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Taxonomic Characterization and Antimicrobial Activity of Actinomycetes Associated with Foliose Lichens from the Amazonian Ecosystems

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Abstract: The study of habitats that have been few explored with purpose of obtaining composts with biotechnological interest that has been produced by the Amazonian's biological diversity it's a strategy that will allow the discovery of important bioactive principles. In microbial biodiversity, the actinomycetes represents the most important bacterial group. They have a filamentous organization that occur in great varieties of substrata and present a great application in pharmaceutical industry. The scope of this work was to analyze taxonomically the actinomycetes associated at lichens and to determine the capability of these bacteria for antibiotic producing. Ten samples of foliose lichens were collected in area of Universidade Federal do Amazonas (UFAM), South Section. For the isolation of actinomycetes were utilized the culture media Yeast Extract-Malt Extract Agar–Starch (ISP-2A), Casein-Starch Agar (CAA), Raffinose-Histidin Agar (RHA) and Water Agar (AA), added with antifungics. Were isolated 71 actinomycetes associated to foliose lichens. The isolated were tested for antimicrobial activity against eight microorganisms-test by the techniques with cultivation in solid medium and broth culture. Among the actinomycetes tested by solid medium 80% shown antimicrobial activity, mainly against *Aspergillus niger*, *Candida albicans* and *Staphylococcus aureus*. In assay by broth cultivation 79% of the actinomycetes inhibited the growth of microorganisms-test, although the higher activities were against *Mycobacterium smegmatis* and *Staphylococcus aureus*. The isolated antimicrobial activity varied from moderate (halo=13 at 18 mm) to high (halo=19 at 35 mm) activity. It was observed that 68% of isolated presented high antimicrobial activity. The identification of the actinomycetes was done by the macro and micromorphological determination, physiologic tests and by the determination of aminoacids from the cell wall, and most of them belonging to genus *Streptomyces*. The microorganisms were preserved by freezing at -20 oC and by preservation of actinomycetes colony directly in water (Castellani's method).

Key words: Amazon Biodiversity, Secundary Methabolites, Antimicrobial Activity, Actinomycetes, Lichens.

INTRODUCTION

In global level is considered that the diversity of microorganisms exceeds in order of some thousands the diversity of plants and animals. Brazil has about 20% of the world biological diversity (SUDAM, 1995; Dias, 1996; Souza et al., 2004) and a considerable portion of this biodiversity is located in Amazonian ecosystems, incommensurable source of raw materials for the most several areas of biotechno logical application.

Among the microbial biodiversity, the actinomycetes represent a bacteria group of filamentous organization,

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many times ramified, whose common characteristic is the formation of aerial and/or vegetative mycelium in some stage of its life cycle (McCarthy; Williams, 1990). The actinomycetes occur in a great diversity of natural and artificial habitats, growing in a large variety of substrata (Williams; Cross, 1974).

The lichen is a symbiotic association between a fungal and a microorganism photosynthetic. The lichen fungal component (mycobiont) is in great majority a fungal of the phylo Ascomycota (above 95%) and, rarely, Basidiomycota. The photosynthetic component (photobiont, also knew ficobionte in allusion to the algae) it is, in general, a Chlorophyta or a cyanobacteria (Seaward, 1977; Nash, 1996).

Even the symbiotic components of the lichens have already been described extensively, however the microbial community that habits these niches still remain not characterized. The lichens constitute a rich reservoir for the isolation of a great variety of actinomycetes diversity, many of them representing an unexplored source, rich in secondary metabolites (González et al., 2005).

In nowadays, more than 70% of the species of the bacteria that cause infections are resistant at least one of the antibiotics commonly used on therapeutics as it is emphasizes for Overbye and Barret (2005). The actinomycetes have especially been useful in the pharmaceutical industry for limitless capacity to produce secondary metabolites with many chemistries structures and biological activities.

The actinomycetes bioprospection in innovative habitats is a strategy that makes possible the discovery of relevant biotechnological bioactive principles, more scientific knowledge about the microbial diversity, better understanding about functions of microbial communities in the environment and knowledge of these interactions with others components of the biodiversity.

This work proposes to characterize taxonomically the actinomycetes isolated from folioses lichens and to determine the capacity of these bacteria in producing antibiotics.

MATERIAL AND METHODS

Samples:

Ten samples of foliose lichen were collected from the trees of Amazon Federal University (UFAM) campus, south sector, (Sul 3º 5’ 56’’ / Oeste 59º 58’56’’), Manaus/AM.

Collect, Packing and Samples Transportation:

The samples were collected with a cleaned metal spatula, individually packing in sterile Petri’s dishes and kept in isotherm boxes, being processed at laboratory.

Isolation of Actinomycetes from Liquens:

Around 300 mg of each lichen were weighted washed twice with sterile distilled water and homogeneized with 30 mL of sterile distilled water. After this, it was made successive dilutions for the actinomycetes isolation. Were inoculated 0,1 mL of dilutions in culture medium Yeast Extract-Malt Extract Agar (ISP-2), Starch-Casein Agar (SCA), Rafinosis-Histidine Agar (RHA) and Water-Agar (WA), added with antifungal cycloheximide (80 μg/mL) (González et al., 2005) or nistatine, and the plates were incubated at 30 oC during 21 days.

Actinomycetes Identification:

Macromorphology and Micromorphology Determination:

The actinobacteria inoculated on Petri’s dishes with media ISP-2, ISP-6 and ISP-7 at 30 oC per until 21 days permitted the colony macromorphology study. The color and production of soluble melaninic pigment were visually evaluated. The micromorphology study of isolated actinomycetes on media ISP-2, ISP-3, ISP-4 and ISP-5, incubated at 30 oC until 21 days, and morphology and spore chains format were evaluated through optic microscopy.

Determination of Amino Acid in Cell Wall:

The study of actinomycetes cell wall consisted in determination of present amino acid type (Staneck; Roberts, 1974). The actinomycetes were cultivated in ISP-2 Broth at 30 oC under shaking at 180 rpm per 72 hours. After this period, the cell mass was filtrated at vacuum and dry at 50 oC per two hours. Were transferred 30 mg of actinomycete dry mass to tube (10 x 90 mm), acidified with 1 mL of HCl 6 N solution and the cell wall was hydrolysed at 100 oC per 16 hours. The insoluble material was removed using one holed eppendorf containing glass wool and washed with 1 mL of distilled water. The filtrated was transferred to

balloon of round bottom and evaporated to remove the acid remaining. Several washes were made until the complete retreat of acid. The material free of the acid was resuspensed in 0.1 mL of distilled water, transferred for eppendorf tubes and stored in freezer until the accomplishment of thin layer chromatography (TLC).

The mobile phase was composed by methanol-water-acid chloridric 6N-piridine (80:26:4:10, v/v) and the stationary phase by cellulose plates with 20 x 20 cm of dimentions (Merck no 5716). In stationary phase were applied, side by side, 2 mL of diaminopimelic acid standard (DAP) at 0.19% (m/v), 2 mL of the hydrolysis of unknown samples and 2 mL of the hydrolysis of known actinomycetes: *Streptomyces olindensis* (DAUFPE 5622), *Streptomyces regensis* (DAUFPE-3053), and *Nocardia asteroides* (DAUFPE-3503). The cube was previously saturated for two hours and run per approximately five hours. The cellulose plate was dry at room temperature, sprinkled with ninhidrine solution at 0.2% m/v, and warmed at 100 oC during five minutes and then visualizated the LL-DAP e *meso*-DAP isomers.

Antimicrobial Activity Characterization:

The isolated actinomycetes were characterizated for antimicrobial activity through solid (Gelose Block Method) and broth media. The microrganisms-test used in this experiment are presented in Figure 1, with the respective growth conditions.

Microorganisms-test	Culture media	Temperature	Period of cultivation
Aspergillus niger (CCT 1357)	Sabouraud Agar	30 °C	72 h
Candida albicans (CCT 0776)	Sabouraud Agar	30 °C	48 h
Staphylococcus aureus (CCT 1352)	Müeller-Hinton Agar	37 °C	24 h
Bacillus subtilis (CCT 1359)	Müeller-Hinton Agar	37 °C	24 h
Listeria monocytogenes	Müeller-Hinton Agar	37 °C	24 h
Pseudomonas aeruginosa (CCT 3971)	Müeller-Hinton Agar	37 °C	24 h
Escherichia coli (CCT 0547)	Müeller-Hinton Agar	37 °C	24 h
Mycobacterium smegmatis (DAUFPE-71)	Müeller-Hinton Agar	30 °C	72 h

Fig. 1: Microrganisms-test used for characterization of antimicrobial activity.

Antimicrobial Activity Assay in Solid Media:

According to Ichikawa et al. (1971), the methodology also known as "Gelose Block Method" consisted in inoculate 0.1 mL of actinomycete suspension in the concentration of 106 to 107 CFU/mL, for the "spread-plate" technique in Petri plates containing 15 mL of the ISP-2 culture medium added with starch. After seven days of incubation at 30 oC, circular gelose blocks of 6 mm diameter were transferred for each plate containing, previously, the test microorganism, obtained by a suspension of standardized cells in approximated concentration of 1,2 x 106 CFU/mL. The plates were incubated respecting the physiologic characteristics of each test microorganism. After the incubation period of the test microorganism, the diameter of the growth inhibition of each block was measured and determinate the antimicrobial activity of the actinomycete.

Antimicrobial Activity Assay in Broth Culture:

The antimicrobial activity by cultivation of actinomycetes in broth was developed with based on methodology describes by Waksman and Woodruff (1941) that consisted in growth the actinomycete under shaking at 150 rpm in MPE broth until a period of 96 hours at 30 oC. The concentration of inoculated cells was 106 a 107 CFU/mL. At ending of the growth period, 10 mL of metabolic liquid was transferred into a paper disk with 6 mm diameter and introduced in Petri plates containing, previously, the test microorganism, obtained by a suspension of standardized cells in approximate concentration of 1.2 x 106 CFU/mL, showed by the "spread-plate" technique. After the incubation period, the inhibition growth halo of microorganisms-test, of each disk, were measured and determinated the inhibitory activity of actinomycetes.

Preservation of Actinomycetes:

The isolated microorganisms were preserved by freezing at -20 oC and by water preservation technique according Castellani (Muro; Luchi, 1989).

RESULTS AND DISCUSSION

Microorganisms Isolation:

From ten samples of foliose lichens, collected on tropical trees of Amazon area, it was isolated a total of 71 actinomycetes (Table 1).

Table 1: Isolation of actinomycetes from liquens in differents culture media.

LICHENS	CULTURE MEDIA ISP-2A	SCA	TOTAL
L1	03	01	04
L2	12	02	14
L3	04	01	05
L4	02	00	02
L5	17	04	21
L6	09	02	11
L7	05	01	06
L8	03	nd	03
L9	03	01	04
L10	01	nd	01
TOTAL	59(83%)	12 (17%)	71(100%)

According to Table 1, these results show that among the four culture media used for the isolation of this filamentous bacteria, the higher efficiency was ISP-2A (83%), followed by SCA (17%), do not being detected none actinomycete growing on RHA or WA, at this experimental conditions. Kitouni et al., (2005), mention that the addition of some sources of carbon and nitrogen as starch, chitin, glycerol, casein, arginine, asparagines, in culture media make favors the growth of actinomycetes/microorganisms isolated of natural substrata in detriment of the nonfilamentous bacteria. Similar observations were verified by Matsuura (1998) for isolation of endophytic actinomycetes.

González (2005) isolated 337 actinomycetes from 25 samples from lichens of three different environments and the isolation rate varied 1 to 45 isolated per lichen, while our work varied 1 to 21 isolated. On the other hand, Cardinalle (2006) studying the microorganisms in nine lichens through molecular techniques obtained only four actinomycetes among 34 bacteria. Those results based the hypothesis that the bacterial communities composition in lichens can be influenced for several biotic and abiotic factors, which it can detach the lichens phylogenetic position, the geographical origin, the substrata, the microhabitat conditions and the pattern of the fungal secondary metabolites (mycobiont).

Identification of Actinomycetes:

The isolated actinomycetes were identificated by genus level with based on the macro and micromorphologic characteristics and in cell wall study. The morphologic characterization was determinate looking the spore chain format, the mycelia colors (aerial and vegetative mycelium) and pigment production in culture media (Table 2).

Table 2: Morphological characterization of actinomycetes isolated from lichens.

Samples	Spore Chain		Colony color	Color of soluble pigments	Melanin Pigment
	Format	Length			
L1-A1	spirales	short	dark grey	-	-
L1-A2	spirales	short	grey	-	-
L1-A3	spirales	larger	light grey	-	-
L1-A4	spirales	larger	brown	-	-
L2-A5	spirales	larger	dark brown	-	-
L2-A6	spirales	larger	brown	-	-
L2-A7	spirales	short	dark brown	-	-
L2-A8	retinaculiaperti	larger	cream-colored	yellow	-
L2-A9	retinaculiaperti	larger	dark cream-colored	yellow	-
L2-A10	*	*	dark grey	-	-
L2-A11	spirales	larger	dark grey	-	-
L2-A12	spirales	middle	light brown	-	-
L2-A13	retinaculiaperti	larger	brown	-	-

Table 2: Continue

L2-A14	rectiflexibles	larger	cream-colored with borders	-	-
L2-A15	rectiflexibles	larger	cream-colored with borders	-	-
L2-A16	retinaculiaperti	larger	brown dark	-	-
L2-A17	spirales	larger	grey	-	-
L2-A18**	*	larger	white with exsudate	-	-
L3-A19	spirales	middle	brown with exsud. amarelo	-	-
L3-A20	spirales	middle	light grey	-	-
L3-A21	spirales	larger	brown	-	-
L3-A22	spirales	larger	brown with grey	-	-
L3-A23	retinaculiaperti	larger	dark grey	-	-
L4-A24	rectiflexibles	larger	grey	red	-
L4-A25	spirales	larger	cream-colored	yellow	-
L5-A26	spirales	larger	brown	-	+
L5-A27	retinaculiaperti	larger	dark grey	yellow	-
L5-A28	spirales	middle	light brown	-	+
L5-A29	spirales	middle	brown	-	+
L5-A30	*	*	white with grey	-	-
L5-A31	retinaculiaperti	larger	cream-colored with brown	-	+
L5-A32**	retinaculiaperti	larger	light grey	yellow	+
L5-A33	spirales	larger	light grey	-	+
L5-A34**	rectiflexibles	larger	cream-colored with orange	-	-
L5-A35	retinaculiaperti	larger	dark grey	-	-
L5-A36	*	*	light grey	yellow	-
L5-A37	spirales	short	dark brown	-	+
L6-A38**	spirales	short	dark brown	-	+
L6-A39	spirales	larger	brown	-	+
L6-A40	spirales	short	brown	-	+
L6-A41	spirales	short	light brown	-	+
L6-A42**	spirales	short	brown	-	+
L7-A43	spirales	short	cream-colored	-	-
L7-A44	spirales	short	cream-colored	-	-
L7-A45	rectiflexibles	larger	brown	-	+
L5-A46	spirales	short	dark brown	-	+
L5-A47	spirales	short	brown	-	+
L5-A48	spirales	short	white with grey	-	-
L5-A49	spirales	short	cream-colored with brown	-	-
L5-A50**	retinaculiaperti	larger	white with yellow	yellow	-
L5-A51	spirales	short	white with grey	-	-
L6-A52	*	*	dark grey	-	-
L5-A53	N I				-
L5-A54	spirales	larger	dark grey	-	-
L5-A55	spirales	larger	dark grey	-	-
L6-A56	spirales	larger	brown	-	+
L6-A57	spirales	larger	dark brown	-	+
L6-A58	spirales	larger	light brown	-	+
L6-A59	spirales	larger	brown	-	+
L6-A60	NI				-
L7-A61	spirales	larger	brown	-	+
L8-A62	spirales	short	light brown	-	+
L7-A63	spirales	larger	brown	-	-
L7-A64	spirales	larger	brown	-	+
L8-A65	spirales	short	brown	-	+
L8-A66	spirales	short	brown	-	-
L9-A67	spirales	short	brown	-	+
L9-A68	spirales	short	brown	-	+
L9-A69	spirales	short	brown	-	+
L9-A70	spirales	short	brown	-	+
L10-A71	spirales	short	brown	-	+

L: Lichen — A: Actinomicete
NI: no identification — * Samples with differentiated morphology
** Exsudate presence — + Strains grew in media ISP6 e ISP7 that present melanin pigmentation.
- no detect

The Table 2 also shows that from 71 actinomycetes isolated, 75% showed the o spore chain format in *spirales*, 14% with the format in *retinaculiaperti* and 8% has *rectiflexibles* chains spore. Only in 3% did not observe the spore chain formation.

The colonies showed mycelium coloration that variated between grey to brown and white to cream-colored. From this actinomycetes in study, 28 samples presented melaninic pigmentation in the medium ISP-6 e ISP-7 and eight presented different diffusible pigmentation. The morphological characteristics and characterization of cell wall amino acid of actinomycetes isolated from foliose lichens indicated that from the total of 71 actinomycetes, 90% are *Streptomyces*, whose constituent of cell wall identified was the LL-DAP acid. Another representative genera was *Nocardia* (4%), identified by presence of meso diaminopimelic acid (*meso*-DAP) in cell wall. O'Leary (1988) describes that *Streptomyces* cell wall exist predominance of LL-DAP and glicine; in *Nocardia*, the constituent that showed predominance are *meso*-DAP, rabinose and galactose. Beyond this actinomycetes, were identified *Streptoverticillium* (1.5%) and *Nocardiopsis* (1.5%) based on the mycelium morphologic characteristics and the conidia disposition (longer conidia chain), respectivally. In micromorphologic observations did not determinated the samples L5-A53 e L6-A60 (3%) should be realize more deep studies for possible identification (Figure 2).

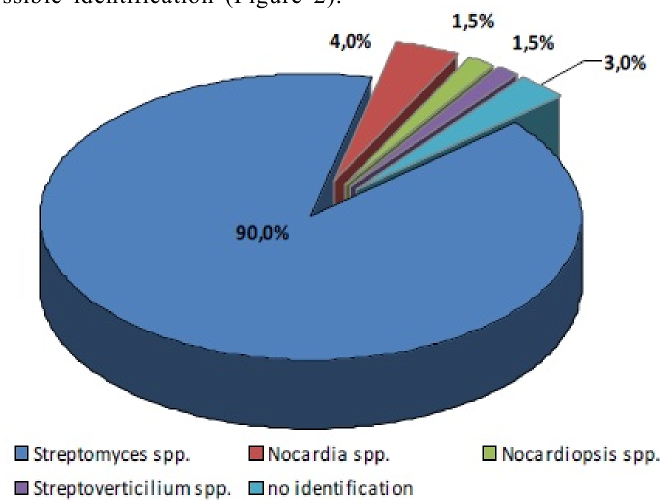

Fig. 2: Identification of actinomycetes genus isolated from foliose lichens.

Determination of Antimicrobial Activity:

The antimicrobial activity analisys of isolated actinomycetes supplied informations about the antimicrobial spectrum. Of the 71 actinomycetes tested 80% presented antimicrobial activity in Gelose Block Method. The average of isolated inhbition halo varied since low (halo = 8 at 12 mm), moderate (halo = 13 at 18 mm) to high (halo = 19 at 35 mm) activity. From this, 68% showed higher antimicrobial activity against the microorganisms-test.

The data referring to the antimicrobial activity of the 71 isolated in Gelose Block against the tested microorganism *(E. coli* CCT0547, *P. Aeruginosa* CCT3971, *S. aureus* CCT1352, *L. monocytogene, B. subitilis* CCT1359, *M. smegmatis* DPUFPE-71, *C. albicans* CCT0776, *A. niger* CCT1357) demonstrated the great majority of the actinomycetes expressed activity against *Aspergillus niger* (65%) and *Candida albicans* (56%), (Figure 3).

However, just only one actinomycete isolated shown activity against *Escherichia coli* and it was not observed antagonism against *Pseudomonas aeruginosa.* Vaara (1993) confirm this results and describes that aproximately 90% of the natural antibiotics should not inhibit organisms Gram-negatives. The reasons for this include, mainly, the external membrane presence in this bacteria that count channel that delayed the antibiotic entrance in cell and of the little hydrofilic composts, and the presence of a lipopolissacaride that produce the antibiotic transmembrane diffusion (Lima, 2006; Nikaido, 1996).

In assay for antimicrobial activity in liquid media MPE, 79% of the actinomycetes showed antimicrobial activity against to the tested microorganisms. The most antimicrobial activity was observed against *M. smegmatis* (62%), followed by *S. aureus* (41%). It was not observed activity against *E. coli.* This result is similar to obtained by the Gelose Block Method, also did not verified inhibition halo against the microorganism *P. aeruginosa* (Figure 4).

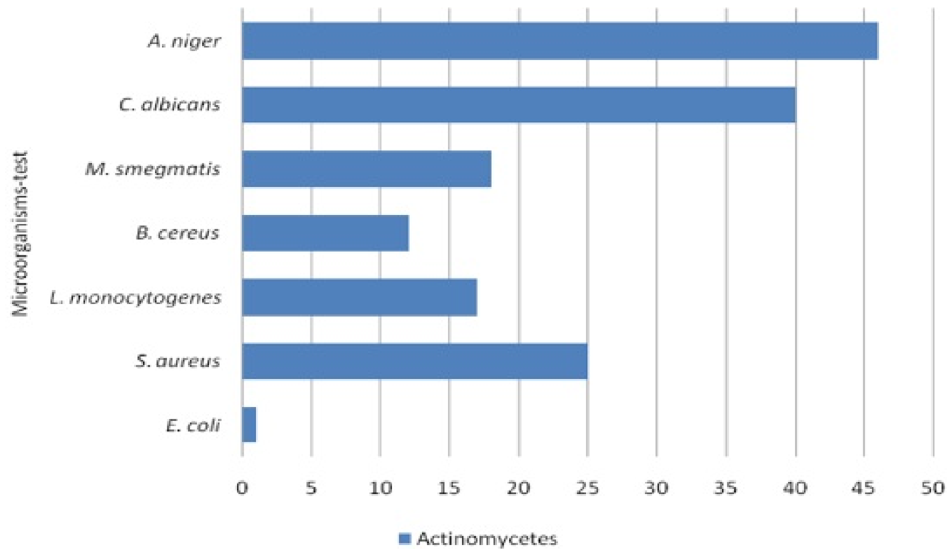

Fig. 3: Test microorganism inhibited by the actinomycetes, in Gelose Block.

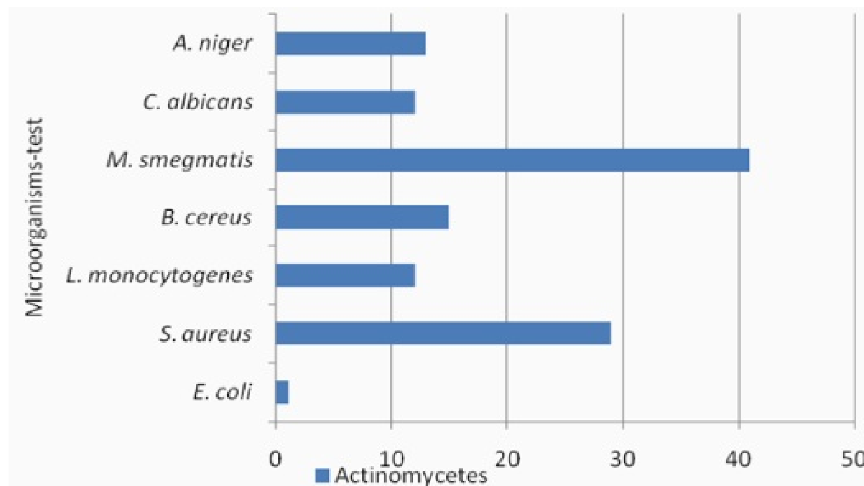

Fig. 4: Test microorganism inhibited by actinomycetes by disk diffusion.

The inhibiton halo average varied from low to high activity. From this, 51% of the isolated showed moderada activity and e 7% present higher antimicrobial activity.

The media MPE demonstrated to be efficient for the bioactive metabolites production. As Huck et al. (1991) the use of yeasts extract, soy bean and other raw complex material could be utilizated as nitrogen source for better production of antibiotics by actinomycetes. Lima (2006) and Ismet et al. (2004) affirmed that the combination of different carbon sources increased the antimicrobial activity in comparision with a only source and that the production increase of bioactive substance was depend of nitrogen source added to the media.

The antimicrobial activity simultaneous detection against filamentous fungi, yeasts and bacteria could suggest the action of more of one antibiotic with different target (González et al., 1999) or the evenctual presence of a new antimicrobial substance capable of cross the cell wall, as much bacterial species as to fungal (Sacramento et al., 2004; Tsvetanova; Prince, 2001; Chino et al., 1996).

The screening for new microorganisms capable to produce substances that can action against bacteria and pathogenic fungi is extremelly relevant, but nowadays the application of actinomycetes has changed to more diverse antimicrobials, like against *Plasmodium* sp. (Castillo et al., 2002, 2003; Pullen et al., 2002).

These results suggest that actinomycetes isolated from lichens are promissors organisms for the discovery of new antibiotics, conquering great importance in pharmaceutic industry.

The biotechnological processes are directly related to the diversity of molecules produced by microorganisms, as result of primary and secondary metabolism, and the conservation of their genetic resources. Besides the manufacturing of new pharmaceuticals and bio-industry products, microbial diversity can be widely used in the Amazon region.

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