ethyl acetate was evaporated on a rotary evaporator (Heidolph Laborota 4000), and the crude extracts were obtained. The crude extracts were weighed to determine the quantity of metabolites produced under different cultural conditions. Subsequently, the crude extracts were individually tested against the test organisms *Candida albicans* and *Mucor miehei* to determine the quantity of the active antifungal metabolites per unit volume of each extract. The results were statistically analyzed using the analysis of variance (ANOVA) test (Duncan's multiple range test) by SPSS version 10 (P=0.001).

RESULTS

Purification and structure elucidation of the active compounds

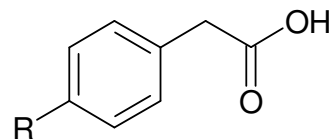
The bulk fermentation was performed in a 50-L fermenter (Biostat U) (working volume: 30 L) using M₂ medium. After harvesting, cell separation and solvent extraction, 5.64 g of the crude extract was obtained from both the mycelium and the liquid phase. The components of the crude extract were purified using silica gel column, preparative TLC and gel exclusion chromatography, yielding two pure compounds

identified as phenylacetic acid and indolyl-3-lactic acid by ^1H , ^{13}C NMR and mass spectrometry and by comparison to the published data.

Phenylacetic acid (1)

Compound 1 was isolated as a colorless, UV-absorbing solid and stained yellow on TLC by anisaldehyde/sulphuric acid spraying reagent. According to its chromatographic properties and spectroscopic data, compound 1 was identified as phenylacetic acid. The result was further confirmed by searching in AntiBase (14) and by comparing its MS and NMR data to those of an authentic sample (19). *p*-Hydroxyphenylacetic acid (2) and phenylacetic acid (1) were isolated from *Taraxacum* roots as natural products, by Power and Browning (20) and as fungal metabolites from *Ophiostoma crassivaginata* (2). Phenyl acetic acid is widespread in higher plants, fungi, *e.g.*, *Aspergillus niger* (19), and bacteria. In microbial antibiotic production, compound 1

was added to the culture of *Penicillium* sp. to increase the production of penicillin G (parasticin) (9, 10).



1: R = H; 2: R = OH

Phenylacetic acid (1): Colorless solid (25.7 mg), UV-absorbing, stained yellow by the anisaldehyde/sulphuric acid spraying reagent. – $R_f = 0.29$ ($\text{CH}_2\text{Cl}_2/3\%$ MeOH). – ^1H NMR (300 MHz, CDCl_3): $\delta = 9.50$ (brs, 1H, OH), 7.30-7.20 (m, 5H, Ar-H), 3.62 (s, 2H, CH_2) (Figure 1a). – ^1H NMR (300 MHz, Acetone- d_6): $\delta = 10.80$ (brs, 1H, OH), 7.50-7.30 (m, 5 H, Ar-H), 3.72 (s, 2H, CH_2) (Figure 1b). – ^{13}C NMR (50 MHz, CDCl_3): $\delta = 178.3$ (CO), 133.2 (C_q), 129.3 (2CH), 128.6 (2CH), 127.3 (CH), 41.1 (CH_2) (Figure 2). – EI MS (70 eV): m/z (%) = 136 ($[\text{M}]^+$, 32), 91 (100), 65 (12) (Figure 3).

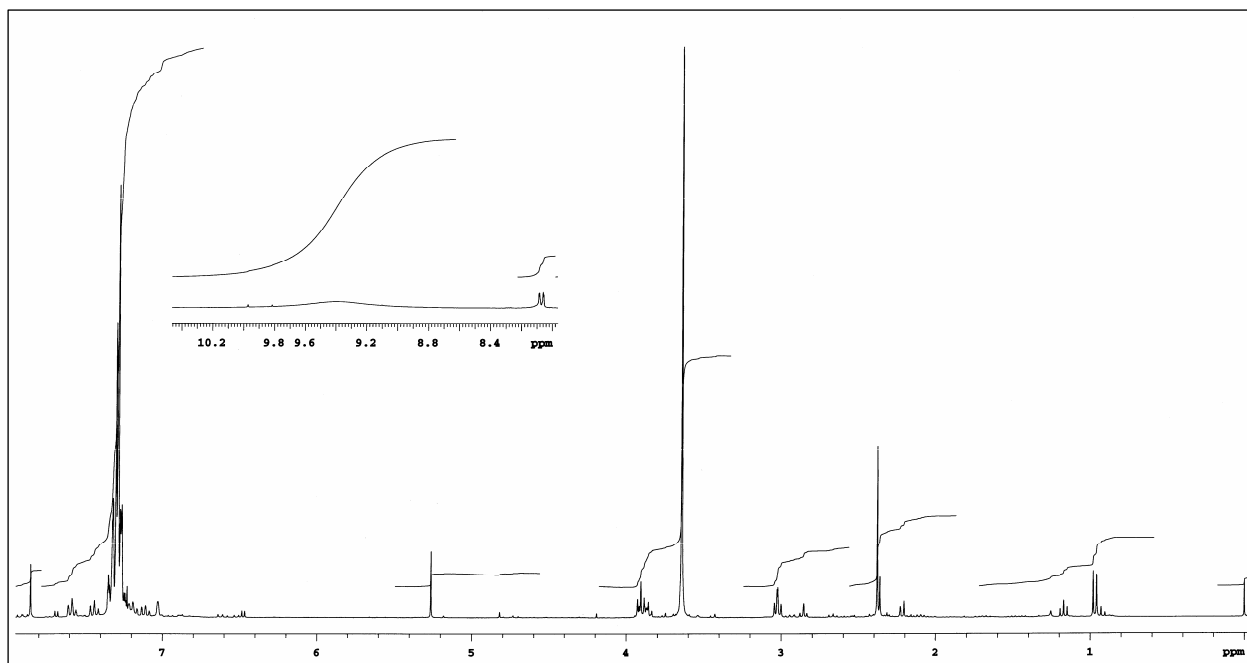


Figure 1a. ^1H NMR (CDCl_3 , 300 MHz) of phenylacetic acid

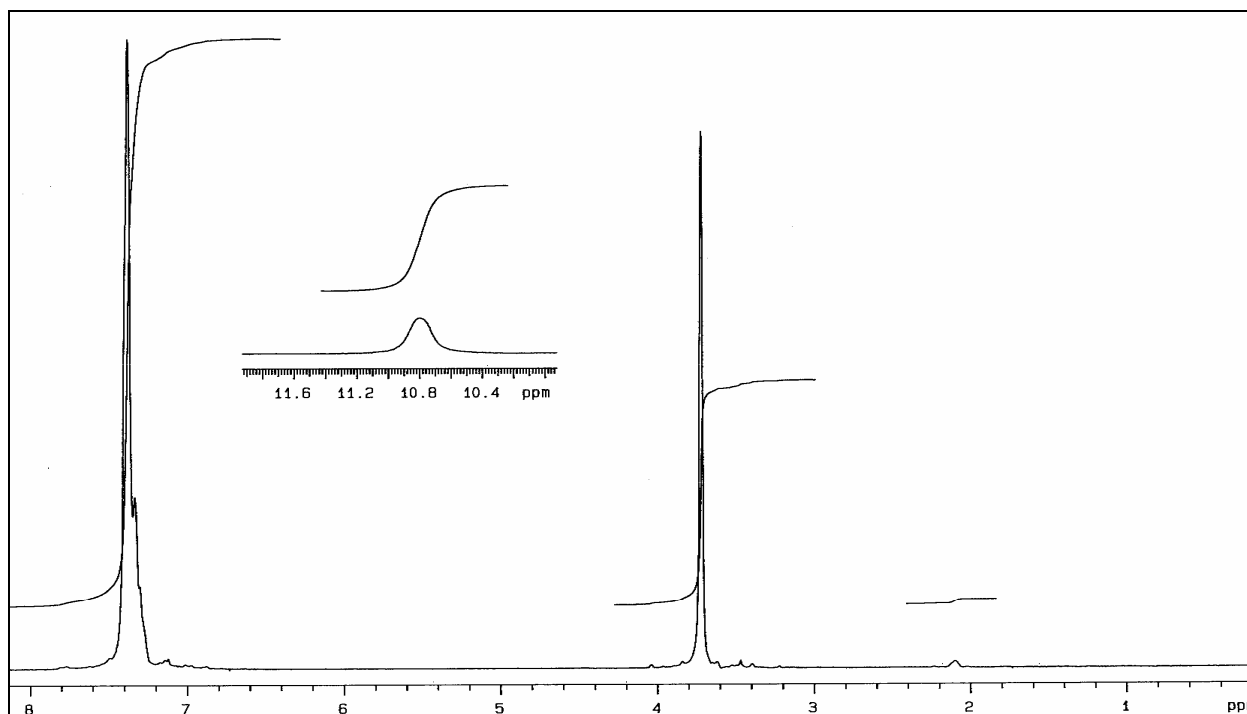


Figure 1b. ^1H NMR (Acetone- d_6 , 300 MHz) of phenylacetic acid

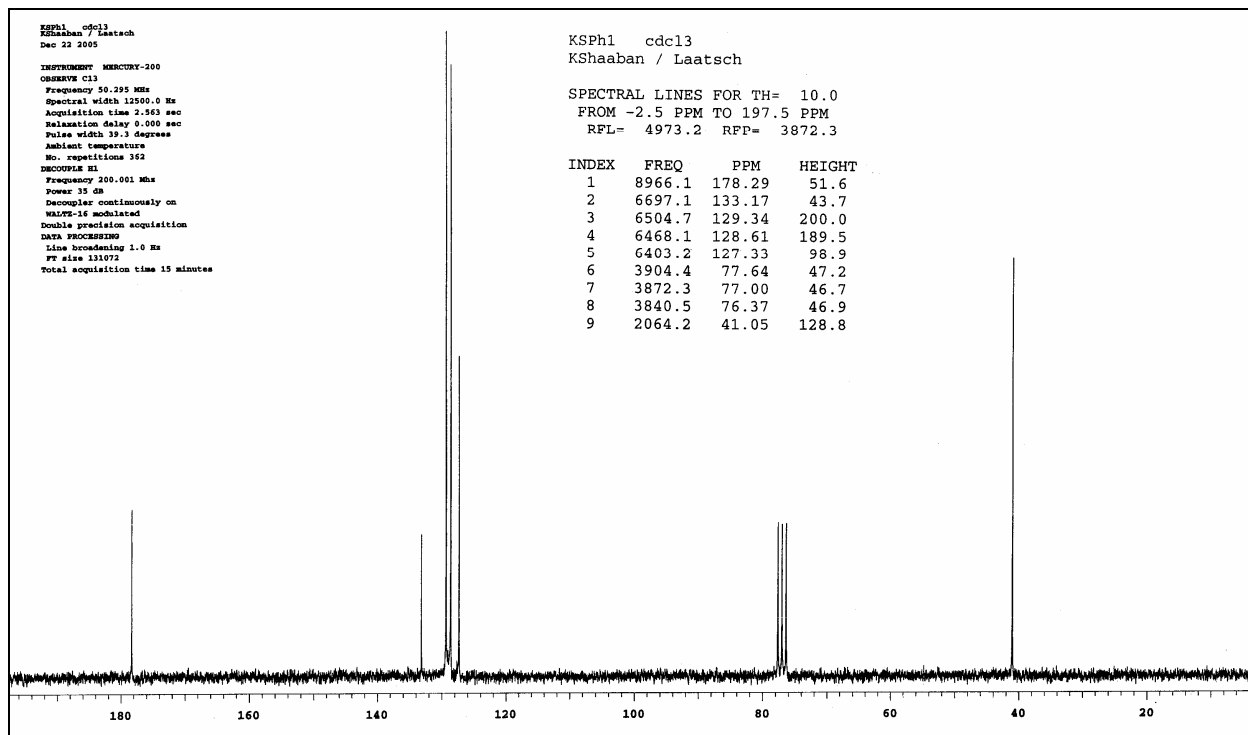
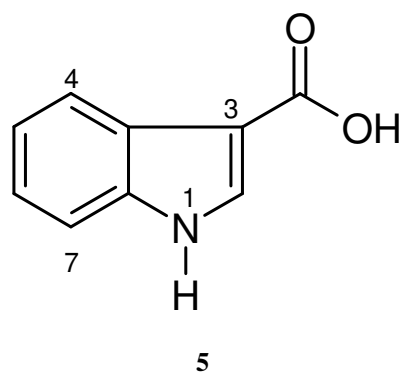
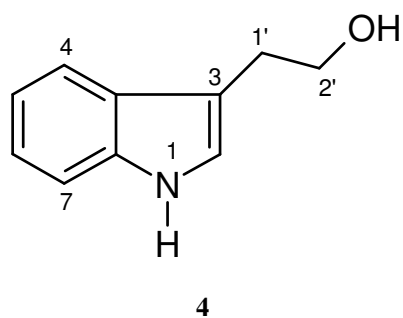


Figure 2. ^{13}C NMR (CDCl_3 , 50 MHz) of phenylacetic acid



Indolyl-3-lactic acid (3): $C_{11}H_{11}NO_3$ (205.2), colorless, moderately polar, UV-absorbing solid (12.8 mg), stained violet by anisaldehyde/sulphuric acid and heating. – $R_f = 0.35$ ($CHCl_3/5\%MeOH$). – 1H NMR ($CDCl_3$, 300 MHz): $\delta = 10.75$ (s br, 1H, NH), 8.31 (s br, 1H, OH), 7.72 (dd, $^3J = 7.9$ Hz, $^4J = 1.1$ Hz, 1H, 4-H), 7.38 (dd, $^3J = 8.3$ Hz, $^4J = 1.1$ Hz, 1H, 7-H), 7.20 (td, $^3J = 7.2$ Hz, $^4J = 1.2$ Hz, 1H, 5-H), 7.10 (td, $^3J = 7.2$ Hz, $^4J = 1.1$ Hz, 1H, 6-H), 7.11 (d, $^3J = 0.8$ Hz, 1H, 2-H), 4.55 (m, 1H, 2'-H), 3.36 (dd, $^2J_{AB} = 11.1$ Hz, $^3J_{AX} = 4.2$ Hz, 1H, 2'-CH_A), 3.26 (dd, $^3J = 6.1$ Hz, 1.1, 1H, 2'-CH_B). – 1H NMR ($DMSO-d_6$, 300 MHz): $\delta = 10.70$ (s br, 1H, NH), 7.55 (dd, $^3J = 7.9$ Hz, $^4J = 1.1$ Hz, 1H, 4-H), 7.32 (dd, $^3J = 8.3$ Hz, $^4J = 1.1$ Hz, 1H, 7-H), 7.11 (d, $^3J = 0.8$ Hz, 1H, 2-H), 7.05 (td, $^3J = 7.2$ Hz, $^4J = 1.2$ Hz, 1H, 5-H), 6.92 (td, $^3J = 7.2$ Hz, $^4J = 1.1$ Hz, 1H, 6-H), 4.45 (s br, 1H, OH), 3.75 (m, 1H, 2'-H), 3.15 (dd, $^2J_{AB} = 11.1$ Hz, $^3J_{AX} = 4.2$ Hz, 1H, 2'-CH_A), 2.65 (dd, $^3J = 6.1$ Hz, 1.1, 1H, 2'-CH_B) (Figure 4). – EI MS (70 eV): m/z (%) = 205 ($[M]^+$, 18), 130 ($[M - (HO-CH-COOH)]^+$, 100) (Figure 5).

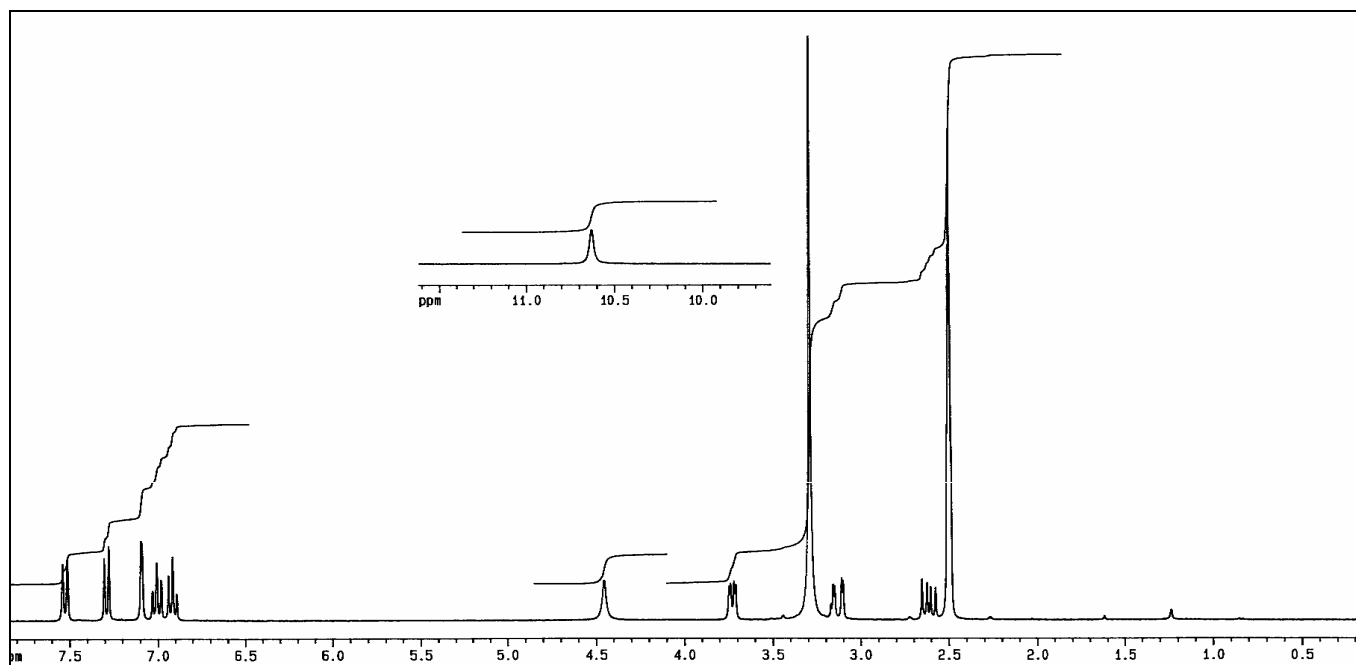


Figure 4. 1H NMR ($DMSO-d_6$, 300 MHz) of indolyl-3-lactic acid

comparable but were significantly different from those of the other culture conditions. A significant increment in yield, to the maximum level, was obtained in the medium D, when the strain was cultured under the CC2 conditions. The relative significance of the other three culture conditions, in terms of

their effects on the yield in the medium D, was CC4>CC3>CC1. In case of the media E and F, the CC3 conditions significantly increased the yield of the crude extracts, to the maximum level, compared to all the other culture conditions (CC1, CC2 and CC4; Table 1).

Table 1. Comparison of the weight of the crude extracts/250 ml culture broth of the strain CTF9 for six media compositions and four sets of culture conditions (expressed as the mean of three replicates \pm S.E)

Culture conditions	Different media compositions					
	Med A	Med B	Med C	Med D	Med E	Med F
CC1	22.65 ^{ey} ± 0.805	26.61 ^{dy} ± 0.352	25.75 ^{dx} ± 1.014	96.66 ^{cz} ± 0.476	166.68 ^{ax} ± 0.644	151.50 ^{by} ± 0.231
CC2	32.593 ^{fx} ± 1.273	51.61 ^{ex} ± 1.951	34.41 ^{ew} ± 0.231	173.06 ^{aw} ± 0.669	37.32 ^{dy} ± 1.544	161.05 ^{bx} ± 1.187
CC3	13.793 ^{ez} ± 0.453	26.62 ^{dy} ± 0.473	26.61 ^{dx} ± 0.929	106.36 ^{cy} ± 1.509	171.63 ^{bw} ± 0.918	183.50 ^{aw} ± 1.097
CC4	64.27 ^{cw} ± 0.274	58.90 ^{dw} ± 1.674	36.55 ^{fw} ± 0.199	150.87 ^{ax} ± 0.502	40.08 ^{ey} ± 1.319	127.29 ^{bz} ± 1.207

Values designated by a, b, c, d, e, and f are for six different media compositions, Values designated by w, x, y, and z are for four different culture conditions, Values designated with the same alphabets are not significantly different, Duncan's (1955) multiple range test at the significance level = 0.001 (Med A: malt extract 10 g, yeast extract 4 g, and glucose 4 g in 1 L of DH₂O, Med B: soya been fat 20 g and mannitol 20 g in 1 L of DH₂O, Med C: glucose anhydrous 10 g, peptone 2 g, yeast extract 1 g, and meat extract 1 g in 1 L of DH₂O, Med D: yeast extract 40 g, glucose 5 g, and CaCl₂ 45 g in 1 L of DH₂O, Med E: trypton 10 g, yeast extract 5 g, NaCl 10 g, and glucose 5 g in 1 L of DH₂O, Med F: glucose 21 g, fish flour 5 g, flour 10 g, MgSO₄ 0.5 g, NaCl 1 g, CaCl₂ 0.5 g, and trace elements 10 ml in 1 L of DH₂O, (trace elements stock solution: FeSO₄ 0.2 g, COCl₂ 0.04 g, CaCl₂ 0.04 g, manganese(II) chloride 0.04 g, zinc sulphate 0.08 g, and sodium borate 0.08 g in 1 L of DH₂O) (CC1: pH 6.5, the incubation temperature of 28°C, shaking at 95 rpm, for 7 days CC2: pH 6.5, the incubation temperature of 35°C, shaking at 110 rpm, for 7 days CC3: pH 7.8, the incubation temperature of 28°C, shaking at 95 rpm, for 7 days CC4: pH 7.8, the incubation temperature of 35°C, shaking at 110 rpm, for 7 days)

Impact of different media compositions and culture conditions on the antimicrobial activity of the strain *Streptomyces* sp. CTF9

Antimicrobial activity of the extracts was determined using two fungal test strains (*Candida albicans* and *Mucor miehei*) to assess the relative concentration of the active metabolites in a crude extract. The different media compositions (A to F) induced comparable antimicrobial responses in an individual set of culture conditions; however, minor variations were observed. In case of the CC1 culture conditions, the optimal response, in respect to antimicrobial activity, was observed in the crude extracts from the media B

and D (Table 2). Under the CC2 culture condition, the medium E induced a significant antimicrobial activity against *Candida albicans*, whereas the medium C induced a significant antimicrobial activity against *Mucor miehei*. Under the CC3 culture conditions, the media B, D and F induced the most significant antimicrobial activity against both test organisms. In case of the CC4 culture conditions, the optimal antimicrobial activity against both test organisms was seen in the crude extracts from the media A and E (Table 2).

Similarly, different culture conditions (CC1 to CC4) also induced comparable antimicrobial responses in a specific culture medium with slight deviations in some cases. In case of

the medium A, the CC4 and CC2 culture conditions showed significant impacts on the antimicrobial activity against both test organisms, compared to the CC1 and CC3 culture conditions (Table 2). In the medium B, the CC3 culture conditions showed a significant impact on the antimicrobial activity, compared to the other culture conditions. In the medium C, the optimal antimicrobial response was observed under the CC2 culture conditions. In the medium D, the culture conditions CC1, CC2 and CC4 showed comparable impacts on the antimicrobial activity against both test organisms; however,

compared to CC3, the antimicrobial activity against *Candida albicans* was weaker, whereas the antimicrobial activity against *Mucor miehei* was stronger. In the medium E, the impacts of the culture conditions CC4 and CC2 on antimicrobial activity were comparable and were more significant, compared to those of CC1 and CC3, against both test organisms. In case of the medium F, the extracts from the culture conditions CC3 and CC4 exhibited significantly higher antimicrobial activity against both test organisms, compared to those from CC1 and CC2 (Table 2).

Table 2. Comparison of the antimicrobial activity of the strain CTF9 at six media compositions and four sets of culture conditions (expressed as the mean of three replicates \pm S.E)

Media / Culture conditions	Antimicrobial activity against test organisms							
	Zone of inhibition (mm)							
	<i>Candida albicans</i>				<i>Mucor miehei</i>			
	CC1	CC2	CC3	CC4	CC1	CC2	CC3	CC4
Med A	0 ^{cy}	17.6 ^{bx}	0 ^{cy}	19.6 ^{aw}	0 ^{dx}	12 ^{cw}	0 ^{dx}	13 ^{aw}
	± 0.000	± 0.333	± 0.000	± 0.333	± 0.000	± 0.577	± 0.000	± 0.000
Med B	13 ^{ax}	0 ^{ez}	16.6 ^{aw}	11 ^{cy}	10.6 ^{bx}	0 ^{dy}	12 ^{bw}	11 ^{bx}
	± 0.000	± 0.000	± 0.333	± 0.000	± 0.333	± 0.00	± 0.000	± 0.000
Med C	0 ^{cx}	11.6 ^{cw}	0 ^{cx}	0 ^{dx}	0 ^{dy}	14.6 ^{aw}	11 ^{cx}	0 ^{cy}
	± 0.000	± 0.333	± 0.000	± 0.000	± 0.000	± 0.333	± 0.000	± 0.000
Med D	11 ^{bx}	10.6 ^{dx}	12 ^{bw}	11 ^{cx}	12.6 ^{aw}	13 ^{bw}	11 ^{cx}	13 ^{aw}
	± 0.000	± 0.333	± 0.000	± 0.000	± 0.333	± 0.000	± 0.000	± 0.000
Med E	0 ^{cx}	19.6 ^{aw}	0 ^{cx}	20 ^{aw}	9.6 ^{cx}	13 ^{bw}	0 ^{dy}	13 ^{aw}
	± 0.000	± 0.333	± 0.000	± 0.000	± 0.333	± 0.000	± 0.000	± 0.000
Med F	0 ^{cy}	0 ^{ey}	12.6 ^{bx}	16.6 ^{bw}	0 ^{dy}	0 ^{dy}	15.6 ^{aw}	11 ^{bx}
	± 0.000	± 0.000	± 0.333	± 0.333	± 0.000	± 0.000	± 0.333	± 0.000

Values designated by a, b, c, d, e, and f are for six different media compositions. Values designated by w, x, y, and z are for four different culture conditions. Values designated with the same letters are not significantly different, Duncan's (1955) multiple range test at a significance level = 0.001 (Med A: malt extract 10 g, yeast extract 4 g, and glucose 4 g in 1 L of DH₂O, Med B: soya bean fat 20 g and mannitol 20 g in 1 L of DH₂O, Med C: glucose anhydrous 10 g, peptone 2 g, yeast extract 1 g, and meat extract 1 g in 1 L of DH₂O, Med D: yeast extract 40 g, glucose 5 g, and CaCl₂ 45 g in 1 L of DH₂O, Med E: trypton 10 g, yeast extract 5 g, NaCl 10 g, and glucose 5 g in 1 L of DH₂O, Med F: glucose 21 g, fish flour 5 g, flour 10 g, MgSO₄ 0.5 g, NaCl 1 g, CaCl₂ 0.5 g, and trace elements 10 ml in 1 L of DH₂O, (trace elements stock solution: FeSO₄ 0.2 g, COCl₂ 0.04 g, CaCl₂ 0.04 g, manganese(II) chloride 0.04 g, zinc sulphate 0.08 g, and sodium borate 0.08 g in 1 L of DH₂O) (CC1: pH 6.5, incubation temperature of 28°C, shaking at 95 rpm, for 7 days CC2: pH 6.5, incubation temperature of 35°C, shaking at 110 rpm, for 7 days CC3: pH 7.8, incubation temperature of 28°C, shaking at 95 rpm, for 7 days CC4: pH 7.8, incubation temperature of 35°C, shaking at 110 rpm, for 7 days)

DISCUSSION

The present work describes the fermentation, isolation, purification and structure elucidation of the antifungal agents phenylacetic acid (1) and indolyl-3-lactic acid (3) from a newly isolated cotton field isolate *Streptomyces* sp. CTF9 in addition

to the culture optimization to examine the appropriate medium components and culture conditions for the maximum yield of these commercially important compounds. Phenylacetic acid, a deamination product of phenylalanine, has been known for its pervasive applications in agriculture, pharmaceutical, and cosmetic industries. It has been known to exhibit a positive

effect on the growth and development of different cereal crops (22). Wightman and Lighty (28) found that phenylacetic acid (1) acts as a natural auxin in the shoots of higher plants, such as barley, corn, tobacco, and tomato. The *in vitro* and *in vivo* antifungal activity of phenylacetic acid has been reported by many studies (7, 11, 15), suggesting that it is a potential agroactive and biocontrol agent. In microbial antibiotic production, phenylacetic acid is added as a precursor to the culture of *Penicillium chrysogenum* to increase the production of penicillin G (9). Phenylacetic acid is also used in perfumes at low concentrations.

Indolyl compounds, including tryptophol (4) and indole-3-carboxylic acid (5) (12, 27), are frequently isolated from bacteria and are characterized by their specific color reactions with anisaldehyde/sulphuric acid (violet) and Ehrlich's (orange) spraying reagents. Compound 5 is known as a fungal metabolite from *Lasiodiplodia theobromae* (1). From the newly isolated cotton field *Streptomyces* sp. CTF9, indolyl-3-lactic acid (3) (12.8 mg) was obtained as colorless solid, showing the same physico-chemical and chromatographic properties as 4 and 5. Compound 3 is often isolated from bacteria, fungi and yeast (14) and exhibits the antimicrobial activity against *Candida albicans*.

Streptomyces can grow on a variety of media and under different culture conditions; however, it is usually helpful to identify the optimal medium, temperature, pH and aeration of the interesting strains for large-scale cultivation. The nutrients and culture conditions play an important role in the onset and intensity of secondary metabolism. To achieve high product yields, it is a prerequisite to identify a proper production medium to be used for an efficient fermentation process (17). The medium composition is usually related to the biosynthesis of antibiotics (4). The role of the medium is to provide nutrients and precursors to the culture (5). Six media compositions (A, B, C, D, E, and F), along with four sets of culture conditions (CC1, CC2, CC3 and CC4), were examined to determine the ability of the *Streptomyces* sp. CTF9 to use

different carbon and nitrogen sources and to identify the optimal culture conditions for the maximum yields of phenylacetic acid and indolyl-3-lactic acid. The cultural optimization studies revealed that different media compositions and cultural conditions have significant impacts on the growth and antimicrobial activity of the strain CTF9: it exhibited promising growth on the culture media D, E and F; however, the maximum yields of the crude extracts were obtained from the medium D under the CC2 culture conditions (Table 1) and from the media E and F under the culture conditions CC1 and CC3 (Table 1). The similar impacts of different media on the growth demonstrate the ability of the strain CTF9 to use various nitrogen sources (including yeast extract, peptone, fish flour, *etc.*) and different carbon sources supplemented in these media. The impacts of other media, such as A, B and C, on the growth of the strain CTF9 was demonstrated by the low yield of the crude extracts, compared to those of the media D, E and F (Table 1). Different quantities of the crude extracts were obtained from the same medium under different culture conditions. In the media A, B, C and D, the culture conditions CC2 and CC4 showed significant impacts on the growth, compared to the culture conditions CC1 and CC3, whereas in the media E and F, the culture conditions CC1 and CC3 showed a significant impact on the growth, compared to CC2 and CC4, indicating the ability of the strain CTF9 to grow in a broad range of temperatures (28 to 35°C) and pH values (6.5 to 7.8). Studies were also conducted on the antimicrobial activity of the crude extracts of the strain CTF9, obtained from different media under different culture conditions, against the test organisms *Candida albicans* and *Mucor miehei*. In general, different media and culture conditions induced similar activities against the test organisms with minor variations, suggesting that the proportion of the active components in the unit weight of the crude extracts is similar. Based on the extensive investigations on different media compositions and culture conditions, we demonstrate that the maximum yield of the antifungal metabolites produced by the strain CTF9

(phenylacetic acid and indolyl-3-lactic acid) can be obtained by culturing the strain on the media D, E or F, at 35°C and pH 7.8.

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