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Actinomycetes from the Coffee Plantation Soils of Western Ghats: Diversity and Enzymatic Potentials

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A B S T R A C T

230 soil actinomycetes were isolated from the coffee plantation of Western Ghats, Karnataka, India along the altitudinal gradients and depths. 24 morphologically distinct species were obtained based on the aerial spore chains and by the sequencing of the 16S rRNA gene. The strains were assigned to the order Micrococcales, and novel orders Pseudonocardiales *ord. nov*., Streptomycetales *ord. nov*., and Streptosporangiales *ord. nov*. The frequently isolated genus was *Streptomyces*, along with rare actinomycetes *Actinomadura*, *Spirillospora*, *Actinocorallia*, *Arthrobacter*, *Saccharopolyspora* and *Nonomuraea*. This study is the first report on *Nonomuraea antimicrobica* as a soil actinomycete. Diversity studies on the distribution of soil actinomycetes indicated significant differences ($P < 0.05$) among Shannon diversity indices of sample group depths along the slope. An attempt was made to correlate the total actinomycete count with soil parameters, by PCA based multiple linear regression (MLR) which significantly correlated ($P \leq 0.0001$) with pH, moisture, available nitrogen and phosphorous. About 91.6% of the isolates screened were found to be potentials for enzymatic activity. The most active enzyme producer *Streptomyces* sp. MH470335 produced 18.51, 1.53, 6.92, 5.62 and 5.15 U/ml for cellulase, pectinase, xylanase, amylase and protease respectively. Plantation soil actinomycetes showing enzymatic activities *in vitro* may indicate the potential for their use as stabilized biocatalysts.

Introduction

Plantation ecosystems are a potential niche for microorganisms. They play a significant role in decomposing and transforming wide variety of complex organic residues in the plantation soils derived from the fallen crop residues and from shade trees. Plantation soil supports actinomycete populations (George *et al.*, 2012) that help to decompose various biomolecules by producing extracellular enzymes. The actinomycetes are aerobic, filamentous Gram-positive bacteria with high G+C content in their DNA.

Bioprospection of underexplored ecosystems have been proven as useful habitats for exploiting numerous bioactive metabolites from novel actinomycetes (Shah *et al.*, 2017). Actinomycetes are known for the production of extracellular enzymes with applications in agriculture and industries (Mukhtar *et al.*,

2017). Though, soil actinomycetes are preferred for their novel bioactive potentials, these organisms have not been explored at greater depths from plantation areas.

Few actinomycetes from plantation regions are potentially rich sources of antimicrobial compounds (Manikkam *et al.* 2014). There are a few reports on the actinomycete diversity from plantation areas, especially from the coffee plantation areas of the southern Western Ghats. Nevertheless, studies are focused to improve the soil quality parameters for coffee productions and estimating the microbial diversity based on shade and open tree canopy types (Velmourougane, 2017). There is no proper documentation on the systematic identification of actinomycetes based on the colony, sporangial characters and 16 S rRNA sequences.

Therefore, the study area was selected in the coffee plantation area of Chikmagalur, southern India which is so far unexplored for the actinomycete isolations and diversity studies. Coffee (*Coffea arabica* L.) is an important plantation crop, cultivated commercially in high altitude regions of southern India.

The heterogeneous tree populations here not only provides a regulated shade system to the coffee canopy, but the characteristic leaf and fruit shedding along with the crop residues favor the buildup of diverse microorganisms (Bagyaraj *et al.*, 2015).

The objectives of this study were focused on the isolation and characterization of soil actinomycetes and studying their interaction with physicochemical properties of soil along the elevation and soil depth gradients in the coffee plantation area and as well to determine their enzymatic potentials.

Materials and Methods

Study site description and sampling

The study was conducted in the coffee plantation area of the Chikmagalur region (13.4333^0N to 75.7500^0E) of Western Ghats, southern India (Fig. 1a) situated at an elevation of 1000 m above mean sea level. The mean temperature and rainfall documented in the study site ranges from 13ºC to 35ºC and 15000 to 20000 mm respectively. To accomplish the aim of the present study, an altitudinal transect with same environmental conditions, except slope positions were determined with an approximate area of five hectares, under cultivation. The area under study was divided into three parts: toe slope (base), back slope (mid) and the summit (top) (Fig. 1b). In each part, two soil profiles *viz.* surface soil (5-15 cm) and sub-surface soil (15-30 cm) was sampled. Five typical major plots (10 m x 10 m) were selected at 0.5 km intervals within the study area. Each major plot was divided into five minor plots of 1m x 1m dimension selected through the five point method (Zhang *et al.*, 2014). Five soil samples were randomly collected from these plots, pooled as composite sample and were air dried at room temperature (25 ºC±2) and preserved in zip locked polyethylene bags. All the soil samples were collected in triplicates.

Isolation and molecular characterization of actinomycetes from coffee plantation soils

Isolation of actinomycetes from soil samples

The isolation of soil actinomycetes was carried out by suspending one gram of dry soil in 100 ml of distilled water. Serial dilutions of soil samples (up to 10^{-5}) were done aseptically and 100 µl suspensions of each dilution were spread evenly over the surface of starch casein agar (SCA) medium

in triplicates supplemented with cycloheximide (100 µg/ml), nystatin (100 µg/ml) and nalidixic acid (50 µg/ml) (Himedia®, Mumbai, India) by the spread plate technique (Kumar *et al.,* 2014). The plates were incubated at 28 °C±2 for two to four weeks. The actinomycete colonies on the plates were counted for each dilution and colony forming units per gram of soil was calculated. The colonies were individually isolated by streak plate technique for purification on ISP2 (International Streptomyces Project type-2, Himedia®, India, 41 g/l) media. The pure cultures were transferred to ISP2 agar slants and maintained at 4 °C for further studies and glycerol (20% v/v) stocks at -20 ºC.

Identification of soil actinomycetes

The purified isolates were identified by morphological characteristics such as the colony morphology and growth pattern under stereo zoom microscope (Lawrence & Mayo®, India). The isolates were observed for the substrate / aerial mycelium and spore chains in methylene blue stain and observed under bright field microscopy (Quasmo™, India) using 100x oil immersion objective. The representative isolates were identified based on Bergey's Manual of Systematic Bacteriology (Goodfellow *et al*. 2012).

Molecular characterization of the isolates involved the extraction of genomic DNA and amplification of 16S rRNA gene by the universal primers 27F and 1492R according to the procedure of Akshatha *et al.* (2014) using Genomic bacterial DNA isolation kit and PCR kit respectively (Chromous Biotech® Pvt. Ltd., Bangalore, India). The sequences of isolates were aligned for the similarity and homology against the reference sequences using the BLAST®>>blasting site provided by NCBI and submitted to the NCBI GenBank submission portal to obtain the accession numbers.

Physico-chemical characteristics of soil

Soil color was determined by Munsell® soil color charts. Soil moisture content and pH were measured gravimetrically and potentiometrically respectively. The soil organic carbon and available nitrogen (Microkjeldahl method), phosphorus (Bray and Kurtz method) and potassium (neutral normal ammonium acetate extraction method) in the samples were analyzed by standard methods (Jones, 2001).

Screening of actinomycete isolates for enzymatic potentials

The actinomycete isolates were screened for their ability to produce extracellular enzymes such as cellulase, xylanase, pectinase amylase and proteases. All the isolates were subjected to the primary screening method. The isolates were inoculated on a suitable medium containing specific substrate (cellulose, pectin, xylan, starch and skimmed milk) by the spot inoculation method followed by incubation for five days. The plates were observed for clear zones surrounding the colonies on agar plates and were measured (Lekshmi *et al.,* 2014). The strains exhibiting positive enzyme activity were selected for secondary screening by shake flask fermentation method (Lekshmi *et al.,* 2014).

Cellulase, pectinase, xylanase and amylase enzyme activities were determined by 3, 5-dinitrosalicylic acid (DNS) assay (Miller, 1959). The universal protease activity assay was used to measure the proteolytic activity, using casein as the substrate (Suthindhiran *et al.,* 2013). The amount of glucose, xylose, polygalacturonic acid, maltose and tyrosine released into the filtrates were measured from the respective standard curves and the enzyme activities were calculated and represented. The specific activity was expressed as enzyme units mg protein^{-1}. The protein content was estimated by Lowry's method

(Lowry *et al.*, 1951) with the protein standard, bovine serum albumin (1 mg /ml). The enzyme activities were expressed in terms of international units (IU). One IU was the amount of enzyme required to release one micromole of substrate (reducing sugar/tyrosine) equivalents in one milliliter of enzyme solution in one minute.

Statistical analysis

The statistical significance of mean differences was determined by the one-way variance of analysis (ANOVA) using SPSS statistical software (version 20.0 for Windows, SPSS, Chicago, IL, USA).

The correlation co-efficient analysis between physico-chemical parameters of soil samples and actinomycetes population relating to two soil profiles along the slope were performed using with principal component analysis (PCA) using PAST (PAleontological STatistics) statistical software version 3.20. The suitability of dataset for PCA was assessed by calculating the correlation coefficients between variables, the determinant of the correlation matrix, KMO measure of sampling adequacy and Bartlett's test of sphericity. The diversity indices measured by Shannon index, Shannon evenness and species richness were performed by PAST. Statistical analysis for enzymatic potentials were studied using analysis of variance (ANOVA) and means were compared for significance using Duncan's multiple Range Test ($P < 0.05$).

Results and Discussion

Isolation and molecular characterization of actinomycetes from coffee plantation soils

A total of 230 actinomycetes (consisting of 24 isolates) were isolated from the plantation study site. The total actinomycete counts in each soils sampled from the toe slope to the summit ranged from $8.30x10^3$ to1. $36x10^6$ cfu/gof dry soil, depth-wise along the slope (Fig. 2a). Maximum isolates were obtained in the back-slope region followed by, toe slope and summit at the surface soil layer. At the sub-surface soil layer, highest count was in the back-slope followed by summit and toe slope (Fig. 2b). These investigations are in compliance with the findings of Velmourougane *et al.* (2017) who documented a similar trend in the distribution of actinobacteria in coffee agroforestry systems and reported that high elevation favored more number of microorganisms than lower elevations. On contrary, Krishna *et al.* (2012) observed that actinomycete populations showed a decreasing trend with increasing depth of soil.

Coffee plantation thrives in well-drained soils rich in humus. It flourishes under a mixed shade canopy of evergreen trees comprising of *Erythrina, Ficus, Artocarpus, Grevillea* etc. The litter composed of dry leaves of coffee and shade trees which forms primary sources of soil organic matter. The litter deposited favors rich soil organic matter and microbial populations (Martins *et al.*, 2018). Therefore, in this study, sampling of such nutrient rich soils would have favored the isolation of soil actinomycetes.

Based on molecular characterization, of 24 actinomycete isolates, 54.2% were identified as *Streptomyces* sp. and 45.8% as non-streptomycetes or rare actinomycetes (Fig. 3**)**. The colony characteristics on ISP2 media, spore morphology and GenBank accession numbers with percent similarity are depicted in Table 1. The isolates comprised of seven genera representing four orders (Micrococcales, Pseudonocardiales *ord. nov*., Streptomycetales *ord. nov*., Streptosporangiales *ord. nov*.). Actinomycetes *Nocardia, Micromonospora, Streptomyces, Rhodococcus* and *Streptosporangium* are reported from rubber and teak plantation soils

based on physiological characterization (George *et al.,* 2012).

Actinomycetes were identified as Streptomycetes and non-streptomycetes from the rubber and coffee plantation soils of Kerala, India based on the phenotypic characteristics (Manikkam *et al.,* 2014). Similar studies were reported from oil palm plantation, Malaysia (Zain *et al.,* 2014; Shariffa-Muzaimah *et al.,* 2015), mulberry and banana plantations (Kawuri, 2016).

Diversity studies on the distribution of soil actinomycetes along the altitudinal gradient

The species diversity values determined using the Shannon index was compared with the Wilcoxon signed-rank test. There was a significant difference ($P < 0.05$) among the Shannon diversity indices of sample group depths along the slope. The range of biodiversity indices of all sampling points is depicted in Table 2. The frequently isolated genus was *Streptomyces*, followed by rare actinomycetes *Actinomadura*, *Spirillospora*, *Actinocorallia*, *Arthrobacter*, *Saccharopolyspora* and *Nonomuraea*.

The frequency of the genera *Streptomyces* and *Actinomadura* was 50% and 32.2%, respectively, whereas, the other genera such as *Spirillospora* (6.32%), *Actinocorallia* (5.08%), *Arthrobacter* (3.3%) and *Nonomuraea* (2.1%) recorded low frequency and *Sachharopolyspora* (0.8%) showed very low frequency. Two genera viz., *Saccharopolyspora* and *Nonomuraea* were found exclusively on summit of both soil profiles. Conversely, *Arthrobacter* on toe slope of both the soil profiles.

The genera, *Spirillospora* and *Actinocorallia* were found in the back slope and summit of both the soil profiles. The species diversities of *Streptomyces* among the back slope isolate were significantly higher ($P < 0.05$) than those of the toe slope isolates and summit isolates. On the contrary, diversity of *Actinomadura* species among back slope isolates was significantly higher ($P < 0.05$) than that of the summit and toe slopes.

So far, no attempt has been made to identify and assign the actinomycetes isolated from the plantation soils to particular order/family/taxa through a systematic approach. Identifications were based on the morphological and sporangial characteristics. This is the first comprehensive report on the identification of plantation soil actinomycetes by 16s rRNA approach. This study is important in reporting *Nonmuraea antimicrobica* as a soil actinomycete, which is otherwise reported as an endophyte from a Chinese medicinal plant (Qin *et al.,* 2009).

Correlation of physico-chemical soil characteristics and total actinomycete count by principal component analysis (PCA)

In this analysis, six sample groups were assessed along eight variables *viz.* pH, soil moisture content (SMC), electrical conductivity (EC), organic carbon (OC), available nitrogen (AN), available phosphorous (AP), available potassium (AK) and total actinomycete count (TAC) for generating PCA biplot. The PCA was used to deduce the correlation between physicochemical parameters of the soil and total actinomycete counts relating to two soil profiles.

The Kaiser–Meyer–Olkin (KMO) measure of sampling efficacy (0.608) and Bartlett's test of sphericity (X^2=88.460, df =21, P<0.0001) showed the suitability of dataset for PCA application. Figure 4 represents the PCA bi-plot with two principal components PC1 and PC2 with 44.571% and 21.093% of variance respectively.

Table.1 Colony characteristics and GenBank accession numbers of actinomycete strains from the coffee plantation soils

Isolate Code	Actinomycete Strain	Accession no.	Similarity %	Colony Characteristics		
				Color	**Pigments**	**Spore characteristics***
CMCS 01	*Streptomyces coelicolor*	KY492192	95	Yellow	Brown	Rectiflexibiles (smooth)
CMCS 02	*Streptomyces hebeiensis*	KY498018	95	Deep grey-yellow-brown	Deep yellow brown	Rectiflexibiles (warty)
CMCS 03	*Streptomyces atrovirens*	KY498019	98	Grey	Greyish green	Spirales (hairy)
CMCS 04	*Streptomyces olivaceous*	KY511125	100	Greyish yellow	Olive colored	Spirales (smooth)
CMCS 05	*Streptomyces spectabilis*	KY555725	100	light reddish brown	Reddish orange	Rectiflexibiles (smooth)
CMCS 06	*Streptomyces clavuligerus*	KY555726	100	Light greyish white	-	Rectuiflexibiles (smooth)
CMCS 07	*Streptomyces griseoplanus*	KY563319	100	Greyed yellow	-	Spirales (Warty)
CMCS 08	*Arthrobacter viscosus*	KY563320	99	Light pink	-	Spherical
CMCS 09	*Streptomyces longisporoflavus*	KY563321	100	Colorless	-	Spirales/retinaculi aperti(smooth)
CMCS 10	*Nonomuraea antimicrobica*	MH392729	100	Brown	-	Spiral spore chains
CMCS 11	*Streptomyces violaceolatus*	MH393284	99	Grey	Blue/violet	Spirales (smooth)
CMCS 12	*Streptomyces rubrogriseus*	MH393288	100	Raspberry red	-	Spirales (smooth)
CMCS 13	*Streptomyces chattanoogensis*	MH393287	100	Pale white	-	Spirales/retinaculiaperti (spiny)
CMCS 14	*Streptomyces mutabilis*	MH393285	100	White	-	Aberrant Spirales/retinaculi aperti (smooth)
CMCS 15	*Actinomadura nitritigenes*	MH470330	99	Brown Mahagony	-	Chain of spore (smooth)
CMCS 16	*A. montaniterrae*	MH470332	99	Dark greyish yellow	Greyish greenish yellow	A chain of four spores (rough)
CMCS 17	*Actinomadura* sp.	MH470334	100	Leathery white	-	Spore chains hooked (smooth)
CMCS 18	*Streptomyces* sp.	MH470335	100	Grey	greyish	Spirales
CMCS 19	*Actinomadura apis*	MH470336	100	Colorless	-	Chain of 3-5 spores (warty)
CMCS 20	*Spirillospora albida*	MH470337	99	Buffy pink	Pale yellow	Spherical spore vesicles
CMCS 21	*Actinomadura rifamycini*	MH472581	100	Brown	Brown pigment-	Chain of 3-8 spores (warty)
CMCS 22	*Saccharopolyspora hattusasensis*	MH472618	100	Pale white	-	Long chain of spores arranged in open loops
CMCS 23	*Actinomadura namibiensis*	MH478193	99	Salmon pink	-	Spiral spore chains (smooth)
CMCS 24	*Actinocorallia libanotica*	MH489087	100	Greyish	-	Spore chains in hooks (warty)

* Based on the Bergey's manual of systematic bacteriology (Goodfellow *et al.* 2012)

Table.2 Species diversity of soil actinomycetes depth wise along the altitudinal gradient

Diversity Indices	TS_s	Su_s	BS_s	TS_ss	Su_ss	BS_ss
Taxa_S	3	6	4	2	6	4
Individuals	35	35	37	17	54	58
Dominance_D	0.7453	0.2718	0.4215	0.5848	0.3951	0.4762
Simpson_1-D	0.2547	0.7282	0.5785	0.4152	0.6049	0.5238
Shannon_H	**0.5063**[a]	**1.501**[b]	**1.015**[c]	**0.6058**[d]	**1.256**[e]	**0.867**[f]
Evenness_e^H/S	0.553	0.7478	0.6902	0.9164	0.5852	0.595
Brillouin	0.4284	1.293	0.8934	0.5136	1.115	0.7897
Fisher_alpha	0.7855	2.084	1.139	0.5887	1.727	0.975
Berger-Parker	0.8571	0.4286	0.5405	0.7059	0.5926	0.5862

P< 0.05 different letter indicates the significant difference by Wilcoxon signed rank test, TS-toeslope, Su-summit, BS-backslope, _s- Surface soil, _ss- subsurface soil

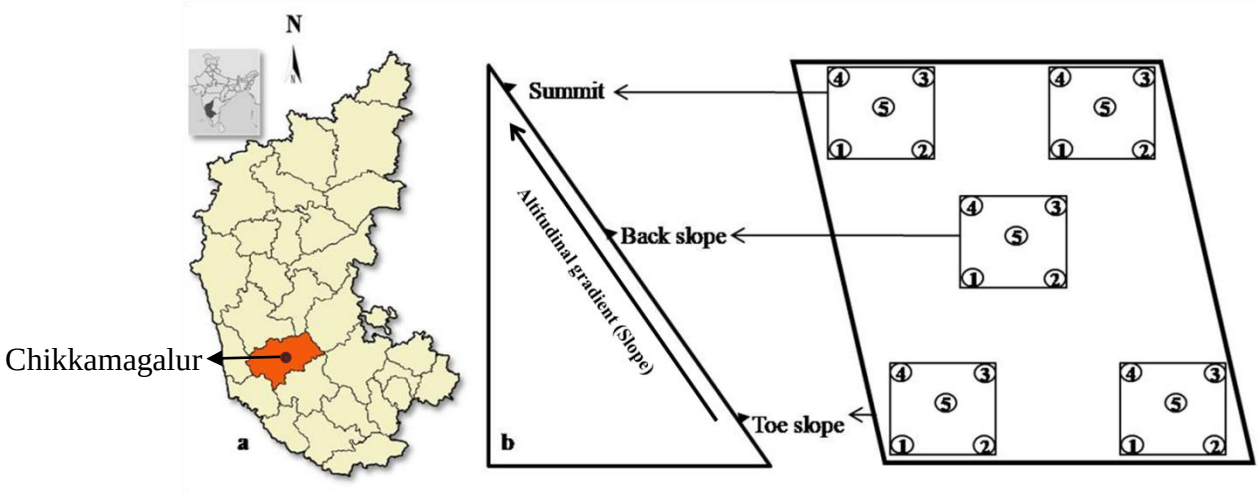
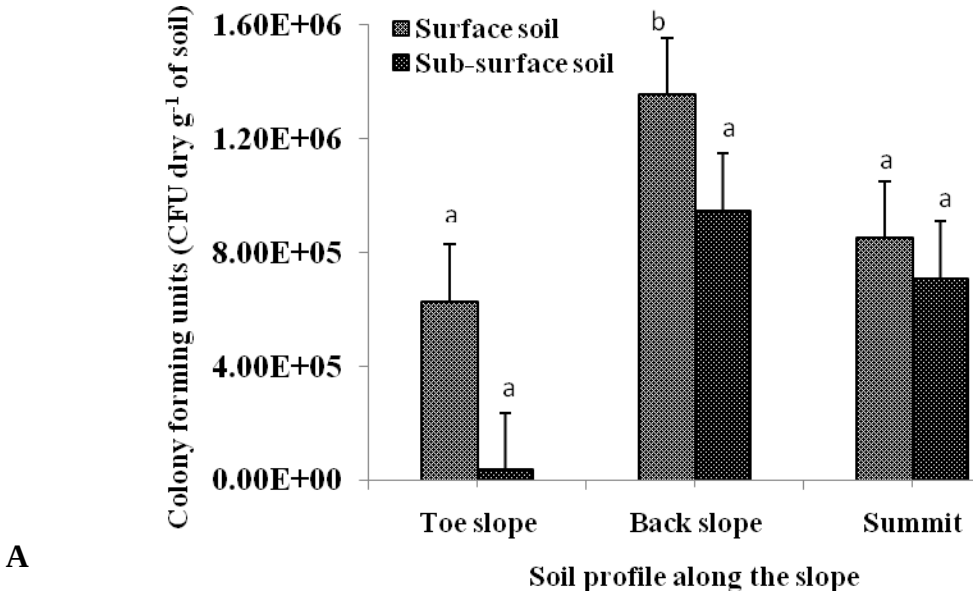
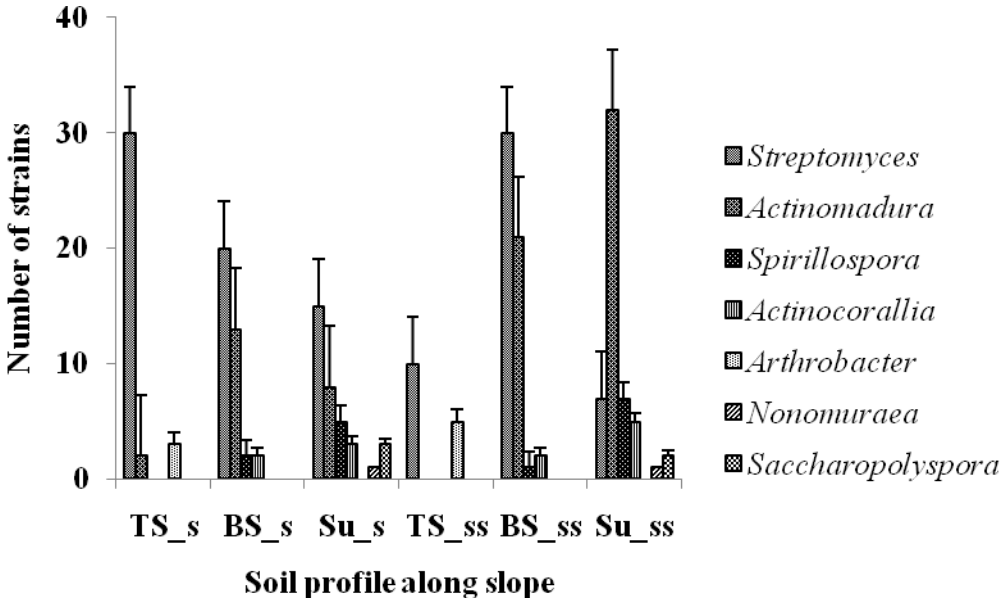


Figure 1 Study Area of coffee plantation soil sampling site in Chikmagalur, Karnataka, India **a.** Map of Karnataka with Chikkamagalur, the plantation sampling site **b.** Five point sampling method with altitudinal gradient



A



B

Figure 2 Actinomycetes recovered from the plantation sampling site along the altitudinal gradient and soil depth

A. Population (mean±SD) of actinomycetes at two soil profiles along the altitudinal gradient. Different letters differ significantly (P<0.05) based on Duncan's Multiple range test

B. Number of actinomycete strains in the soil profiles along an altitudinal gradient TS- Toe slope, BS – Back slope, Su- Summit, s-surface, ss-sub surface

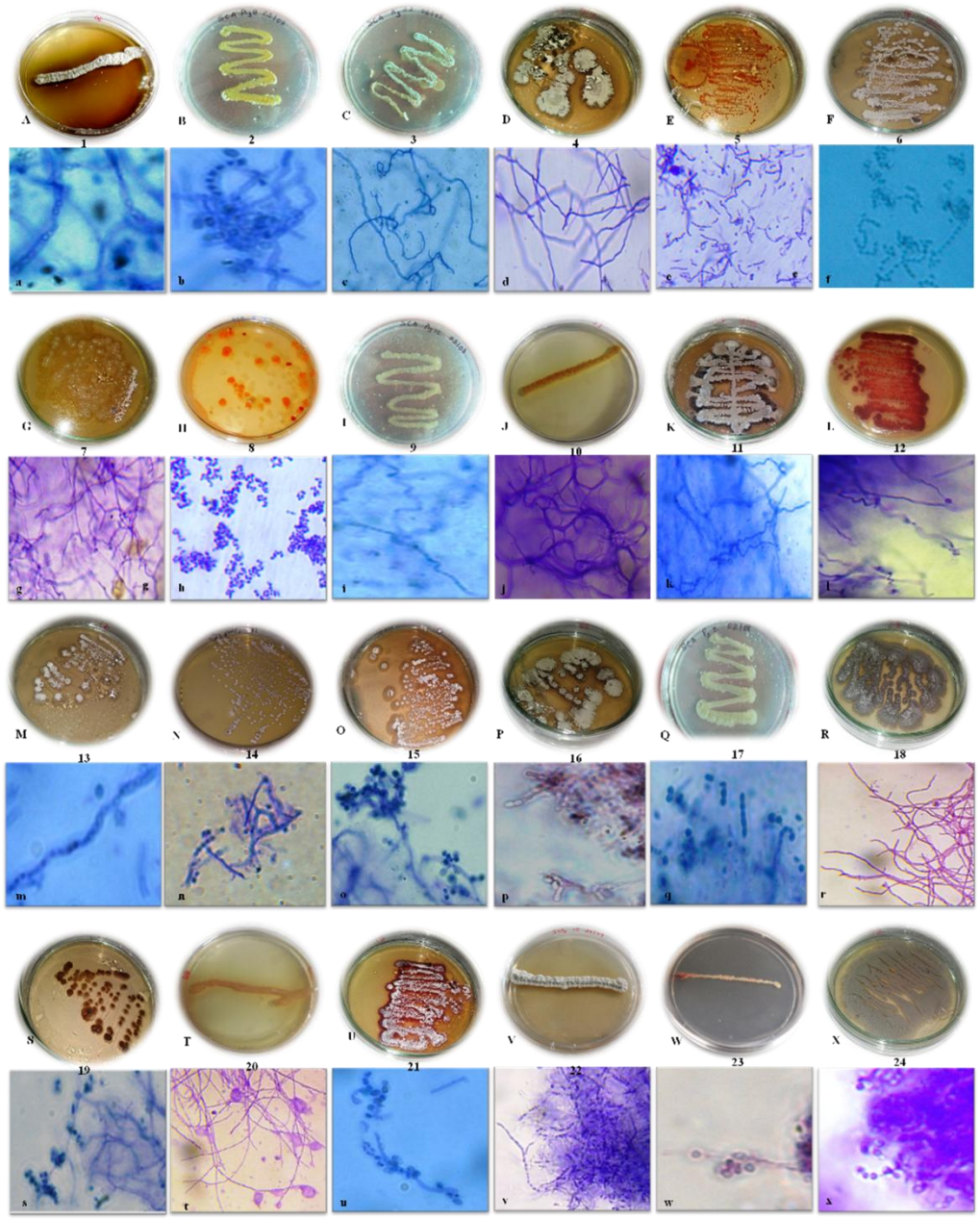


Figure 3 Plantation soil actinomycete strains on ISP-2 medium and their aerial spores
1. *S. coelicolor* 2. *S. hebeiensis* 3. *S. atrovirens* 4. *S. olivaceous* 5. *S. spectabilis* 6. *S. clavuligerus* 7. *S. griseoplanus* 8. *A. viscosus* 9. *S. longisporoflavus* 10. *N. antimicrobica* 11. *S. violaceolatus* 12. *S. rubrogriseus* 13. *S. chattanoogensis* 14. *S. mutabilis* 15. *A. nitritigenes* 16. *A. montaniterrae* 17. *Actinomadura* sp. 18. *Streptomyces* sp. 19. *A. apis* 20. *S. albida* 21. *A. rifamycini* 22. *S. hattusasensis* 23. *A. namibiensis* 24. *A. libanotica*; Letters in upper case represents on plate colony; Letters in lower case represents light microscopy photographs at 100X magnification.

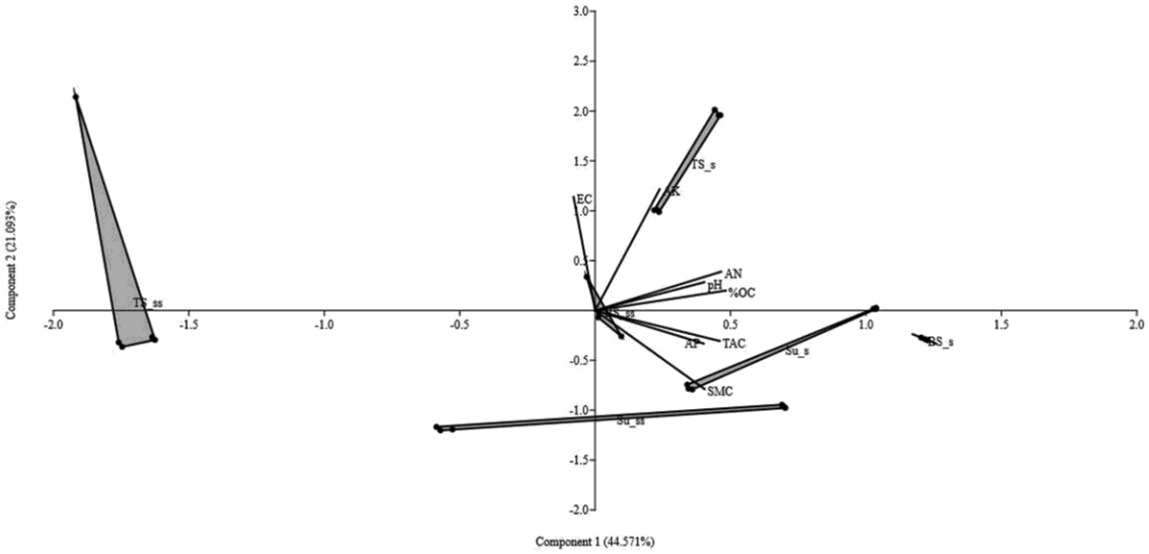


Figure 4 Biplot of principal component analysis (PCA)
TAC-total actinomycetes count, SMC- soil moisture content, EC- Electrical conductivity, OC- Organic carbon, AN- Available nitrogen, AP- Available phosphorus, AK- Available potassium, TS-toe slope, Su-summit, BS-back slope, _s- Surface soil, _ss- subsurface soil

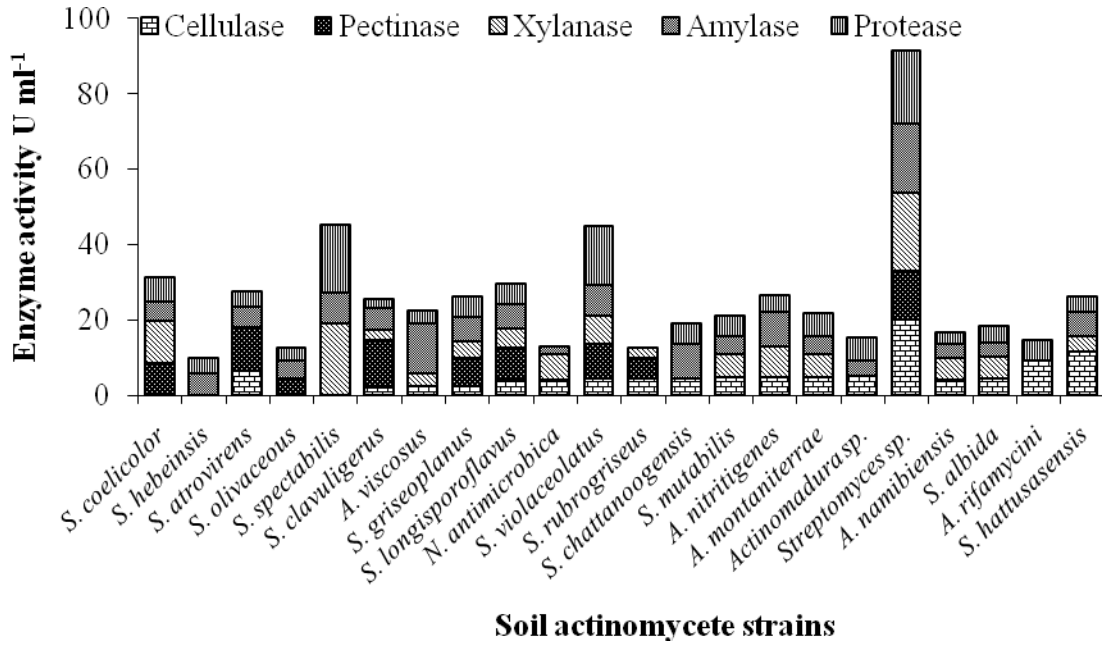


Figure 5 Enzyme activity (U/ml) of actinomycete isolates
Each value represents the mean ± SEM of triplicate experiments

The TAC variable correlated with variables AN, AP and SMC through positive loadings for PC1. The correlated variable AK and EC with positive loadings and high contributions for PC2 is associated with a separate variable group. Along with SMC and %OC with negative PC2 loadings and high contribution to PC2- these variables contrasts the slope position (i.e. Toe slope, summit and back slope). The surface soil groups, i.e. TS_s have positive PC2 scores and a content abundance of EC and AK variables compared with other groups with negative scores and SMC and %OC content abundance compared with the groups along the slope. Thus, SMC, %OC, EC and AK are responsible for group distinction along the slope. The outcome of PCA based MLR analysis demonstrated that PC1 (P=<0.0001) and PC2 (P= 0.014) had a significant effect on TAC and considered for 47.4% and 45.78% of total variation in the TAC to subsurface soil and surface soils. Previous investigations have shown significant or no correlations between soil properties and actinomycete populations. Jehangir *et al.* (2012) reported no correlation between the physicochemical parameters of forest soil versus actinomycete populations. But, investigations by Priyadarshini *et al.* (2013) on the physico-chemical properties *viz.* pH, moisture, organic matter, nitrogen and phosphorous content in paddy field soils showed significant correlation with actinomycete populations.

Screening of actinomycete strains for enzymatic potentials

The actinomycetes isolated from coffee plantation soil exhibited remarkable enzymatic potentials. The results of the preliminary screening revealed that 62.5%, 33.3%, 54.2%, 70.8% and 79.2% of the strains showed cellulase, pectinase, xylanase, amylase and protease enzymatic potentials respectively, while 20.8% of the strains were found to produce all the enzymes. During the secondary screening, all tested isolates showed enzyme activities, between 0.360 to 18.51 U/ml for Cellulase, 0.86 to 1.53 U/ml for pectinase, 0.65-6.92 U/ml for xylanase, 0.26-5.62 U/ml for amylase on day 6 at 50^{o}C at pH 7.0 and 2.63 -5.15 U/ml for protease on day 6 at 30^{o} C at pH 7.0 and the results are represented in Figure 5. The optimum enzyme activity was exhibited by *Streptomyces* sp. for all enzyme activities studied. Earlier reports on the enzymatic potentials of actinomycetes indicate that approximately 90% of the isolates possessed one or more potentials (Lekshmi *et al.*, 2014; Singh and Roymon, 2018). The complexity of plantation habitats comprising a cool temperature and shade with special lighting conditions that in turn contributes to the potential to produce a wide range of enzymes by actinomycetes.

Actinomycetes, especially *Streptomyces*, are reported from the plantation habitats. From this study, it is clearly indicated that coffee plantation system can be considered as an important resource for screening useful enzyme producing actinomycetes. Further, investigations are required to elucidate the potentials of these organisms for enzyme production by biotechnological approaches. These isolates may be effectively used in large scale production for commercial, industrial and pharmaceutical applications in the coming future.

Currently we are pursuing the antibiotic potential of soil actinomycetes, as they are known to be novel antibiotic sources.

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Conflict of interest

The authors declare that there is no conflict of interest involved in this study.

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