

Short Communication

Preliminary study on some actinomycetes and evaluation of their potential antagonism against fungal pathogens

Zahaed Evangelista-Martínez

Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco, A.C.
Unidad Sureste. Calle 30 No. 151 (7 y 7A). Col. García Ginerés. CP 97070. Mérida,
Yucatán. México

ABSTRACT

Aims: Control of microbial pathogens by using antagonistic microorganisms is a promising alternative to chemical fungicides. The objective of the present study was to isolate and characterize soil actinomycetes and to their inhibitory activity against some fungal plant pathogens.

Place and Duration of Study: National Park "El Chico", Hidalgo State, and Laboratory of the Southeast Unit of CIATEJ, Yucatán, México, between June 2010 and May 2011.

Methodology: Actinomycete species were isolated from six composite soil samples using microbiological standard procedures. All isolates were phenotypically characterized.

Antagonistic isolates were selected according to the inhibitory growing of *Fusarium* sp. and *Candida albicans*. Afterwards, a new evaluation for the isolates selected was done against *Helminthosporium* sp., *Curvularia* sp., and *Aspergillus niger*. Actinomycetes were identified performing an analysis of the 16S rDNA gene sequence.

Results: 164 actinomycete strains were characterized by morphological and biochemical features. Six of them, inhibited the growth of *Fusarium* sp. and *C. albicans* from 5 to 10 mm distance in between the actinomycete's colony growth border of fungal or yeast. A growing reduction from 50 to 83 % in the *in vitro* antagonism assays was observed for *Helminthosporium* sp., *Curvularia* sp., and *Aspergillus niger*. Results in disc diffusion assays suggested an inhibitory growing capacity of CACIA-1.46HGO for *P. capsici*, this behavior could be due to the production of diffusible compounds related to secondary metabolism, hydrolytic enzymes, or both of them. Four antagonistic isolates were identified into *Streptomyces* genus and one as *Microbacterium* sp. through 16S rDNA gene sequence.

Conclusion: Actinomycetes could be potentially a control tool to prevent several fungal commercial plants diseases. However, *in situ* isolate evaluations are suggested to be investigated.

Keywords: antagonism; actinomycetes; biocontrol; *Streptomyces*, fungal pathogen

1. INTRODUCTION

Pathogenic microorganisms affecting plant health are a major and chronic threat to food production and ecosystem stability worldwide [1]. For decades, control and manage of fungal plant diseases has been dependent on the chemical synthetic fungicides. However, constant use of fungicides may cause accumulation of toxic compounds potentially hazardous to humans and environment.

Tel.: 01 52 9999202671.

E-mail address: zevangelista@ciatej.net.mx

Biological control of fungal plant diseases using native microorganisms is a promising alternative to chemical fungicides [2]. The biocontrol agents are not limited to a specific group, and given the microbial diversity of soil ecosystems it is probable that the full spectrum of potentially effective strains has barely been explored [3]. Nowadays, a variety of biopesticides based on bacteria and fungi are registered and available on the market and have shown potential to act as effective biocontrol agents of plant pathogens, by suppressing its growth and promote overall plant vigor and yield [4].

The actinomycetes group enclosed species of gram positive bacteria broadly distributed in both terrestrial and aquatic ecosystems, and well-known for the production of many important bioactive metabolites [5]. The diversity and activity of terrestrial *Streptomyces* species have been estimated on different ecosystems, such as beach and dune systems [6], saline farmlands [7], and tropical soils [8].

Biological control products based on actinomycetes have been developed to suppress the growth of pathogenic microorganisms in fields. Many species has been isolated from different ecosystems, and evaluated *in vitro* and *in vivo* against species of *Aspergillus*, *Fusarium*, *Trichoderma*, *Curvularia oryzae*, *Pyricularia oryzae* and *Bipolaris oryzae* [9,10]. Thus, it is important to search and to identify new isolates adaptable to different environments and soil conditions. Hence the importance of characterize and identify potential biocontrol agents previously to study the mechanism behavior to diseases control. For the above, the objective of this study was to isolate and characterize soil actinomycetes and to evaluate their antagonism against selected pathogen fungus.

2. MATERIAL AND METHODS

2.1 Study site and sample collection

Soils for actinomycetes isolation were collected from a mesophyll mountain forest localized at "El Chico", a National Park in Hidalgo State, Mexico. Six locations were sampled between coordinates 20°10'10" to 20°13'25" north latitude, 98°41'50" to 98°46'02" west longitude (altitude 2426-2949 m). In turn, for each location six subsamples were taken from the soil surface (10 cm depth) into an area of 10 m in diameter to prepare a composite sample [11]

2.2 Fungal strains

Aspergillus niger NRRL-3 and *Fusarium* sp. (CDBB:1172) were obtained from National Culture Collection, CINVESTAV-IPN, México. *Curvularia* sp and *Helminthosporium* sp were isolated from Chrysanthemums plants, and *Phytophthora capsici* was obtained from infected plants of *Capsicum chinense*.

2.3 Isolation of actinomycetes

Soil samples were pretreated for 1 hour at 70° C. 10 g of the soil was deposited in 100 ml of sterile distilled water and serial dilutions up to 10⁻⁴ were inoculated onto ISP2 and ISP3 agar media [12], supplemented with nalidixic acid (12.5 µg ml⁻¹) and cyclohexamide (50 µg ml⁻¹). Incubation was carried out at 29°C for 2 to 4 weeks. Isolated strains were stored as Preservation Stocks (PS) of spore or cells at -20°C in 20% (w/v) glycerol. General Inoculums (GI) of spore or cells were prepared from PS to make a turbid suspension of 0.5 McFarland standards (10⁸ CFU ml⁻¹).

2.4 Phenotypic characterization

Morphological and physiological characterizations were based on International Streptomyces Project Methods [12]. Enzymatic activity of lipase [13], protease [14] and L-asparaginase [15] were determined in Petri dishes.

2.5 *In vitro* antagonistic activity

An initial antagonistic screening over *Fusarium* sp. (CDBB:1172) and *Candida albicans* by dual culture and streaking assays on Petri dishes were carried out to select antagonistic actinomycetes. A growth inhibition zone between the borders of the actinomycete and fungi/yeast greater than 5 mm was the criteria to choice strains. Then, the antagonistic activities of isolates were evaluated by dual culture assays against *Aspergillus niger* NRRL-3, *Fusarium* sp., *Curvularia* sp. and *Helminthosporium* sp. Initially, 2 μ L of the GI of spore's solution was inoculated near the border of a PDA Petri dish, and incubated for 0 and 5 days at 29°C before fungus inoculation. Afterward, an agar block (6 X 6 mm diameter) with fungal mycelium was placed in the center of the dish, and maintained for additional 7 days at 29°C. "Zero days" of incubation contemplates the simultaneous inoculation of the actinomycete and fungus. The percentage of inhibition (PI) was determined using the formula: $PI (\%) = [FR-AR]/[FR] \times 100$, where FR represents the fungal growth radius (mm) of a control culture and AR the distance (mm) of the fungal growth in the direction of actinomycetes [16]. All experiments were done by triplicate.

2.6 Production of diffusible substances by *Streptomyces* sp. CACIA-1.46HGO

Antifungal activity of diffusible substances produced by strain CACIA-1.46HGO were evaluated by placing in the center of a PDA Petri dish an agar plug (6 mm diameter) taken close to the border of a 14-old days colony culture grown on ISP2 agar medium, together with two agar blocks (6 X 6 mm) with actively growing mycelium of *Phytophthora capsici*. Dishes were maintained for 7 days at 29° C. As positive control, an agar plug with the streptomycete mycelium growth was used. Assays were done by triplicate.

2.7 Determination of extracellular chitinase, cellulase and xylanase activity.

Extracellular activity for chitinase, cellulase and xylanase enzymes were carried out on Petri dishes of ISP9 agar medium supplemented with 1 % chitin from shrimp shells, 1 % carboxymethyl cellulose and Whatman No. 1 filter paper, and 1% xylan from birchwood, respectively. 2 μ L of the GI of each isolate were inoculated on the dishes and incubated for 14 days at 29° C. The enzymatic activity was detected using gram's iodine [17]. The ratio "diameter of the hydrolytic halo/diameter of the colony" was calculated to determine the potency (Enzymatic Index) of the isolates for enzyme production. Assays were done by duplicate.

2.8 Molecular identification by analysis of the 16S rDNA sequence

Actinomycetes were identified by sequence analysis of 16S rRNA gene with oligonucleotides fD1 and rD1 [18]. Partial sequences were analyzed for homology using the BLASTN program and the non-redundant GeneBank database (<http://blast.ncbi.nlm.nih.gov/>). Phylogenetic analysis was carried out at Phylogeny.fr web page: <http://www.phylogeny.fr/version2.cgi/index.cgi>. The partial 16rRNA gene sequences obtained in the present study were deposited in GenBank database under accession JQ400108, and JX534230 to JX534234.

2.9 Statistical Analysis

115 A Two-way ANOVA was used to determine differences between the selected actinomycetes
 116 and their antagonistic activity, and the effect of pre-inoculation time over the PI value.
 117 Statistical analysis was done using statistical package Statgraphics Centurion version XV
 118 V16.2.04 (Statpoint Technologies, Inc.) and statistical significance at $P=0.05$ was used.

119

120 3. RESULTS AND DISCUSSION

121

122 3.1 Phenotypic characterization

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124 One hundred sixty-four putative actinomycetes were isolated with a predominance of spore
 125 producer isolates that could be likely due to isolation conditions [19]. All isolates were
 126 characterized by morphology, physiology and carbon source assimilation (data not shown).
 127 The antagonism exerted for the actinomycete isolates over *Fusarium* sp. and *Candida*
 128 *albicans* was used to select six isolates and evaluate their antagonistic activity (Table 1).
 129 Morphologically five isolates showed well-developed substrate mycelium, filamentous,
 130 branched and mostly unfragmented with a smooth appearance that develops to an aerial
 131 mycelium that either appeared floccose, granular or powdery. The sixth isolate (CACIA-
 132 1.29HGO) develops smooth, circular, convex, non-filamentous, transparent and yellow
 133 colonies with gram positive short-rod cells. Colors of colonies were also noted.
 134 Physiologically, some isolates degrade starch, casein and not at all produce melanin. The
 135 extracellular enzymatic activity of asparaginase and protease were also detected; CACIA-
 136 1.49HGO was the unique strain to express lipolytic activity. Enzymatic activity determination
 137 is a relevant characteristic of soil microorganisms considering that in terrestrial habitats the
 138 actinomycetes are excellent saprophytes and prolific producers of extracellular enzymes for
 139 initial decomposition of organic material, N-mineralization and assimilation of extracellular
 140 nitrogen sources [20]. Also, actinomycetes are producers of important industrial enzymes
 141 such as chitinases, cellulases, amylases, xylanases and pectinases [21].

142

143 Phenotypic characterization of selected isolates was fulfilled by evaluate the assimilation of
 144 carbon sources and its role in the mycelium development and spore production
 145 (Supplementary Table S1).

146

147 Table 1. Characterization of soil actinomycetes isolates

148

	CACIA- 1.9HGO	CACIA- 1.29HGO	CACIA- 1.46HGO	CACIA- 1.49HGO	CACIA- 2.22HGO	CACIA- 3.23HGO
Characteristics on ISP2						
SM ^a	White	Yellow	Brown	Beige	Brown	White
AM ^b	White	-	White	White	White	White
S ^c	Gray	-	Gray	Grey	Gray	Brown
Diffusible pigments	None	-	Yellow light	None	Violet	None
Growth	F ^d	S-R ^e	F	F	F	F
Test						
Gram	+	+	+	+	+	+
Starch	+	+	+	+	+	+

Casein	+	+	+	-	+	+
Melanin	-	-	-	-	-	-
Extracellular activity						
Lipase	-	-	-	+	-	-
Asparaginase	+	+	+	-	+	+
Gelatinase	+	-	+	+	+	+
Antifungal activity †						
<i>Fusarium</i> sp.	+	+	+	+	+	+
<i>C.albicans</i>	+	+	+	+	+	+

† Positive (+), considers at least 5 mm of inhibition of growth between the margin of fungal growth in the direction of the actinomycete colony. ^aSM, color of substrate mycelium; ^bAM, color of aerial mycelium; ^cS, color of spore mass; ^dF, filamentous; ^eS-R, short-rod.

3.2 Phylogeny of actinomycetes

Analysis of partial nucleotide sequence of 16S rRNA gene indicated that five isolates matched entries resembling *Streptomyces* and *Microbacterium* species (95 to 99% proximity). Analysis of the CACIA-2.22HGO ribosomal sequence showed a closest proximity with a *Streptomyces* strain (identity of 84 %), which suggests that this isolate could be part of a novel actinomycete group (Supplementary Table S2). Therefore, the taxonomic position of the above strain remains uncertain. Phylogenetic tree further confirms the above results (Figure 1). Four isolates were sorted into *Streptomyces* cluster and CACIA-2.22HGO was clearly constrained in a separate branch. CACIA-1.29HGO is sorted into *Microbacterium* group, which is confirmed by its morphology that fits to members of the genus [22].

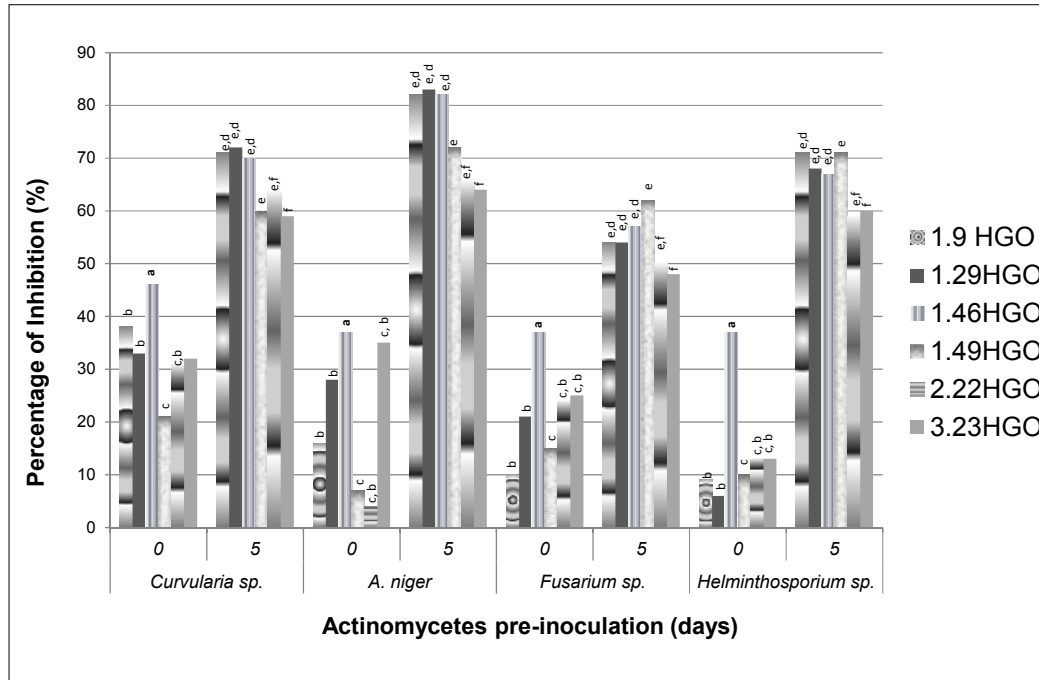


Fig. 2. Inhibition of fungal growth (PI) by actinomycetes. Values represent means of three replication, Bars with the same letter are not significantly different (P= 0.05).

3.4 Preliminary evaluation of antifungal compounds

Considering that over 50 % of antimicrobial compounds produced by microorganisms have been isolated from actinomycetes, particularly from *Streptomyces* [24], a preliminary assay was carried out to evaluate if the antagonistic activity of *Streptomyces* sp. CACIA-1.46HGO could be related to the accumulation of compounds diffusible to the agar medium, e.g. secondary metabolites and/or hydrolytic enzymes. Figure 3 showed the inhibition of mycelium of *Phytophthora capsici* by the action of diffusible substances contained in the agar block (right panel), with a PI of 15.9 % (SD $\pm$ 0.2). An agar block with the streptomycete mycelium was used as control (center panel, PI of 13.4 % $\pm$ 0.02).

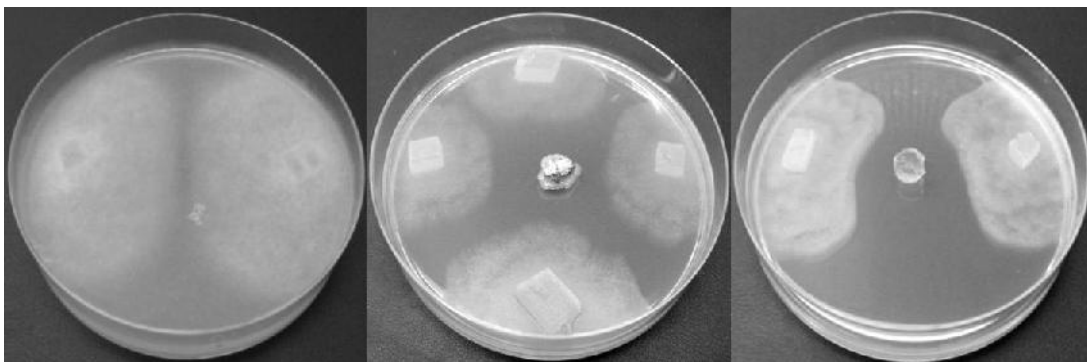


Fig. 3. Inhibitory activity of metabolites diffused into the agar media of growth from a fourteen days culture of *Streptomyces* sp CACIA-1.46HGO against *Phytophthora capsici*. Left, control Petri plate with *P. capsici*; center, inhibition of fungal growth by

a disc of agar with streptomycete mycelium; right, inhibition of fungal growth by diffused metabolites from a disc of agar media.

In order to advance in the identification of the antagonist compounds, Petri plate assays were done to detect the extracellular enzymatic activity of chitinase, cellulase and xylanase. The Enzymatic Index (EI) for each strain was calculated (Table 2 and Supplementary Figure S1). Results showed that *Streptomyces* sp. CACIA-1.9HGO, CACIA-1.46HGO and CACIA-3.23HGO express a high level of extracellular activity for chitinase, cellulase and xylanase, which suggest that at least the chitinase activity contribute to the antagonism exerted against fungal pathogens.

Table 2. Enzymatic Index of streptomycetes isolates

	CACIA-1.9HGO	CACIA-1.46HGO	CACIA-3.23HGO
Cellulase ^a	5.8±0.6	5.5±0.5	4.3±0.3
Cellulase ^b	6.2±0.5	5.1±0.4	5.2±0.1
Xylanase ^c	6.9±0.0	6.1±0.1	3.1±0.2
Chitinase ^d	6.7±1.2	6.9±1.2	4.7±0.2

^a Enzymatic activity with Carboxymethyl cellulose (CMC); ^b Enzymatic activity with Whatman 1 filter paper. Isolates CACIA-1.29HGO, CACIA-1.49HGO and CACIA-2.22HGO do not growth on the substrates used.

In conjunction, the results exposed above suggest that actinomycete isolates have the potential to be used as agents to control plant diseases such as, root rots on chili by *P. capsici* or leaf blight on ornamentals caused by *Helminthosporium* or *Curvularia* species. In this sense, many species of *Streptomyces* are known to be potent producers of many antibiotics against soil-borne pathogens [25,26]. Furthermore, chitinase actinomycete producers have received attention due to its use as a biocontrol agent [27]. Also, *Streptomyces* strains have been evaluated to reduce the incidence of seed pathogen fungi *Aspergillus* spp in maize [28], and to antagonize human pathogens such as *Candida albicans* and *A. niger* [29].

4. CONCLUSION

The present study shows that soil actinomycetes isolated from the National Park "El Chico" holds potential biocontrol activities against fungal pathogens. These members of actinomycetes were isolated from an ecosystem characterized by the presence of oyamel, cedar, oak, pine and tlascal trees with epiphytes and ferns, which are mainly located in mountains, canyons and sites where conditions of humidity are favorable for decomposition of plant litter and residues to support a high microbial abundance at the topsoil's surface.

ACKNOWLEDGEMENTS

This study was supported by FOMIX Conacyt-Hidalgo grant No. 97444 (Characterization and Conservation of Germplasm Bank of Actinomycetes isolated from "El Chico" National Park, Hidalgo México). *Curvularia* sp., *Helminthosporium* sp. and *P. capsici* were kindly provided by Dr Alberto Uc-Varguez. I acknowledge to Diana Baigts PhD for the technical helpful in the statistical analysis and English correction.

250 **COMPETING INTERESTS**

251

252 "Author has declared that no competing interests exist".

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Carbon Source*	CACIA-1.9HGO		CACIA-1.29HGO		CACIA-1.46HGO		CACIA-1.49HGO		CACIA-2.22HGO		CACIA-3.23HGO	
	G †	S ‡	G	S	G	S	G	S	G	S	G	S
None	+/-	-	+/-	-	+/-	-	+/-	-	+	-	+/-	-
D-Glucose	+	+	+	-	+	+	-	-	+	+	+	+
Sucrose	+/-	-	+	-	+/-	-	-	-	+	-	+	-
L-Xylose	+	-	-	-	-	-	+	-	+	+	-	-
D-Xylose	+	+	+	-	+	-	-	-	+	+	+/-	-
Maltose	+	+	+	-	+	-	+	-	+	+	+	+
D-Arabinose	+	+	+	-	+	+	+	-	+	+	+	+
D-Lactose	+	+	+	-	+	+/-	-	-	+	+	+	+
D-Raffinose	+	+	+	-	+	+/-	-	-	+	-	+	+/-
D-Cellobiose	+	+	+	-	+	+	-	-	+	-	+	+/-
D-Fructose	+	+	+	-	+	+	-	-	+	+	+	+/-
L-Rhamnose	+	+	+	-	+/-	-	-	+	+	+	+	+/-
Manitol	+	+	+	-	+	+	-	-	+	-	+	+/-
Myo-Inositol	+	+	+	-	-	-	+	+	+	-	+	+/-
Glycerol	+	+	+	-	+	-	+	+	+	+	+	+

* ISP9 medium + 1% (w/v) of carbon source. † G, growth of substrate mycelium: (+), positive utilization and vigorous develop of mycelium substrate; (+/-), positive utilization and little develop of mycelium substrate; (-), negative utilization and no develop of mycelium substrate. ‡ S, Sporulation: (+), vigorous spore formation; (+/-), weak spore formation; (-), no spore formation.

Table S2. Blast matches for the closest 16S rRNA gene sequence of the actinomycetes isolates

16S rDNA gene sequence match by BLASTN						
Isolates	Sequence ID	Related organism	Sequence ID	Bits	(%)	References*
CACIA-1.9HGO	JX534230	<i>Streptomyces</i> sp. P911A	EU977303	605	95	Smith et al., 2008
CACIA-1.29HGO	JX534231	<i>Microbacterium</i> sp. FI1010	JQ691553	1478	99	Sanchez et al., 2012
CACIA-1.46HGO	JQ400108	<i>Streptomyces</i> sp. BA2	EF494203	1240	99	Yilmaz et al., 2008
CACIA-1.49HGO	JX534332	<i>Streptomyces longisporoflavus</i> NRRL_ISP-5165	EF178687	1186	97	Unpublished
CACIA-2.22HGO	JX534233	Unknown	-	-	-	-
CACIA-3.23HGO	JX534234	<i>Streptomyces olivochromogenes</i> A018	AB369121	1137	99	Lee et al., 2008

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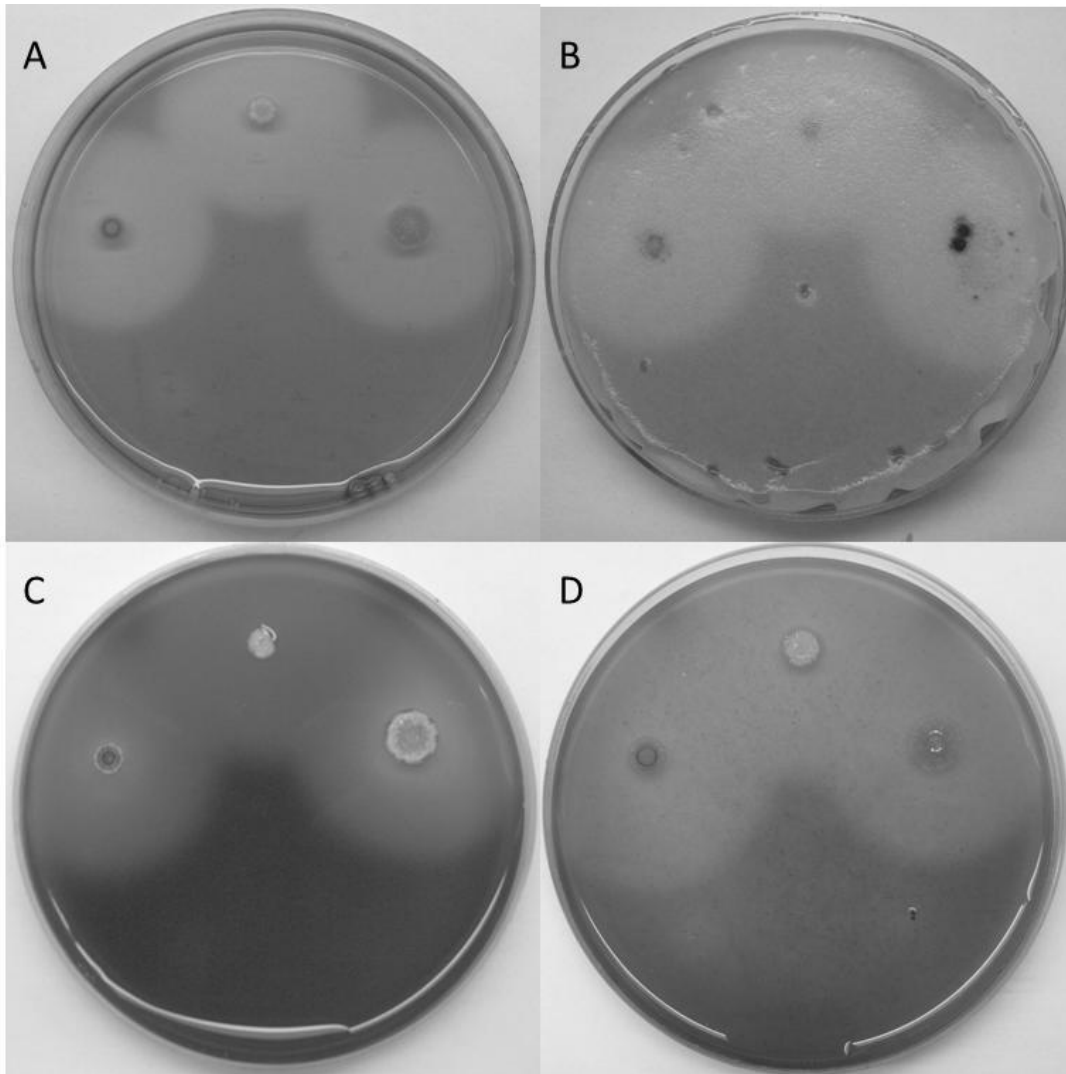


Fig. 2. Extracellular enzymatic activity of actinomycetes. Left, CACIA-3.23HGO; center, CACIA-1.9HGO; right, CACIA-1.46HGO. A, Cellulase activity on carboxymethyl cellulose; B, Cellulase activity on Whatman No.1 paper; C, Xylanase activity; D, Chitinase activity.