

ANTIOXIDANT, ENZYME INHIBITORY AND ANTIFUNGAL ACTIVITIES OF ACTINOMYCETES ISOLATED FROM *CURCUMA LONGA* RHIZOSPHERE

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ABSTRACT

Objective: To screen the actinobacterial strains associated with root rhizosphere of *Curcuma longa* for antioxidant, enzyme inhibitory and antifungal potential and to identify the potent strain TRA-1.

Methods: The strains were isolated on starch casein agar (SCA). The antioxidant, enzyme inhibitory and antifungal activities were studied using standard methods. Identification of active strain TRA-1 was carried out by studying morphological and cultural characteristics and by employing 16S rRNA sequencing.

Results: The detected phenolic content and total antioxidant activities were in the range of 1µg/ml to 80 µg/ml and 0.198 to 1.088 each. Diphenyl picrylhydrazyl (DPPH) and hydroxyl radicals scavenging activity and capacity to chelate metal ion and reduce ferric iron were found in 11, 14, 6 and 12 strains respectively. The activities of α-amylase and α-glucosidase were inhibited by crude extracts of twelve and three isolates and the extracts of ten isolates inhibited lipase activity. Nine isolates inhibited the growth of one or more test fungal pathogens. All test pathogens were sensitive to TRA-1. The active strain TRA-1 was identified as *Streptomyces albus*. The active metabolite of TRA-1 showed 41.43% DPPH scavenging activity, 54.48% and 34.46% respective inhibition in α-amylase and lipase activity and was highly active against *C. neoformans* (35 mm) followed by *A. niger* (18 mm).

Conclusion: The results of the study showed remarkable antioxidant, enzyme inhibitory and antifungal potential of isolated strains. The potent strain *Streptomyces albus* TRA-1 can be used as a promising source for isolation of pharmaceutically important bioactive compounds.

Keywords: Antioxidant, Inhibition, Enzyme, Antifungal, *Streptomyces albus*.

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INTRODUCTION

In past few decades, the search for natural products derived from plants, animals and microorganisms has been intensified due to their applications in the treatment of numerous human related diseases. So far, more than a million natural products have been discovered by using various drug screening programs. More than 22,500 bioactive compounds have been extracted from microorganisms and among these, actinomycetes derived products contributes a major share (45%) followed by fungi (38%) and unicellular bacteria (17%) [1].

Actinomycetes are the most ecologically and biotechnologically valuable prokaryotes being responsible for the production of about half of the discovered bioactive metabolites including antibiotics [2], antitumor and immunosuppressive agents [3] and enzymes [4]. Recently, the antioxidant and anti-diabetic activities of secondary metabolites isolated from *Streptomyces* and *Nocardopsis* species have been reported [5]. *Streptomyces* sp are known as most promising candidates as they are producers of about more than 80% of secondary metabolites identified from actinomycetes. However, the immense biotechnological potential of actinomycetes has led to extensive search on isolates from normal terrestrial habitats and resulted in an associated increase in the number of compounds being rediscovered due to a high rate of redundancy in the strains isolated [6]. A mathematical model designed to estimate the number of undiscovered antimicrobial agents from the genus *Streptomyces* predicts that the decline of finding such agents is due to the declining efforts in screening more microorganisms and not due to a lesser number of undiscovered antimicrobial agents [7] and thus, the search for microorganisms and new bioactive compounds has gained importance. In this regard, the root rhizosphere soil of medicinal plants could be a better option of environment for isolation of actinomycetes with new biologically active metabolites.

Curcuma longa (Turmeric), categorized under Zingiberaceae family, is one of the important spice crops in India and extensively used as

therapeutic agent for over 6000 y in Ayurveda, Unani and Siddha medicinal systems. Rhizome portion of *C. longa* (L) is used as an antiseptic, antileukemic, and antihelminthic, anti bronchitic, candidicidal agent, as CNS stimulant, fungicide, pesticide and also shows antitumor, antiaging, antioxidant and antimutagenic activities. The characteristics of rhizome to display all these activities is due to the presence of an array of chemical substances including, cineole, hydroxymethyl anthraquinone, terpinol, turmerone, ascorbic acid, azulene, caffeic acid, caprylic acid etc. These substances are expected to be released in some extent during rhizodeposition and provide important substrates for the growth of associated soil microbial community. The microorganisms which are resistant to these substances are able to metabolize them during their growth and are expected to produce novel bioactive compounds.

Oxidative stress is a deleterious process generated due to excess production of reactive oxygen species [ROS] and plays important role in development of vascular complications in diabetes particularly in type II diabetes [8]. Under these situations the patients exhibits lower immune response, making them immunocompromised. Hence, apart from metabolic acute complications and systemic late complications, onset of many infections caused by opportunistic pathogens is commonly observed in patients suffering from diabetes. The synthetic antioxidants such as butylated hydroxy anisole (BHA) and butylated hydroxy toluene (BHT) are known to induce carcinogenic effects in living cell. The knowledge regarding emergence of new diseases and multiple drug resistance to human pathogenic bacteria and fungi, toxic nature of ROS, harmful side effects associated with available synthetic drugs and complications raised due to diabetes, have opened a new vista for the search of new antiradical, antidiabetic and antimicrobial agents from natural sources. The present research work describes isolation of actinomycetes from root rhizosphere of *C. longa* (L) and their screening for antioxidant, enzyme inhibitory and antifungal activities. The identification of potential actinomycete strain is also reported.

MATERIALS AND METHODS

Sample collection and isolation of actinomycetes

Rhizospheric soil was collected from the agricultural field having previous 15 y history of turmeric cultivation in Nanded, M. S. India. Briefly the plants were uprooted and the soil adhered to roots was collected in sterile polyethylene bags and stored at 4 °C until processed. For the isolation of actinomycetes, 10 g oven dried soil was suspended in 90 ml of sterile distilled water and used for preparing tenfold serial dilution. 0.1 ml of each dilution (10^{-3} to 10^{-7}) was spread in triplicates on SCA plates [9] supplemented with 50 µg/ml of each nystatin, cyclohexamide and tetracycline. The plates were incubated at 30 ± 2 °C for 7 d and fourteen morphologically distinct, well-isolated colonies of actinomycetes were selected for further study.

Source of bioactive substance from actinomycetes

Briefly, the selected actinomycetes were grown individually in soybean casein digest broth supplemented with 1% dextrose (Hi Media) on rotary shaker at 30 °C and 120 rpm for 7 d. After incubation, the broths were centrifuged at 10,000 rpm for 30 min at 4 °C. The supernatants were used as a crude source of metabolites from respective actinomycetes and tested for the presence of a compound with biological activity.

Screening of actinomycetes for antioxidant properties

The selected crude extracts of actinomycetes were screened for antioxidant compounds by using DPPH radical scavenging assay [10], determining total phenol content [11], total antioxidant activity [12], hydroxyl radical scavenging activity [13], ferrous reducing assay [14] and assay for metal chelation capacity [15]. Ascorbic acid was used as a standard antioxidant for DPPH, total antioxidant activity and hydroxyl radical scavenging assay, phenolic contents were determined using catechol as a standard and tannic acid and EDTA were used as positive controls for ferrous reducing assay and metal chelation assay respectively.

Screening of actinomycetes for presence of enzyme inhibitors

The *in vitro* α -glucosidase inhibition assay was used to test the inhibition activity of selected actinomycetes [16]. The assay mixture consisted of 100 µl phosphate buffer (0.1 M, pH 7.2), 100 µl p-Nitrophenyl- β -D-glucopyranoside (5 mM in 0.1M phosphate buffer, pH 6.9) and the 500 µl crude extract from different actinomycetes. The reaction was initiated after 5 min preincubation of assay mixture at room temp by adding the crude α -glucosidase solution from yeast (25 µl). This was followed by 10 min incubation at 28 °C. After incubation, the absorbance was recorded at 405 nm on microtitre plate reader and compared with the control (phosphate buffer solution in place of the crude extract). Acarbose was used as standard α -glucosidase inhibitor, and the intensity of inhibition (%) was calculated as follows:

$$\text{Inhibition (\%)} = \frac{A-B}{A} \times 100$$

Where, A is the absorbance of control at 405 nm and B is the absorbance in the presence of extract.

The assay mixture used for detecting the presence of α -amylase inhibitors involves 500 µl crude extracts of each isolate and 500 µl sodium phosphate buffer (0.02 M, pH 6.9) containing α -amylase solution (0.5 mg/ml). The reaction was initiated after pre incubation of 10 min at 37 °C by adding 500 µl starch (1% in 0.02 M phosphate buffer, pH 6.9). The whole reaction mixture was incubated for 10 min at 37 °C and the reaction was terminated by adding 1 ml dinitro salicylic acid reagent. The tubes were kept in boiling water bath for 5 min and cooled to room temp. The rate of inhibition was calculated by noting the amount of glucose released at 540 nm in presence and absence of extracts [17]. Acarbose was used as a standard α -amylase inhibitor and inhibition was determined as follows:

$$\text{Inhibition (\%)} = \frac{A-B}{A} \times 100$$

Where, A is the absorbance of control at 540 nm and B is the absorbance in the presence of extract.

Lipase inhibitory activity of crude extracts were tested by mixing 1000 µl of each extract, 3 ml of olive oil emulsion (5% olive oil emulsified with 10% gum arabic) and 1 ml lipase (from *A. niger*, Hi Media) solution (1.56 mg/ml) followed by 30 min incubation at 37 °C. The control was prepared by replacing crude extract with phosphate buffer from the reaction mixture. The fatty acids liberated were estimated by titrating contents of reaction mixture against 0.05 M NaOH and using phenolphthalein (0.1% ethanolic) as pH indicator [18]. Lipase inhibition activity (%) of extracts was calculated as follows:

$$\text{Lipase inhibition (\%)} = \frac{A-B}{A} \times 100$$

Where, A is the enzyme activity in control and B is the activity in the presence of extract.

Screening of actinomycetes for antifungal activity

Antifungal activity of crude extracts of selected actinomycetes was assessed by performing agar well diffusion assay. The test fungi (*Aspergillus niger*, *Aspergillus flavus*, *Cryptococcus neoformans* and *Trichophyton rubrum*) availed from School of life sciences culture facility centre, S. R. T. M. University Nanded, were inoculated in antifungal assay broth (Hi Media) and incubated for 48 h at 30 °C. 0.1 ml of active culture of each test fungus was spread on the surface of sterile potato dextrose agar and wells of 5 mm were made using sterile cork borer. 100 µl crude extract of each isolate was transferred into wells, and the plates were kept for incubation at 30 °C for 48–72 h. Nystatin (50 µg/ml) was used as positive control. Each test was performed in triplicate and antifungal activity was expressed as the mean of the diameter of zone of inhibition formed around the wells.

Identification of potential bioactive compound producer strain

The strain TRA-1 showing good antioxidant, enzyme inhibitory and antifungal activities was selected as a potential strain and was identified based on morphological characterization and using 16S rRNA sequencing. The inclined cover slip culture technique was adapted to study morphological and sporulation pattern of TRA-1 on SCA after growing at 30 °C for 7 d. National Bureau standards (NBS) colour name charts were used to record the colour of spore mass and aerial and substrate mycelia [19]. Chemotaxonomic studies relating type of amino acids and sugar content present in whole cell hydrolysates were performed as mentioned earlier [20, 21]. The strain TRA-1 was identified at the molecular level by 16S rRNA sequencing after isolating the DNA using phenol-chloroform extraction method. The primers used for PCR analysis were 8 F: 5'AGAGTTT GATCCTGGCTCAG-3' and 907 R: 5'CCGTC AATT CMTTTRAGTTT-3'. The PCR amplification conditions used for the study were 94 °C for 3 min, 53.2 °C for 50 s, 72 °C for 60 s and final extension step of 71.5 °C for 3 min. The PCR product was purified by PEG-NaCl method and sequenced on ABI 3730XL sequencing machine. The forward and reverse sequences were assembled through DNA Baser V35.0 software and aligned with other closely related taxa retrieved from GenBank database. Comparison of 16 S ribosomal RNA gene sequence of TRA-1 and other known bacterial sequences was done using NCBI BLASTn. The reference sequences required for comparison were downloaded from <http://www.ncbi.nlm.nih.gov/genebank>. The sequence alignment was done by using CLUSTAL W, a multiple sequence alignment programs, gaps were removed manually and pairwise evolutionary distances were computed using the Kimura 2-parameter model [22]. The bootstrap analysis was done using 1000 replicates of original dataset and used for constructing the phylogenetic tree using Neighbor-joining method [23].

RESULTS AND DISCUSSION

Root rhizosphere soil is nutritionally rich niche providing different growth factors to associated microflora. Isolation of various groups of actinomycetes from the rhizosphere of different plant species have been reported previously [24]. Turmeric is an important member of Zingiberaceae family that have proved its immense potential in treating various ailments and has antitumor, anti aging, antioxidant and antimutagenic activities. In the present study, 14 strains of actinomycetes isolated from root rhizosphere of *C. longa* were selected for screening various biological activities.

Antioxidant activities of actinomycetes

Radical scavenging ability of antioxidant compounds was evaluated extensively by using stable free radicals containing DPPH molecule. Of the total isolates selected for study, ten showed DPPH scavenging activity and was found in a range of 12.15% to 69.88 %. The strain TRA-3a showed the highest scavenging activity whereas it was least found in strain TRA-30. However, the recorded activities of all strains were lower than the ascorbic acid (100 µg/ml, 87.83%) which was used as a standard antioxidant compound. (fig. 1)

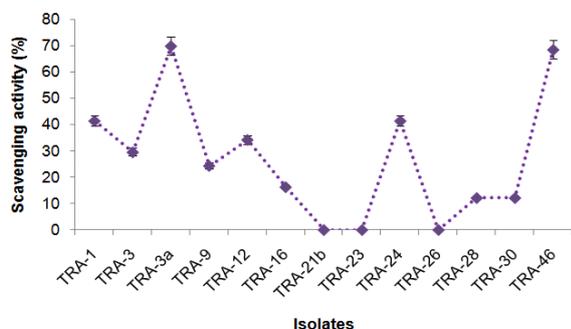


Fig. 1: DPPH radical scavenging activity of actinomycetes isolated from *C. longa* root rhizosphere. Each value represents mean±standard deviation (SD) of three independent replicates. Error bar indicates SD

The results on detected phenol content and total antioxidant activities in crude extracts of selected actinomycetes are shown in fig.2. The antioxidant properties of bioactive compounds are mainly due to their redox properties that play an important role in absorbing and neutralizing free radicals [25]. The phenolic content found in crude extracts of selected actinomycetes was in the range of 1µg/ml to 80 µg/ml. The higher phenol content and antioxidant activity were observed in TRA-26 extract. Phenol content in antioxidant compounds is positively correlated with their antioxidant activities [26] and also acts as a key element for deciding the antioxidant efficacy of secondary metabolites [27]. The higher total antioxidant activity found in strain TRA-26 also confirmed the interdependence of phenolic content and antioxidant capacity of a secondary metabolite.

The scavenging of hydroxyl radical was more in extracts of strain TRA-21. The activities were moderate in TRA-1 and TRA-3a extracts, whereas least observed in strain TRA-46 as shown in fig. 3.

The reducing power of selected actinomycetes extracts was examined in terms of their ability to reduce Fe³⁺ to Fe²⁺ and the results are presented in fig.4. Of the total isolates, 11 showed the capacity to reduce ferric ion as indicated by an increase in absorbance at 700 nm over the control. The strain TRA-1 showed highest reducing power followed by TRA-26 and TRA-3. The least activity was recorded in strain TRA-12 while it was absent from strains TRA-9 and TRA-28.

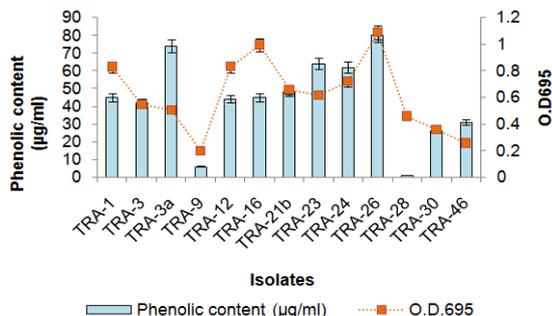


Fig. 2: Phenol content and total antioxidant activity of actinomycetes isolated from *C. longa* root rhizosphere. Each value represents mean±SD of three independent replicates. Error bar indicates SD

The reports on metal chelation capacity of actino bacterial secondary metabolites were least found in published literature. In the present study, activity was studied in terms of chelation of iron and was found only in six extracts (fig.5). The chelation capacity was more in TRA-46 which was comparable to standard chelating agent EDTA (60%, 100 µg/ml). Iron chelators play an important role to relieve the oxidative stress generated due to iron overload. The accumulation of toxic iron concentration can cause tissue damage leading to heart failure, hyperthyroidism, liver failure and early death [28].

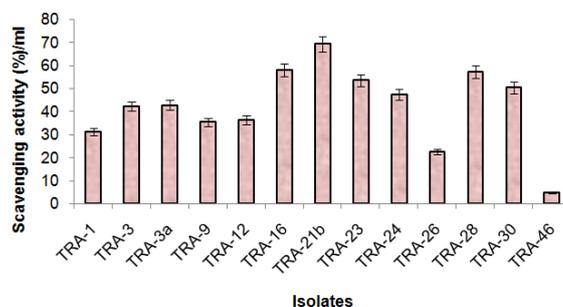


Fig. 3: Hydroxyl radical scavenging activity of actinomycetes isolated from *C. longa* root rhizosphere. Each value represents mean±SD of three independent replicates. Error bar indicates SD Ascorbic acid (500 µg/ml) showed 80.64% radical scavenging activity

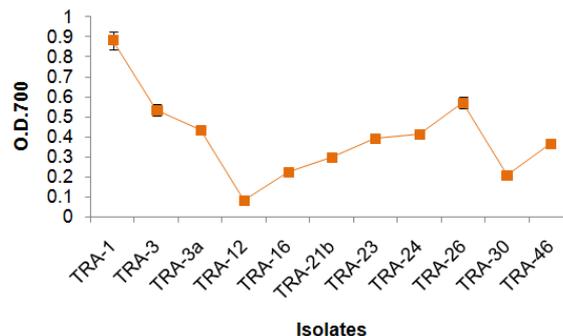


Fig. 4: Reducing the power of actinomycetes isolated from *C. longa* root rhizosphere. Each value represents mean±SD of three independent replicates Error bar indicates SD The reducing activity of tannic acid (50 µg/ml) in terms of O. D.700 was 1.343

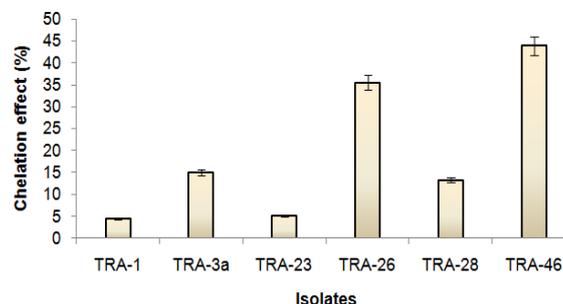


Fig. 5: Metal chelation capacity of actinomycetes isolated from *C. longa* root rhizosphere. Each value represents mean±SD of three independent replicates. Error bar indicates SD Enzyme inhibitory activities of actinomycetes

The results of α-glucosidase, α-amylase and lipase inhibition assay for selected actinomycetes are shown in fig.6. The crude extract of TRA-26 showed a significant inhibition in α-glucosidase activity

followed by TRA-12 and TRA-1 extracts. Similarly, the activity of α -amylase was reduced greatly in the presence of extracts of TRA-30, TRA-28 and TRA-46 respectively. α -amylase activity inhibition was moderate in TRA-1, TRA-16 and TRA-21 extracts whereas it was least observed in strain TRA-3a. The enzymes α -glucosidase and α -amylase break down the carbohydrate into absorbable monosaccharides [29]. The inhibitor of these enzymes delays the absorption of ingested carbohydrates and thus lowers the rise in blood glucose level. Hence, such compounds are good drug targets for reducing the disorders such as diabetes mellitus.

Lipases play a significant role in the development of obesity leading to type II diabetes and cardiovascular diseases. Lipase inhibitors bind to intestinal lipases [30] preventing the hydrolysis of dilatory triglycerides to mono glycerides and fatty acids [31]. This reduces the absorption of fats resulting in lessened obesity. In the present study, extracts of nine isolates showed lipase inhibition activity with the highest inhibition found in the presence of TRA-9 extract (65%). Orlistat was used as a standard lipase inhibitor. Although lower than orlistat (100 μ g/ml, 80.92%) and studied at primary level, the inhibition shown by TRA-9 crude extract was noticeable.

Antifungal potential

The antifungal activities of selected strains of actinomycetes are shown in table 1. Of the 14 isolates, TRA-16 and TRA-1 highly inhibited the growth of *A. niger* and *A. flavus* respectively. *C. neoformans* was sensitive to three isolates with the highest zone of inhibition (35 mm) produced in the presence of strain TRA-1 extract (fig.7). *T. rubrum* was inhibited by only one isolate with maximum inhibition (13 mm) caused by strain TRA-1.

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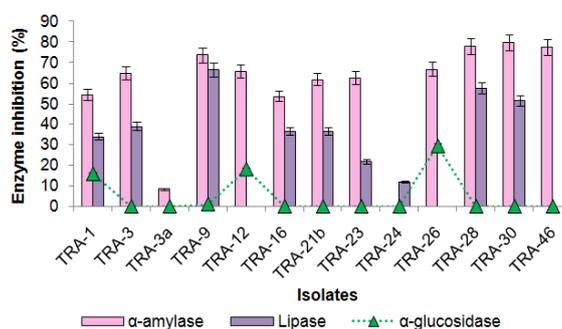


Fig. 6: Enzyme inhibitory activities of actinomycetes isolated from *C. longa* root rhizosphere. Each value represents mean \pm SD of three independent replicates. Error bar indicates SD Acarbose (100 μ g/ml) showed 76.25% and 62.59% inhibition in α -amylase and α -glucosidase activity. Orlistat (100 μ g/ml) showed 80.92% inhibition in lipase activity

Table 1: Antifungal efficacy of crude extract of selected isolates, each value represents mean \pm SD of three independent replicates

Isolate	Name of the test pathogen/Zone of inhibition (mm)			
	<i>A. niger</i>	<i>A. flavus</i>	<i>C. neoformans</i>	<i>T. rubrum</i>
TRA1	18 \pm 1.2*(78.26)	12 \pm 0.69(70.58)	35 \pm 0.71(87.5)	13 \pm 0.165(72.22)
TRA3	6 \pm 0.29(54.54)	8 \pm 0.13(61.53)	10 \pm 0.19(66.66)	0.00(0)
TRA16	29 \pm 0.49(85.29)	6 \pm 0.26(54.54)	0.00(0)	0.00(0)
TRA21	14 \pm 0.83(73.68)	0.00(0)	0.00(0)	0.00(0)
TRA24	10 \pm 0.77(66.66)	8 \pm 0.49(61.53)	0.00(0)	0.00(0)
TRA26	6 \pm 1.47(54.54)	0.00(0)	0.00(0)	0.00(0)
TRA30	10 \pm 0.32(66.66)	0.00(0)	0.00(0)	0.00(0)
TRA54	9 \pm 0.94(64.28)	0.00(0)	4 \pm 0.03(44.44)	0.00(0)
Nystatin	22.4 \pm 0.69(76.19)	18.9 \pm 2.24(79.07)	19.3 \pm 1.75(79.42)	17.2 \pm 0.34(77.47)

*Fig. in parenthesis represents percent antifungal activities.

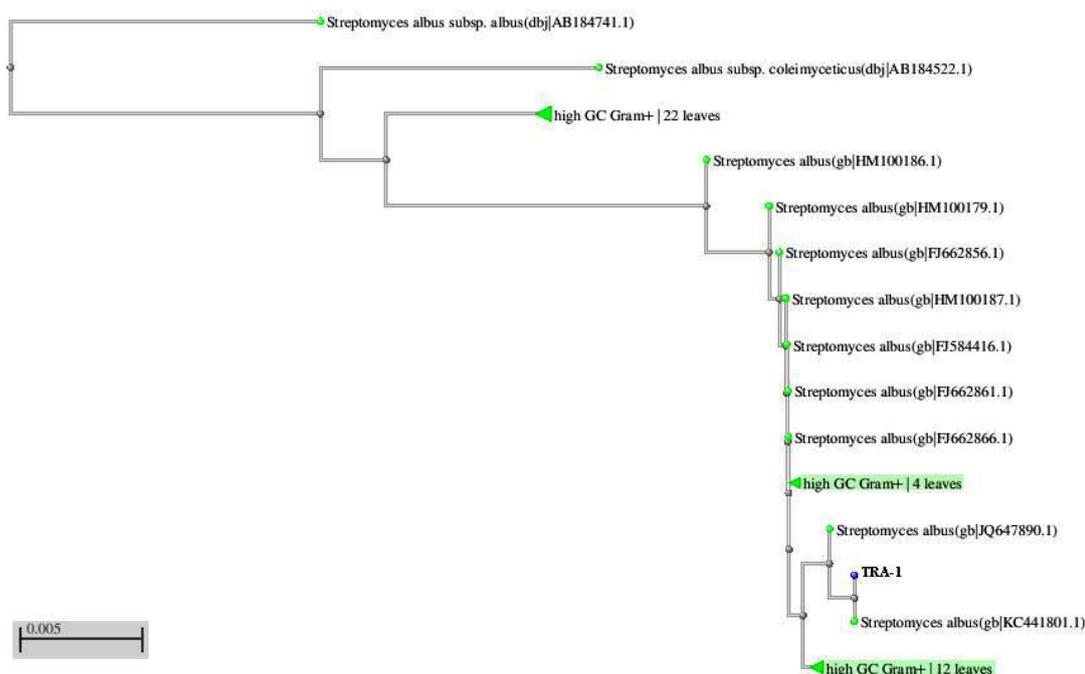


Fig. 8: Phylogenetic tree based on 16S rRNA gene sequence showing taxonomic relatedness between TRA-1 and related members of genus *Streptomyces*

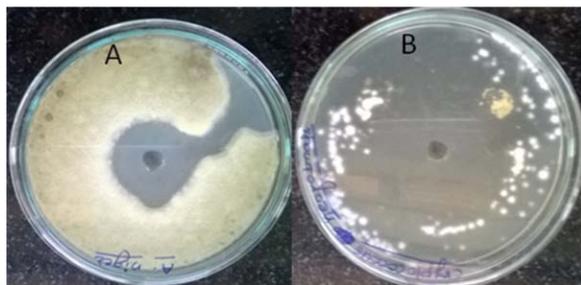


Fig. 7: Antifungal activity of TRA-1 extract against *A. niger* (A) and *C. neoformans* (B) by agar well diffusion method

Characterization of TRA-1

The actinomycetes strain TRA-1 isolated from the rhizosphere of *C. longa* was identified as *Streptomyces* genus according to its ability to form substrate and aerial mycelium and form spiral spore chains. The isolate produced colonies with light pink spore mass on SCA with pale yellow reverse. The cell wall chemo type I was designated by observing the presence of LL-Diaminopimelic acid and without any characteristic sugar pattern in cell wall hydrolysate. Further characterization of TRA-1 was done by 16S rRNA analyses. The isolate showed 99% homology with existing species of *S. albus* (KC441801.1). The taxonomic position of the isolate is shown in fig. 8.

CONCLUSION

In the present study 14 actinomycetes, strains associated with root rhizosphere of *Curcuma longa* have been screened for antioxidant, enzyme inhibitory and antifungal potential. All the isolated strains have the capacity to produce one or more biologically active substances. The potent strain *Streptomyces albus* TRA-1 with abilities to produce antioxidant, enzyme inhibitory and antifungal compounds could be used as a promising strain for the development of pharmaceutically important drugs.

CONFLICT OF INTERESTS

All authors have no conflict to declare.

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