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Characterization and phylogenetic affiliation of *Actinobacteria* from tropical soils with potential uses for agro-industrial processes

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ABSTRACT. Secondary metabolites produced by *Actinobacteria* of tropical soils represent a largely understudied source of novel molecules with relevant application in medicine, pharmaceutical and food industries, agriculture, and environmental bioremediation. The present study aimed to characterize sixty-nine *Actinobacteria* isolated from compost and tropical soils using morphological, biochemical, and molecular methods. All the isolates showed high variation for morphological traits considering the color of pigments of the aerial and vegetative mycelium and spore chain morphology. The enzymatic activity of amylase, cellulase, and lipase was highly variable. The amylase activity was detected in 53 (76.81%) isolates. Eighteen

isolates showed enzymatic index (EI) > 4.0, and the isolates ACJ 45 (*Streptomyces curacoi*) and ACSL 6 (*S. hygroscopicus*) showed the highest EI values (6.44 and 6.42, respectively). The cellulase activity varied significantly ($P \leq 0.05$) among the isolates. Twenty-nine isolates (42.02%) showed high cellulase activity, and the isolates ACJ 48 (*S. chiangmaiensis*) and ACJ 53 (*S. cyslabdanicus*) showed the highest EI values (6.56 for both isolates). The lipase activity varied statistically ($P \leq 0.05$) with fourteen isolates (20.29%) considered good lipase producers (EI > 2.0). The isolate ACSL 6 (*S. hygroscopicus*) showed the highest EI value of 2.60. Molecular analysis of partial 16S rRNA gene sequencing revealed the existence of 49 species, being 38 species with only one representative member and 11 species represented by one or more strains. All species belonged to three genera, namely *Streptomyces* (82.61%), *Amycolatopsis* (7.25%), and *Kitasatospora* (10.14%). The present results showed the high biotechnological potential of different *Actinobacteria* from tropical soils.

Key words: Actinomycetes; Morphological traits; Enzymatic activity; 16S rRNA gene; Molecular identification

INTRODUCTION

The phylum *Actinobacteria* (*Actinomycetes*) is an ancient bacterial group branched off from the other prokaryotes very early in the evolutionary process (Battistuzzi et al., 2004; Ventura et al., 2007). *Actinobacteria* comprise an ecologically diverse group ubiquitously distributed in various natural environments as free-living, pathogens, and endophyte symbionts (Hardoim et al., 2015). They are distinguished as Gram-positive bacteria, normally aerobic, non-acid fast and with a high GC content in their DNA, varying to less than 50% in a few species to more than 70% in some genera (Lewin et al., 2016).

Actinobacteria exhibit a high level of diversity of biochemical features, such as the production of a wide variety of secondary metabolites and extracellular enzymes with relevant applications in different fields (Suneetha et al., 2011; Barka et al., 2015; Li et al., 2016). Although near half of the bioactive molecules with different uses in medicine, industrial processes, agriculture, and environmental bioremediation are produced by *Actinobacteria*, it represents only a small fraction of the overall metabolites already identified in this bacterial group (Abid et al., 2016). New enzymes selected based on enzymatic index (EI) criterion have been extensively explored commercially by the food, textile, and biofuel industry. Amylase, lipase, cellulase are some examples of enzymes isolated from *Actinobacteria* and currently used in the global market (Sathya and Ushadevi, 2014). Thus, the understudied *Actinobacteria* from tropical soils may represent a promising new source of secondary metabolites for many purposes.

The taxonomy of *Actinobacteria* has been a subject of intense debate. Traditionally, morphological traits as growth pattern and mycelia type are the main characteristics used to define order, genera, and species in this group, and biochemical tests using enzyme activities are used for identifying new bioactive metabolites. Recently, molecular data based on DNA sequencing of the 16S rRNA gene assumed an important role on systematic of the phylum *Actinobacteria* (Ventura et al., 2007).

In the present study, morphological traits, enzymatic activities, and partial sequencing of the 16S rRNA gene were used for characterizing *Actinobacteria* isolated from Brazilian tropical soils.

MATERIAL AND METHODS

The study was performed with sixty-nine bacterial isolates deposited at the Embrapa Maize and Sorghum Multifunctional Culture Collection (CCMF-CNPMS) that were previously identified as *Actinobacteria* based on their growth morphology (Table 1).

Table 1. Collection location (origin and location sites), the year at which the material was collected, and identification of the *Actinobacteria* isolates.

Origin	Location site	Year	Identification
Cerrado soil with high level of phosphate at the Experimental Station of Embrapa	Sete Lagoas	2000	ACSL 1A, 2, 8
Cerrado soil from the Experimental Station of Embrapa	Sete Lagoas	2001	ACSL 12, 16A
Cerrado soil from the Experimental Station of Embrapa	Sete Lagoas	2004	ACSL 485, 490, 495, 509, 517
Cerrado soil from the Experimental Station of Embrapa	Sete Lagoas	2014	ACSL 1B, 6, 13, 16B, 18B, 22, 23, 27B
Maize Rhizospheral soil from the Experimental Station of Embrapa	Sete Lagoas	2004	ACSL 7, 18A, 25, 27A, 50, 53, 54, 64B, 67, 77, 82, 91
Organic farming from the Experimental Station of Embrapa	Sete Lagoas	2002	ACSL 432, 448, 449, 450, 453, 457, 470
Cerrado soil planted with eucalyptus	Sete Lagoas	2001	ACSL 64A, 80, 83, 85, 93, 115
Cerrado soil planted with eucalyptus and pinus woods	Sete Lagoas	2003	ACSL 404
Cerrado soil from the Fazenda Santa Rita Experimental Station	Prudente de Morais	2006	ACPM 641
Cerrado soil from the Fazenda Santa Rita Experimental Station	Prudente de Morais	2007	ACPM 5, 29, 31, 38, 66
Cerrado soil planted with peanuts from the Fazenda Santa Rita Experimental Station	Prudente de Morais	2002	ACPM 346, 363, 364
Agricultural Cerrado soil from the Experimental Station of Embrapa	Jaíba	2001	ACJ 66, 76
Cerrado degradated soil	Jaíba	2001	ACJ 1, 17, 26, 29
Mata seca	Jaíba	2001	ACJ 36, 43, 45
Protected area	Jaíba	2001	ACJ 48, 49, 51, 52, 53
Compost	Papagaios	2013	ACP 35
Compost	Capim Branco	2015	ACCB 1

The isolates were grown in the agar glycerol-asparagine (AGA) medium [1 g/L L-asparagine, 10 g/L glycerol, 1 g/L KH_2PO_4, 15 g/L agar, 1 mL/L micronutrient solution (0.1 g $FeSO_4 \times 7H_2O$, 0.1 g $MnCl_2 \times 4H_2O$, 0.1 g $ZnSO_4 \times 7H_2O$, qsp 100 mL deionized water)] according to Pridham and Lyons (1961) and supplemented with 0.03 g/L cycloheximide. After inoculations, the plates were incubated for 14 days at 28°C.

Morphological characterization

The morphological analysis was performed according to Shirling and Gottlieb (1966). The following growing cultural parameters were evaluated: the color of the vegetative and aerial mycelium, and changes in the color of the medium around the colony.

For the micromorphological analysis, the microculture technique was used according to Holt et al. (1994). After the incubation period, the coverslip was removed and placed on another sterile microscopic slide containing 10 µL Amann lactophenol and the edges were sealed with colorless nail enamel. The spore chain morphology was observed under the optical microscope Olympus BX 60 (Olympus Optical Co. Ltd., Tokyo, Japan) with 1000X magnification, and photographed with a digital camera (Leica DFC 490, Leica Microsystems Inc., Buffalo Grove, IL, USA).

Molecular characterization

The DNA extraction, PCR, and DNA sequencing were made according to Lana et al. (2012). The 16S rRNA gene was amplified using the universal primers 8F (5'-AGA GTT

TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') (Turner et al., 1999). The sequencing was made with the PCR primers and the internal primers 515F (5'-GTG CCA GCM GCC GCG GTA A-3'; Turner et al., 1999) and 902R (5'-GTC AAT TCI TTT GAG TTT YAR YC-3'; Hodkinson and Lutzoni, 2009). In the primers, the letters Y; M; R; and I represent the nucleotides cytosine or thymine; adenine or cytosine; adenine or guanine; and a modified guanine, respectively.

The DNA sequences were generated in the Applied Biosystems 3500xL Genetic Analyzer (Applied Biosystem, Foster City, CA, USA). The nucleotide sequences were edited using the Sequencher 4.1 program and compared in the GenBank database (http://www.ncbi.nlm.nih.gov/) through the BLAST N program (Altschul et al., 1997) located at the NCBI (National Center for Biotechnology Information).

Phylogenetic analysis

The phylogenetic analysis was performed by the neighbor-joining method using the Molecular Evolutionary Genetics Analysis version 5 (MEGA5) program (Tamura et al., 2011).

Enzymatic activity

To test the enzymatic activity of amylase, cellulase, and lipase, disks of cultures on agar plate were inoculated in triplicate in a completely random design and incubated at 28°C for 10 days (amylase and cellulase) and 72 h (lipase). The EI was estimated by the following equation: EI = ratio of halo diameter/ratio of colony diameter. The isolates were classified as non-producer (EI = 0), low producer ($0 > EI \leq 2$), middle producer ($2 > EI \leq 4$), and high producer ($EI > 4$). The data were analyzed for significance ($P<0.05$) using the Scott-Knott test.

Amylase

The amylase production was determined as described by Coon et al. (1957) modified by the addition of 6.6% soluble starch. The isolates were inoculated on the starch agar medium (6.6 g/L soluble starch, 0.5 g/L sodium chloride, 3 g/L meat extract, 1 g/L peptone casein, 15 g/L agar with the pH adjusted to 7.0). After culture growth, 10 mL Lugol's solution (5 g iodine, 10 g potassium iodide in 100 mL distilled water) was diluted to 1:10 and added to the dishes. The amylase production was detected by the formation of a light yellow zone around the colony corresponding to the discoloration of the medium.

Cellulase

The cellulase production was tested in the culture medium supplemented with carboxymethylcellulose (CMC) as the sole carbon source (3 g/L $NaNO_3$, 1 g/L K_2HPO_4, 0.5 g/L $MgSO_4$, 0.5 g/L KCl, 10 mg/L $FeSO_4.7H_2O$, 10 g/L CMC, 15 g/L agar with the pH adjusted to 7.0) according to Lewis (1988). After incubation, 10 mL 0.5% Congo red dye was added to each plate, incubated for 15 min at room temperature and washed with NaCl (5 M). Afterward, the excess of solution was discarded, and 10 mL NaCl solution (1 M) was added to each plate and incubated for 30 min at room temperature. The production of the enzyme was observed by the discoloration of the medium, which forms an orange zone around the colony.

Lipase

Lipase production was tested according to Savitha et al. (2007) using a culture medium with the following composition: 5 g/L peptone, 1 g/L yeast extract, 4 g/L sodium chloride, 15 g/L agar, 31.25 mL/L olive oil, 0.01 g/L Rhodamine B, pH 7.0. Disks of the cultured isolates were inoculated in the medium, and after the incubation period, the plates were exposed to ultraviolet radiation to observe the formation of blue stained halos around the positive colonies, which is the parameter used to indicate the activity of the enzyme (Colen, 2006).

RESULTS

In this study, the analysis of morphological traits, enzyme activities, and molecular sequencing revealed a high level of variability among sixty-nine isolates of *Actinobacteria* from composts and tropical soils.

Morphological characterization

The analyses of the four main morphological traits used for *Actinobacteria* identification were highly variable (Table 2). Besides these characteristics, the production of classic pulverulent mycelium and radial growth confirmed the identity of the isolates as *Actinobacteria*. All sixty-nine morphospecies produced vegetative mycelium on the AGA medium with evident variation in the pattern of development, color, and pigment production (Figure 1). The release of soluble pigments on the AGA medium was observed in 50.72% of the isolates and varied from yellow (28.57%), light brown (40%), and dark brown (28.57%) to black (2.86%).

The micromorphological features of the bacterial colonies were assessed with a light microscope to determine the presence, absence, and morphology of the spore chain compared with valid criteria reported in the Bergey's Manual of Systematic Bacteriology (Holt et al., 1994) (Table 2). The morphological structure of the spore chain varied depending on the isolate, and was classified as straight (10.14%), retinaculum apertum (28.99%), spiral (27.54%), and flexuous (33.33%) (Figure 2). The phylogenetic tree constructed using morphological and biochemical data showed all species grouped together (data not shown).

Molecular characterization and phylogenetic analysis

Partial sequences of the 16S rRNA gene, ranging in length from 1210 to 1400 nucleotides, were determined for 69 isolates. The molecular characterization based on nucleotide comparisons of the 16S rRNA gene with nucleotide sequences deposited in the GenBank (accession numbers: KY585931 to KY585999) confirmed the morphological identity of the isolates as *Actinobacteria* (Table 3). A total of forty-nine taxa were identified distributed among the following three genera: *Streptomyces* (82.61%), *Amycolatopsis* (10.14%), and *Kitasatospora* (7.25%).

The phylogenetic tree constructed by the maximum likelihood, evolutionary distance, and maximum parsimony methods with the MEGA5 program (Tamura et al., 2011) generated two distinct clades with *Amycolatopsis* separated from *Streptomyces* and *Kitasatospora* (Figure 3). The clade formed by the genera *Streptomyces* and *Kitasatospora* was divided

Table 2. Morphological characteristics of the *Actinobacteria* isolates from composts and tropical soils from different collecting places of Brazil.

Isolate	Macromorphology					Micromorphology
	Aerial mycelium			Vegetative mycelium	Pigments	Spore chain type
	Center	Edge	Uniform			
ACSL 1A	Yellow	Gray		Beige	-	Retinaculum apertum
ACSL 1B			White	Black	Black	Retinaculum apertum
ACSL 2	Cream	Yellow		Cream	-	Spiral
ACSL 6			Dark brown	Dark brown	-	Retinaculum apertum
ACSL 7	Beige	White		Cream	-	Spiral
ACSL 8	Beige	White		Beige	-	Flexuous
ACSL 12			Light brown	Dark brown	Light brown	Flexuous
ACSL 13			Dark brown	Dark brown	-	Retinaculum apertum
ACSL 16A			Cream	Cream	-	Straight
ACSL 16B	Beige	White		Cream	-	Spiral
ACSL 18A			Cream	Cream	Yellow	Retinaculum apertum
ACSL 18B			Gray	Dark brown	Dark brown	Straight
ACSL 22			White	Dark brown	Yellow	Flexuous
ACSL 23	White	Gray		Light brown	Light brown	Flexuous
ACSL 25			Cream	Light brown	-	Flexuous
ACSL 27A			Cream	Cream	-	Spiral
ACSL 27B			Gray	Beige	Light brown	Retinaculum apertum
ACSL 50			Brown	Brown	Dark brown	Flexuous
ACSL 53			Beige	Beige	-	Spiral
ACSL 54			Ocher	Ocher	-	Flexuous
ACSL 64A			Beige	Beige	Light brown	Spiral
ACSL 64B			Light brown	Light brown	Yellow	Retinaculum apertum
ACSL 67	Brown	Gray		Dark brown	Light brown	Flexuous
ACSL 77			Light brown	Light brown	Light brown	Flexuous
ACSL 80			Gray	Dark brown	Dark brown	Straight
ACSL 82			Beige	Beige	-	Spiral
ACSL 83			Cream	Cream	Yellow	Retinaculum apertum
ACSL 85			Gray	Dark brown	Dark brown	Straight
ACSL 91			White	Dark brown	Yellow	Flexuous
ACSL 93	Dark gray	Brown/purple		Dark brown	-	Retinaculum apertum
ACSL 115			White	Light brown	Light brown	Spiral
ACSL 404	Cream	Yellow		Cream	-	Spiral
ACSL 432	Dark brown	Gray		Brown	Light brown	Spiral
ACSL 448			White	Beige	Light brown	Spiral
ACSL 449			White	Dark brown	Yellow	Flexuous
ACSL 450			Cream	Cream	Yellow	Flexuous
ACSL 453	Beige	White		Cream	-	Spiral
ACSL 457			Beige	Beige	-	Flexuous
ACSL 470	Light brown	Gray		Brown	-	Flexuous
ACSL 485	Dark brown	Light brown		Brown	Dark brown	Retinaculum apertum
ACSL 490	Yellow	White		Cream	-	Flexuous
ACSL 495	Yellow	Gray		Beige	-	Flexuous
ACSL 509	Beige	Gray		White	-	Retinaculum apertum
ACSL 517			Beige	Beige	-	Straight
ACPM 5			Gray	Light brown	-	Spiral
ACPM 29			Dark brown	Dark brown	Light brown	Retinaculum apertum
ACPM 31	Dark brown	Light brown		Dark brown	Light brown	Flexuous
ACPM 38	White	Gray		Beige	-	Flexuous
ACPM 66	Gray	White		Light brown	Dark brown	Flexuous
ACPM 346			Ocher	Ocher	-	Straight
ACPM 363	Beige	White		Cream	-	Spiral
ACPM 364			Gray	Dark brown	-	Flexuous
ACPM 641			Beige	Light brown	Light brown	Retinaculum apertum
ACJ 1	Beige	White		White	-	Retinaculum apertum
ACJ 17	White	Pink		Beige/pink	-	Spiral
ACJ 26			White	Ocher	Yellow	Spiral
ACJ 29	Beige	White		Cream	-	Spiral
ACJ 36	Dark brown	Light brown		Brown	Dark brown	Retinaculum apertum
ACJ 43	Dark brown	Light brown		Brown	Dark brown	Retinaculum apertum
ACJ 45			Light brown	Light brown	Light brown	Spiral
ACJ 48			Brown	Brown	Dark brown	Retinaculum apertum
ACJ 49	Gray	White		Brown	-	Retinaculum apertum
ACJ 51			Light brown	Light brown	Yellow	Retinaculum apertum
ACJ 52	Light brown	White		Light brown	Light brown	Flexuous
ACJ 53			White	Cream	-	Retinaculum apertum
ACJ 66	Beige	White		Cream	-	Spiral
ACJ 76			White	Dark brown	Yellow	Flexuous
ACP 35	Dark gray	Light gray		Dark brown	Dark brown	Straight
ACCB 1			Cream	Cream	-	Flexuous

(-) indicate absence.

into two separated subgroups. There was a clear separation between species, except for two *Streptomyces* strains grouped together within *Kitasatospora*. The branches were well supported with a bootstrap value above 70%.

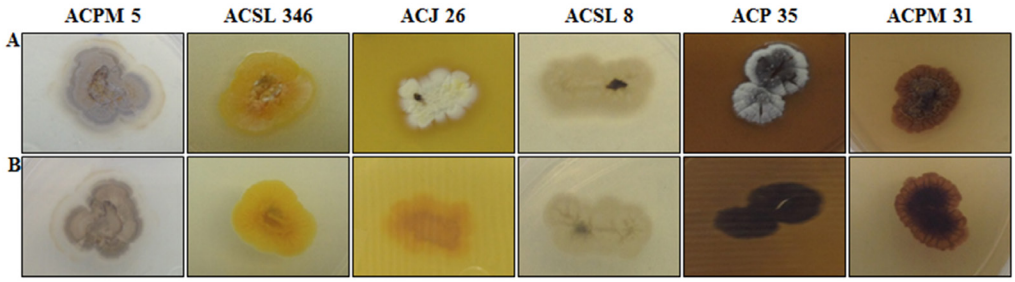

Figure 1. Morphological characteristics of representative *Actinobacteria* isolates from composts and tropical soils from different collecting places of Brazil. **A.** aerial mycelium; **B.** substrate mycelium.

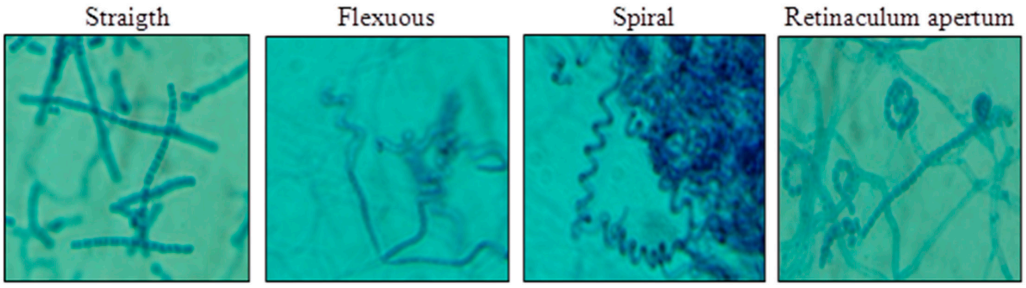

Figure 2. Spore chain morphology of *Actinobacteria* isolates from composts and tropical soils.

Enzymatic activity

The data of enzymatic activity for amylase, cellulase, and lipase are shown in Table 4. It was observed that 95.65% of the isolates were able to produce at least one of the three enzymes studied, even though they were not classified as good potential producers, that is, those that presented EI ≤ 2.0. The production of amylase, cellulase, and lipase was observed in 76.81, 79.71, and 40.58% of the isolates, respectively. The isolates ACSL 93 (*A. echigonensis*), ACSL 76 (*S. yunnanensis*), and ACBB 1 (*S. variabilis*) did not show enzymatic activity (EI = 0) for any of the three enzymes tested (Table 4).

Amylolytic activity

Concerning amylase, the amylolytic activity was indicated by the presence of a yellow halo around the colonies (Figure 4a). The relationship between the halo and the colony diameters determined the EI. The EI was statistically significant ($P \leq 0.05$) among the isolates (Table 4). Sixteen isolates (23.19%) did not produce a halo. Thus, they were considered non-amylase producers. All seven species identified as belonging to the genus *Amycolatopsis* (10.4%) did not show amylolytic activity.

Table 3. Molecular characterization of the *Actinobacteria* isolates from composts and tropical soils from different Brazilian collecting places.

Isolate	GenBank accession No.	Species	Similarity (%)	Isolate	GenBank accession No.	Species	Similarity (%)
ACSL 1A	KY585949	*Streptomyces seymenliensis*	99	ACSL 450	KY585946	*A. bullii*	98
ACSL 1B	KY585960	*S. massasporeus*	99	ACSL 453	KY585958	*S. galbus*	99
ACSL 2	KY585933	*S. chartreusis*	99	ACSL 457	KY585981	*A. pretoriensis*	99
ACSL 6	KY585976	*S. hygroscopicus*	100	ACSL 470	KY585944	*S. pseudovenezuelae*	100
ACSL 7	KY585992	*S. galbus*	99	ACSL 485	KY585964	*S. psammoticus*	100
ACSL 8	KY585974	*S. sporocinereus*	100	ACSL 490	KY585977	*A. kentuckyensis*	100
ACSL 12	KY585963	*Kitasatospora atroaurantiaca*	99	ACSL 495	KY585979	*A. lexingtonensis*	99
ACSL 13	KY585937	*S. hygroscopicus*	100	ACSL 509	KY585980	*S. deserti*	99
ACSL 16A	KY585957	*S. purpeofuscus*	99	ACSL 517	KY585931	*S. phaeochromogenes*	98
ACSL 16B	KY585934	*S. galbus*	99	ACPM 5	KY585962	*S. olivochromogenes*	99
ACSL 18A	KY585996	*S. longwoodensis*	99	ACPM 29	KY585969	*S. scabiei*	99
ACSL 18B	KY585951	*S. phaeochromogenes*	98	ACPM 31	KY585941	*S. phaeopurpureus*	99
ACSL 22	KY585986	*S. yunnanensis*	99	ACPM 38	KY585942	*S. rishiriensis*	99
ACSL 23	KY585972	*S. indiaensis*	100	ACPM 66	KY585984	*S. sioyaensis*	99
ACSL 25	KY585988	*Amycolatopsis rifamycinica*	99	ACPM 346	KY585953	*S. endophyticus*	99
ACSL 27A	KY585995	*S. lydicus*	99	ACPM 363	KY585940	*S. galbus*	99
ACSL 27B	KY585943	*S. corchorusii*	99	ACPM 364	KY585935	*K. viridis*	99
ACSL 50	KY585998	*S. sampsonii*	99	ACPM 641	KY585959	*S. lannensis*	100
ACSL 53	KY585990	*K. paracochleata*	99	ACJ 1	KY585945	*S. ossamyceticus*	100
ACSL 54	KY585991	*S. sasae*	99	ACJ 17	KY585947	*S. bangladeshensis*	99
ACSL 64A	KY585999	*S. coacervatus*	99	ACJ 26	KY585978	*S. capoamus*	99
ACSL 64B	KY585987	*S. griseoruber*	99	ACJ 29	KY585967	*S. galbus*	99
ACSL 67	KY585994	*S. phaeopurpureus*	100	ACJ 36	KY585975	*S. psammoticus*	100
ACSL 77	KY585997	*K. phosalacinea*	99	ACJ 43	KY585970	*S. psammoticus*	99
ACSL 80	KY585938	*S. phaeochromogenes*	98	ACJ 45	KY585954	*S. curacoi*	99
ACSL 82	KY585989	*K. paracochleata*	99	ACJ 48	KY585971	*S. chiangmaiensis*	100
ACSL 83	KY585936	*S. longwoodensis*	99	ACJ 49	KY585948	*A. rhabdoformis*	100
ACSL 85	KY585983	*S. phaeochromogenes*	99	ACJ 51	KY585968	*S. griseoruber*	100
ACSL 91	KY585993	*S. yunnanensis*	99	ACJ 52	KY585973	*S. yaanensis*	100
ACSL 93	KY585966	*A. echigonensis*	99	ACJ 53	KY585932	*S. cyslabdanicus*	99
ACSL 115	KY585939	*S. thioluteus*	99	ACJ 66	KY585965	*S. galbus*	99
ACSL 404	KY585950	*S. chartreusis*	100	ACJ 76	KY585955	*S. yunnanensis*	99
ACSL 432	KY585961	*S. novaecaesareae*	99	ACP 35	KY585956	*S. laculatispora*	99
ACSL 448	KY585952	*S. sioyaensis*	99	ACCB 1	KY585985	*S. variabilis*	100
ACSL 449	KY585982	*S. yunnanensis*	99	-	-	-	-

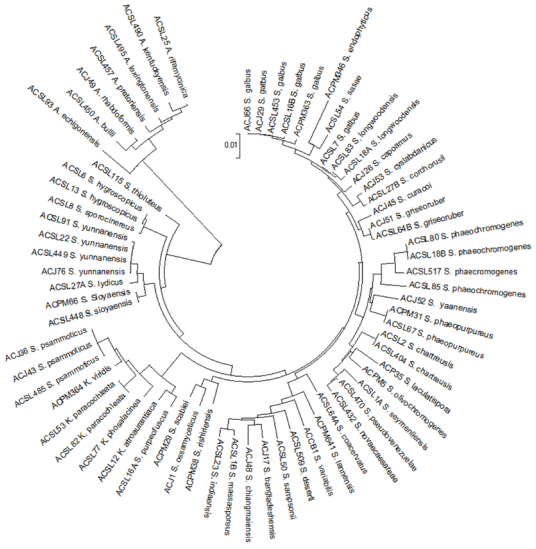

Figure 3. Dendrogram based on partial sequences of the 16S rRNA gene of the *Actinobacteria* isolates from composts and tropical soils.

Table 4. Enzymatic index (EI) for amylase, cellulase, and lipase activity of the *Actinobacteria* isolates.

Isolate	Species	Enzymatic index		
		Amylase	Cellulase	Lipase
ACSL 1A	*S. seymenliensis*	2.20^{i}	3.36^{e}	2.17^{b}
ACSL 1B	*S. massasporeus*	2.67^{h}	3.42^{e}	0.00
ACSL 2	*S. chartreusis*	1.52^{k}	5.55^{b}	0.00
ACSL 6	*S. hygroscopicus*	6.42^{a}	4.00^{e}	2.60^{a}
ACSL 7	*S. galbus*	5.10^{d}	3.78^{e}	0.00
ACSL 8	*S. sporocinereus*	5.43^{c}	3.67^{e}	1.22^{e}
ACSL 12	*K. atroaurantiaca*	4.81^{d}	2.93^{f}	1.46^{d}
ACSL 13	*S. hygroscopicus*	4.87^{d}	5.33^{b}	0.00
ACSL 16A	*S. purpeofuscus*	0.00	4.17^{d}	0.00
ACSL 16B	*S. galbus*	4.13^{f}	4.58^{d}	0.00
ACSL 18A	*S. longwoodensis*	3.40^{g}	3.83^{e}	0.00
ACSL 18B	*S. phaeochromogenes*	2.93^{h}	4.33^{d}	0.00
ACSL 22	*S. yunnanensis*	3.15^{g}	0.00	0.00
ACSL 23	*S. indiaensis*	3.22^{g}	5.13^{c}	0.00
ACSL 25	*A. rifamycinica*	0.00	4.39^{d}	2.02^{b}
ACSL 27A	*S. lydicus*	4.31^{f}	0.00	1.66^{c}
ACSL 27B	*S. corchorusii*	5.00^{d}	5.50^{b}	0.00
ACSL 50	*S. sampsonii*	3.13^{g}	3.93^{e}	0.00
ACSL 53	*K. paracochleata*	1.65^{k}	3.44^{e}	0.00
ACSL 54	*S. sasae*	3.42^{g}	0.00	2.23^{b}
ACSL 64A	*S. coacervatus*	3.60^{g}	2.86^{f}	2.12^{b}
ACSL 64B	*S. griseoruber*	1.13^{m}	2.73^{f}	2.20^{b}
ACSL 67	*S. phaeopurpureus*	3.21^{g}	3.92^{e}	2.16^{b}
ACSL 77	*K. phosalacinea*	2.14^{i}	4.21^{d}	1.79^{c}
ACSL 80	*S. phaeochromogenes*	1.24^{l}	0.00	0.00
ACSL 82	*K. paracochleata*	6.00^{b}	3.75^{e}	0.00
ACSL 83	*S. longwoodensis*	2.77^{h}	4.92^{c}	0.00
ACSL 85	*S. phaeochromogenes*	0.00	4.28^{d}	0.00
ACSL 91	*S. yunnanensis*	2.71^{h}	0.00	0.00
ACSL 93	*A. echigonensis*	0.00	0.00	0.00
ACSL 115	*S. thioluteus*	2.37^{i}	0.00	0.00
ACSL 404	*S. chartreusis*	1.98^{j}	3.90^{e}	1.52^{d}
ACSL 432	*S. novaecaesareae*	4.93^{d}	3.17^{f}	0.00
ACSL 448	*S. sioyaensis*	2.61^{h}	0.00	0.00
ACSL 449	*S. yunnanensis*	3.27^{g}	1.78^{g}	0.00
ACSL 450	*A. bullii*	0.00	3.98^{e}	1.68^{c}
ACSL 453	*S. galbus*	1.96^{j}	0.00	0.00
ACSL 457	*A. pretoriensis*	0.00	4.42^{d}	1.47^{d}
ACSL 470	*S. pseudovenezuelae*	4.00^{f}	4.33^{d}	0.00
ACSL 485	*S. psammoticus*	4.52^{e}	4.53^{d}	1.51^{d}
ACSL 490	*A. kentuckyensis*	0.00	2.89^{f}	0.00
ACSL 495	*A. lexingtonensis*	0.00	3.20^{f}	0.00
ACSL 509	*S. deserti*	2.29^{i}	4.27^{d}	0.00
ACSL 517	*S. phaeochromogenes*	2.55^{h}	5.70^{b}	2.07^{b}
ACPM 5	*S. olivochromogenes*	3.29^{g}	3.58^{e}	2.07^{b}
ACPM 29	*S. scabiei*	5.52^{c}	5.08^{c}	2.10^{b}
ACPM 31	*S. phaeopurpureus*	3.39^{g}	4.07^{d}	2.00^{b}
ACPM 38	*S. rishiriensis*	2.83^{h}	4.47^{d}	0.00
ACPM 66	*S. Sioyaensis*	4.18^{f}	0.00	0.00
ACPM 346	*S. endophyticus*	3.07^{g}	3.56^{e}	0.00
ACPM 363	*S. galbus*	2.62^{h}	5.06^{c}	2.27^{b}
ACPM 364	*K. viridis*	0.00	0.00	1.93^{b}
ACPM 641	*S. lannensis*	1.90^{j}	2.75^{f}	1.37^{e}
ACJ 1	*S. ossamyceticus*	5.14^{d}	4.42^{d}	0.00
ACJ 17	*S. bangladeshensis*	4.28^{f}	5.08^{c}	0.00
ACJ 26	*S. capoamus*	3.29^{g}	3.83^{e}	1.75^{c}
ACJ 29	*S. galbus*	2.17^{i}	4.75^{c}	0.00
ACJ 36	*S. psammoticus*	0.00	5.00^{c}	0.00
ACJ 43	*S. psammoticus*	4.51^{e}	1.83^{g}	0.00
ACJ 45	*S. curacoi*	6.44^{a}	4.17^{d}	1.13^{e}
ACJ 48	*S. chiangmaiensis*	0.00	6.56^{a}	0.00
ACJ 49	*A. rhabdoformis*	0.00	0.00	1.58^{d}
ACJ 51	*S. griseoruber*	3.45^{g}	4.55^{d}	2.23^{b}
ACJ 52	*S. yaanensis*	0.00	4.58^{d}	2.13^{b}
ACJ 53	*S. cyslabdanicus*	0.00	6.56^{a}	2.08^{b}
ACJ 66	*S. galbus*	2.03^{j}	3.55^{e}	0.00
ACJ 76	*S. yunnanensis*	0.00	0.00	0.00
ACP 35	*S. laculatispora*	5.70^{c}	3.67^{e}	0.00
ACCB 1	*S. variabilis*	0.00	0.00	0.00

Mean values followed by the same letters do not differ by the Scott-Knott test at 5% probability.

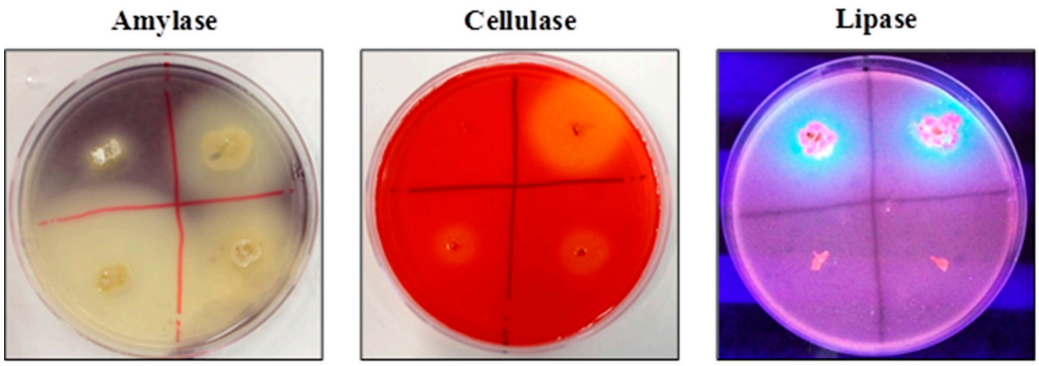

Figure 4. Enzymatic activity of amylase, cellulase, and lipase on agar plate demonstrated by the halo formation around the bacterial colonies.

The results of the EI analysis are shown in Table 4. Sixteen isolates did not produce amylase, and the isolates with $0 > EI \leq 4$ were classified as low and middle potential producers of amylase, and isolates with $EI > 4.0$ were identified as the highest enzyme producers. Sixteen isolates of genus *Streptomyces* and two of *Kitasatospora* were classified as high amylase producers ($EI > 4.0$). The isolates ACJ 45 (*S. curacoi*) and ACSL 6 (*S. hygroscopicus*) showed the highest EI (both with $EI = 6.44$).

Cellulolytic activity

The cellulolytic activity was determined by the formation of lighter staining halos (orange) around the colonies against a red background (Figure 4b). The EI for cellulolytic activity varied significantly ($P \leq 0.05$) among the *Actinobacteria* isolates. Fourteen isolates (20.29%) were not able to form a halo on the CMC plate assay. Therefore, they were considered as non-cellulase producers. Twenty-nine isolates (42.02%) were identified as good cellulase producers ($EI > 4.0$) (Table 4). The isolates ACJ 48 (*S. chiangmaiensis*) and ACJ 53 (*S. cyslabdanicus*) showed the highest EI (both with $EI = 6.56$).

Lipolytic activity

The lipase activity was measured by the formation of blue staining halos around the colonies against a purple background (Figure 4c). The lipase production varied significantly ($P \leq 0.05$) among the isolates evaluated (Table 4). Fourteen isolates (20.29%) showed the highest EI values ($EI > 2$), and 41 (59.42%) did not show enzymatic activity. The isolate ACSL 6 (*S. hygroscopicus*) showed the highest EI value ($EI = 2.60$).

DISCUSSION

In this study, the analysis of morphological traits and enzyme activity revealed a high level of variability among sixty-nine isolates of *Actinobacteria* from composting and tropical soils, and molecular sequencing enabled the identification of the isolates at the species level.

Morphological characterization

The analysis of morphological characteristics based on valid criteria described in the Bergey's Manual of Systematic Bacteriology (Holt et al., 1994) confirmed all isolates as *Actinobacteria*. The high morphological diversity and growth habit, characterized by the formation of highly differentiated hyphae and branched aerial mycelium with formation of spores, have been extensively used as valid criteria for identification of genera and species of the phylum *Actinobacteria* (Ventura et al., 2007; Suneetha et al., 2011; Barka et al., 2015). The grouping of six morphological characters and three enzymatic parameters showed the existence of fifty-seven different morphospecies. Twelve isolates distributed into five different groups and showing the same morphological pattern were considered to represent reisolates of the same sample. In the present study, only the AGA medium was used for bacterial growth. However, it is necessary to point out that the parameter color of the pigment used as a criterion for discriminating among the Actinobacterial isolates may change considerably depending on various cultural factors such medium composition, pH, and temperature of incubation (Holt et al., 1994).

Enzymatic activity

Enzymes of microbial origin present a great variety of catalytic activities with many applications in various industrial and biotechnological processes, especially in the textile and food industry (Luz et al., 2016). In the present study, the enzymatic activities of amylase, cellulase, and lipase were examined on agar media containing starch, carboxymethylcellulose, and olive oil as substrates, respectively. All but three isolates showed the ability to produce at least one of the three enzymes tested, proving their potential for industrial applications. Members of the three genera identified in the present study are of great importance due to their ability to produce compounds for medical, pharmaceutical, and agricultural purposes.

Amylase activity

The microbial amylase (EC 3.2.1.1) is among the most relevant classes of enzymes due to the wide range application in industrial biotechnological processes such as processed food, fermentation, and pharmaceutical purposes (de Souza and Oliveira Magalhães, 2010; Adrio and Demain, 2014).

The EI has been used a fast tool for selecting and comparing the enzyme production among different bacterial isolates (Carrim et al., 2006; Castro et al., 2014). Fungaro and Maccheroni (2002) suggested that EI greater than 1.0 are a reliable indicator for the presence of enzymes excreted by microorganisms, while EI $\geq$ 2.0 is considered good indicator for potential enzyme production by a bacterium (Lealem and Gashe, 1994). However, while for lipase the variation in EI is minimal (~2.0), for cellulase and lipase the scale of EI values vary between >2.0 to >6.0. In the current EI scales, differences of only 0.1 in the scale used to measure enzymatic activity represent a great difference in the potential for enzyme production. In our study, we introduced a new grade of variation in the scale for selecting bacterial species candidates for application in biotechnological processes. In our study, isolates with EI values of 2 > EI $\leq$ 4 were considered middle producer, and only EI values above 4.0 were considered high producers. Thus, the isolates with EI values >4 were selected for further studies aiming at biotechnological applications.

The production of amylase by microorganisms is very sensitive to incubation temperature. Minotto et al. (2014), performing the enzymatic characterization of endophytic *Actinobacteria* from tomato plants, observed that the highest EI value recorded for starch degradation was 6.46 when the microorganisms were incubated at 28°C. Also, results of EI values by Karanja et al. (2010) varied between 3.4 and 5.2 for starch degradation by *Streptomyces* sp isolates from soil in Kenya. In our study, 32 isolates showed similar results for amylase production with EI values varying between 3.0 and 6.44 using a different medium, and the same incubation temperature (28°C).

In our study, none of the seven *Amycolatopsis* isolates showed amylase activity corroborating the study by Ding et al. (2007). In fact, the absence of amylase activity is one important diagnostic feature of this genus of *Actinobacteria*.

Cellulase activity

Cellulose, xylan, and lignin (lignocellulose) are the three primary constituents of plant biomass (Yu et al., 2017). These are the most abundant biopolymers in the planet, therefore, the main renewable resources that can be used to produce glucose and proteins, industrial fertilizer, biofuel, and compost (Ramírez and Calzadíaz, 2016). Recently, cellulolytic enzymes of bacterial origin have received special attention of the bioenergy industry due to the higher bacterial growth, sustainability, and environmental impact compared with non-renewable fossil fuel counterparts. However, the industrial-scale breakdown of lignocellulosic plant biomass into simple sugars that can be converted into biofuels is one of the major barriers to the lignocellulosic ethanol production (Lewin et al., 2016).

In Kenya, Karanja et al. (2010) found EI values between 3.4 and 5.2 for starch degradation by *Streptomyces* sp isolated from soil. In Brazil, Minotto et al. (2014), analyzing *Actinobacteria* isolated from tomato cultivated in Cerrado soil, and Silva et al. (2015), studying *Streptomyces* isolated from rhizosphere soil from semi-arid climates, found highest EI values of 4.04 and 6.90, respectively. In our study, 31 isolates of *Streptomyces* presented EI values between 4.0 and 6.56 indicating their high biotechnological potential uses for large-scale industrial enzyme production and composting.

In our study, two *Streptomyces* (ACP 35 and ACCB 1) from compost did not produce cellulases. Rodrigues (2006) found that 9% of *Streptomyces* isolates were not able to degrade cellulose. Considering that the amount of cellulose in the composting process is higher than that available in other soils, the ability to degrade cellulose confers to *Streptomyces* an important role in the composting processes. Thus, the *Streptomyces* isolates with the highest EI observed in the present study may be tested for degrading lignocellulose in composting.

Lipase activity

The enzyme lipase (EC 3.1.1.3) is an important catalyst in biotechnology due to its wide versatility. Lipase can be applied in different industrial processes such as food processing, oils and fats, detergent manufacturing, drug synthesis, cosmetics, and many other products. Microbial lipases have also been used in grease trap waste for the treatment of heavy oils and grease (Bornscheuer, 2002).

The diverse characteristics of lipases produced by microorganisms appear to have evolved to guarantee the fast and efficient access of the microorganism to different sources of

organic matter (Roveda et al., 2010). Therefore, this group of enzymes is very attractive for industrial applications and recycling processes of lipid-rich compounds (Bornscheuer, 2002).

Despite the knowledge on lipid metabolism in many species of *Streptomyces*, studies on lipolytic activity in this group are still incipient. Mohamed et al. (2015), evaluating the lipase activity of streptomycetes from soil samples of Taif (Saudi Arabia), detected lipolytic activity in 91.3% of the isolates. Regarding the EI, Karanja et al. (2010) observed values varying between 3.0 and 4.2 for the lipolytic activity of *Streptomyces* isolated from soils in Kenya.

In the present study, the highest EI value for lipase was only 2.60, which was lower than the lowest EI value measured by Karanja et al. (2010). These results may be due to the close relationship between the abundance of lipid compounds in the environment and the specific characteristics of the enzymes produced by each microorganism (Gomes et al., 2007). Nowadays, the use of immobilized lipase or whole cell catalysts is one of the most promising methods to produce renewable and environmentally friendly alternative biofuels compared to the non-renewable fossil combustible (Yan et al., 2014).

Molecular characterization

The molecular data analysis based on the partial sequencing of the 16S rRNA gene enabled the identification of a total of 49 species, being 38 represented by only one isolate and 11 molecular species with more than one strain. Four of the twelve reisolates identified by the morphological traits were confirmed by the molecular analysis.

The three genera of *Actinobacteria* identified comprise two distinct families of the order *Actinomycetales*. The genus *Amycolatopsis* belong to the family Pseudonocardiaceae and the genera *Streptomyces* and *Kitasatospora* are classified in the family Streptomycetaceae. However, the three genera are very closely related genetically, and many strains have been misidentified by different authors as belonging to any of these three genera (Ward and Bora, 2015).

Cluster analysis of the morphological and biochemical data grouped the three genera together (data not shown). However, considering only the molecular data, two clusters were observed with the genus *Amycolatopsis* separated from *Streptomyces* and *Kitasatospora*. These results using different criteria reinforce the idea that these subgroups of the phylum *Actinobacteria* may be recognized as representing a complex of intimately related species, thus difficult to be separated into distinct taxa with the current methods used for species and genus identification. Even studies using detailed molecular data analysis of the 16S rRNA gene, a consensus does not exist about the status of various taxa among systematists of the phylum *Actinobacteria*. In fact, the variations within the 16S rRNA genes of the Actinobacterial group, even in the variable regions, are insufficient to clarify doubts concerning the identification of species and to estimate the species divergence within a genus or the phylogenetic relationships among genera (Kämpfer et al., 2014); this is the case for the genus *Kitasatospora* proposed by Omura et al. (1982), which the identity as a valid genus has been changed within years (Zhang et al., 1997; Girard et al., 2014). Bacterial strains identified as belonging to *Kitasatospora* and Streptomyces exhibit similar lifestyle and morphological traits (Ichikawa et al., 2010). In fact, the differentiation between *Kitasatospora* and *Streptomyces* strains is only based on the composition of peptidoglycan in the cell wall. Therefore, the presence of LL-isomer of diaminopimelic acid (DAP) in the aerial mycelia and meso-DAP in the vegetative mycelia of *Kitasatospora* is the main criterion used to discriminate strains between *Kitasatospora* and *Streptomyces*, while the latter display LL-DAP in both the aerial and vegetative mycelia

(Zhang et al., 1997; Takahashi, 2017). Likewise, representatives of the genus *Amycolatopsis* and other six genera in the family *Pseudonocardiaceae*, which was proposed based on the 16S rRNA sequence analysis, vary greatly in their morphology and other phenotypic characteristics (Embley et al., 1988). However, the current members of Pseudonocardiaceae contain meso-DAP in their cell wall, and the presence of arabinose and galactose sugars in whole-cell hydrolysates is used as accepted criterion for the diagnosis and species identification in this group (Embley et al., 1988).

Comprehensive comparative studies including protein-coding gene sequences with higher phylogenetic resolution and genome-based studies are needed to clarify the relationships and species delineation within the Streptomycetaceae (Kämpfer et al., 2014).

CONCLUSION

The ability to degrade several substrates reveals that isolates from the genus *Streptomyces*, *Kitasatospora*, and *Amycolatopsis* of the phylum *Actinobacteria* have high biotechnological potential uses and may be used in future studies intended to new sources for enzyme production.

Conflicts of interest

The authors declare no conflict of interest.

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