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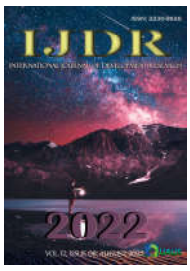

RESEARCH ARTICLE OPEN ACCESS

EVALUATION OF THE ANTIMICROBIAL POTENTIAL OF ACTINOMYCETES ISOLATED FROM MANGROVE SOILS IN THE MUNICIPALITY OF SÃO JOÃO DE PIRABAS, PARÁ, BRAZIL

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ABSTRACT

The present work aimed to characterize mangrove-derived actinomycetes strains isolated from São João de Pirabas mangrove in Pará State of Brazil and evaluate their antimicrobial action against clinical importance bacteria. Sixteen isolated actinomycetes strains were evaluated by their morphology and biochemical features and antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella* sp. and *Enterococcus faecalis*. They presented mixed morphological characteristics and different biochemical profiles which reinforce diversification on mangrove-derived actinomycetes strains. Colony growth and pigment production were evaluated in Czapek Dox agar medium enriched with 0,5 nystatin which was satisfactory for this study. Also, the isolates showed high and interesting broad-spectrum antibacterial inhibition against pathogenic Gram-positive and Gram-negative bacteria leading nosocomial infections worldwide by the two confrontation methods employed. Based on these evidences, São João de Pirabas mangrove in Amazon tropical region is a rich and prospective source for actinomycetes owing important antibacterial compounds.

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INTRODUCTION

Actinomycetes consist of a vast group of free-living, Gram-positive and prokaryotic organisms that survive under immensely diverse conditions. They are widely present in aquatic and terrestrial ecosystems such as lakes, oceans, plants, animals and soils (Barka *et al.,* 2016; Rahlwes *et al.,* 2019; Yi *et al.,* 2016) and that is why they are considered ubiquitous. The Actinobacteria phylum represents one of the largest taxonomic units composed by 6 classes which own 29 orders, 67 families, 391 genera and approximately 3900 different known species (Yadav *et al.,* 2018). This group of microorganisms has morphological and reproductive characteristics similar to fungi with which they were for a long time mistaken as they display filamentous growth generating structures similar to fungal hyphae and mycelia that by means of specialization gives rise to spores (Barka *et al.,* 2016).However, based on functional differences and internal cellular organization, actinomycetes were properly classified as bacteria afterwards (Li *et al.,* 2016).

Actinomycetes produce pigments of different colors that could help in taxonomic classification and genera identification (Barka *et al.,* 2016; Goodfellow and Haynes, 1984). Although pigmentation does not generate major impacts on microbial growth, it makes actinomycetes competitiveness against other microorganisms more effective therefore helps in their survival in the environment (Sharma *et al.,* 2014). Actinomycetes colonies could express white, cream, limestone, gray, violet, orange, yellow, brown (Shouce and Bhati, 2019) and possibly other colors. Actinomycetes main habitat is the soil wherein they play important roles on interacting with other living beings and the environment itself due to their ability to produce natural compounds as a survival strategy (Barka *et al.,* 2016; Jose *et al.,* 2021). Antitumor, immunosuppressive, insecticide, herbicide, and especially antimicrobial activity substances produced from their secondary metabolism have already been characterized (Jose *et al.,* 2021), however, their complete productive capacity looks to be still poorly known. The *Streptomyce s*genus is the most prevalent and stands out as a source for high-impact natural products as it is responsible for more than two-thirds of all commercially available

antibiotics from natural origin (Barka *et al*., 2016). However, important bioactive compounds presenting biotechnological potential produced by other genera are also being increasingly assessed nowadays (Jakubiec-Krzesniak *et al*., 2018; Jose *et al*., 2021). The mangrove is a coastal ecosystem present in tropical and subtropical zones presenting unique conditions, and it consists of an intertidal transition environment in which the marine and terrestrial biomes interact (Giri *et al.,* 2010; Ottoni *et al.,* 2021). The Brazilian mangrove areas represent 7% of the worldwide mangrove area. In Brazil, mangrove zones extend from North to the South, nevertheless, only four federative units concentrate about 85% of national mangrove area: Maranhão (46%), Pará (22%), Amapá (9%) and Bahia (7%) (Diniz *et al.,* 2019; Ottoni *et al.,* 2021).This ecosystem provides highly relevant services to humanity such as water purification, coastal protection against storms, erosion control, biological filter, reservoir for species, availability of raw materials and food, carbon capture and cultural services relatedto tourism, education and research (Donato *et al.,* 2011; Souza *et al.,* 2018; Ottoni *et al.,* 2021). In contrast, the constant human action on this environment harms its notorious ecological role, bringing damages to the communities linked to it. There are several reports about actinomycetes appearance in mangrove soil sediments (Abdin *et al.,* 2018; Palla *et al.,* 2018;Lwin *et al.,* 2020; Nivetha *et al.,* 2021),but despite that there is a lack of research that aims at evaluating the mangrove-derived actinomycetes potential antimicrobial products in the Brazilian Amazon which is a great and rich ecosystem (Oliveira, 2018).

The current world faces one of the greatest health threats in terms of antimicrobial resistance. According to the World Health Organization (WHO), 2019, antimicrobial resistance occurs when drugs lose their ability to inhibit microbial infectious agents, making the fight against infectious diseases much more difficult. Consequently, about 1.27 million people died directly from bacterial infections in 2019.Moreover, superbugs had an indirect role in another 4.95 million deaths in that year, exceeding mortality ratesby severe diseases such as acquired immunodeficiency syndrome (AIDS) and malaria (Murray *et al.,* 2022), and this scenario tends to worsen in the coming years. In 2017, the WHO published a list of resistant bacteria that are highly threatening to human health which would serve as a guide for the search, discovery and urgent development of new antibiotics. In the list, bacteria were labeled into three priority categories: critical priority (for example, *Escherichia coli*, *Klebsiella pneumonia* and other enterobacteria), high priority (for example, *Staphylococcus aureus* and *Enterococcus faecium*) and medium priority (for example, *Streptococcus pneumoniae*). The need for new sources of potentially antimicrobial compounds makes the exploration for new resources needed. In this context, actinomycetes stand out sincethese microorganisms are historically important sources for antibacterial substances (Genilloud, 2017; Jose *et al.,* 2021). Hence, the search for wild actinomycetes strains owning such potential is relevant especially those inhabitingharsh and poorly explored ecosystemsgiven that particular conditions of the environment influence the microbial populations biodiversity leading to the expression of different products by them (Lipton, 2007). Thus, it is necessary that the mangrove, an ecosystem of peculiar characteristics (Wu and Jiang, 2012; Liang *et al.,* 2006), gets investigated for bioprospecting on actinomycetes potentially sources for new antimicrobials, especially the mangroves of the Amazon tropical region which have received little focus by researchers. So, the present study aimed at isolating actinomycetes strains from São João de Pirabas mangrove in Pará, Brazilian Amazon in order to characterize them morphologically and biochemically as well as to evaluate their antimicrobial activity against resistant bacteria of clinical importance.

MATERIALS AND METHODS

Compliance With Ethical Standards: The current study did not require approval by Ethics Committee since its samples were obtained from São João de Pirabas mangrove soil, Brazil, in accordance with Federal Law 12.651/2012 which allows free movement of people in Permanent Protection Areas as long as there is no generation of negative impactsinit.

Mangrove location: To carry out the study, 10 soil samples were collected from four different points of the mangrove, resulting in a total of 40 soil samples, in the city of São João de Pirabas, Pará, Brazil (Figure 1):

- **Point 1:** Lat: 0° 45' 57"; Long: 47° 10' 06" W. Samples from this location were coded as SJP 1, SJP 2, SJP 3, SJP 4, SJP 5, SJP 6, SJP 7, SJP 8, SJP 9 and SJP 10;
- **Point 2:** Lat: 0° 46' 00"; Long: 47° 10' 07" W. Samples from this location were coded as SJP 11, SJP 12, SJP 13, SJP 14, SJP 15, SJP 16, SJP 17, SJP 18, SJP 19 and SJP 20;
- **Point 3:** Lat: 0° 46' 02"; Long: 47° 10' 05" W. Samples from this location were coded as SJP 21, SJP 22, SJP 23, SJP 24, SJP 25, SJP 26, SJP 27, SJP 28, SJP 29 and SJP 30;
- **Point 4:** Lat: 0° 46' 05"; Long: 47° 10' 06" W. Samples from this location were coded as SJP 31, SJP 32, SJP 33, SJP 34, SJP 35, SJP 36, SJP 37, SJP 38, SJP 39 and SJP 40.

Samples were collected from the first soil superficial centimeters (± 10 cm) from the points which were about 30 meters away by each other (Rocha, 2008), by taking into consideration the size and accessibility of the mangrove zone. It was preferred to pick up samples from areas presumably free of direct anthropic action. Also, it is known that seasonality is a factor that could influence the frequency of microbial populations in soils (Costa *et al.,* 2012), so it is worth noting that the soil samples pick up took place on November 3, 2021, a period marked by rains in the Pará State, Brazil. Thereafter, the samples were packed into polyethylene bags which were stored in a thermal box and transported to the Applied Microbiology Laboratory in the State University of Pará (LabMicro CCBS – UEPA).

Samples Processing: About 1 g of each soil sample was diluted into 9 mL of sterile 0.9% NaCl in sterile glass test tubes. After homogenization, the dilutions were centrifuged at 2500 RPM for three minutes (Oliveira, 2018). Then, supernatant fractions were used for streaking Czapek Dox agar Petri plates enriched with 0.5% nystatin which is an antifungal capable of inhibiting the possibly contaminating fungi growth,by disposable loop using a single streak method (Azuma, 2011; Costa, 2012).

***Growth, Isolation and Characterization of Actinomycetes*:** The streaked Petri plates were incubated at ± 35ºC for 96 hours in a bacteriological humid chamber (Costa, 2012) for bacterial growth and initial assessment of the colonies morphological features such as colony size, mucoid or dry aspect, flat or raised surface and effective attachment to the medium (Sathi *et al.,* 2001 apud Silva *et al.,* 2019). Then, the colonies displaying these typical actinomycetes features were submitted to the Gram method in order to visualize their micromorphological shape and dye affinity and the actinomycete-like ones were isolatedinnew Czapek Dox agar Petri plates for further evaluation.

Morphological Characterization: After 72 hours incubation and good growth, characteristics such as aerial mycelium color, vegetative mycelium color, pigments production and colony aspect were evaluated in accordance with Shirling and Gottlieb (1966). For micromorphological characterization, the actinomycetes colonies were evaluated by Gram method and the microculture method (coverslip method) in order to check mycelia formation and their reproductive structures. In this regard, the isolates were soaked into sterile 0.9% NaCl and streaked on Sabouraud agar by Drigalski loop by the spread plate method. Three coverslips inclined at 45º were inserted into each Sabouraud agar medium Petri plate and they were incubated at ± 35ºC in a bacteriological humid chamber for a period of 72 hours for the growth of bacterial structures on the coverslips (Williams *et al.,* 1989). Thereafter, cover slips were removed from the medium and were overlaid on slides and flooded with methylene blue dye along with distilled water in the proportion of 1:1, then, they were evaluated by light microscopy under 1000x magnification.

Biochemical Characterization: From the colonial growth and isolation, the actinomycetes were submitted to the biochemical profile evaluation by means of analytical substrates in culture media in order to assess their ability to produce specific enzymes and check the presence of motility structures. The biochemical features evaluated were catalase production, citrate utilization, phenylpyruvic acid production, sugars fermentation (glucose, sucrose and lactose) and/or ferrous sulfate degradation (H_2S production), urea degradation and cell motility. In this regard, Simmons Citrate agar, Phenylalanine agar, semi-solid Tryptone Soy agar (TSA), Triple Sugar Iron agar (TSI), Urea broth as well as 3% hydrogen peroxide solution were utilized (Arimateia and Neto, 2017; Laborclin, 2019; Good fellow *et al.,* 2012). Biochemical tests were read after 48 hours incubation andtheir analysis was based on the color change of the media employed, except for catalase utilization test which was readby viewing the immediate appearance of bubbles in the reaction.

Antimicrobial Activity: The pathogenic bacteria (test strains) employed were *Klebsiella* sp., *Escherichia coli*, *Enterococcusfaecalis* ATCC® 29212 and *Staphylococcus aureus* ATCC® 25923 which make up the list of resistant pathogens to antibacterials (WHO, 2017). The first two were isolated from urine samples from patients treated at a health center linked to the Applied Microbiology Laboratory, and the last two are commercialized standard strains. The antibacterial activity evaluation of actinomycetes strains was performed by two methods: (i) the direct confrontation (agar well diffusion method) between the bacteria in which two agents are streaked directly on the Petri plate; and (ii) the indirect confrontation (disk diffusion method) in which paper discs impregnated by the bacterial agent are employed to be evaluated in a plate previously streaked with a selected pathogenic bacterium. The inhibition halos formed from the two methods were assessed using a ruler to measure their diameter after plates incubation at ± 35ºC.

Direct Confrontation Method: In the direct confrontation method, the actinomycetes strains were streaked on Czapek Dox agar Petri plates enriched with 0.5% nystatin by disposable loop and they were incubated for 24 to 72 hours in a bacteriological humid chamber for the growth of bacterial colonies to be employed in the confrontation. The test agents with known pathogenicity and resistance properties were streaked on Mueller-Hinton agar medium Petri plates and perforated wells were made in the same medium for the addition of same-size fragments from the grown culture of actinomycetes. The Mueller-Hinton agar plates containing both test strains and actinomycetes colony same-size fragments were incubated at ± 35ºC in a bacteriological humid chamber for 24 to 72 hours for bacterial growth evaluation and inhibition zones formation (Nguyen *et al.,* 2018).

Indirect Confrontation Method: In the indirect confrontation, the paper discs employed were produced by their impregnation in bacterial suspension of 0.9% sterile NaCl solution containing diluted actinomycetes (105 ufc/mL), in which the discs were emerged and kept for 12 hours at ± 35ºC in a bacteriological humid chamber. The test strains were streaked in Mueller-Hinton agar medium Petri plates and the previously impregnated discs were punched onto the medium (Bauer *et al.,* 1966).

RESULTS AND DISCUSSION

Isolation of Actinomycetes: Forty mangrove soil samples were prepared under the conditions described above. The growth of several bacteria was observed on all Czapek Dox agar Petri plates, but only 16 colonies representing 16 isolates were selected for further evaluation.

Actinomycetes Strains Characterization

Morphological Characterization: Regarding the macromorphological characteristics, most of the isolated colonies presented aerial myceliacolor (front side) ranging from white to yellow (Plate 1) and vegetative myceliacolor (reverse side) ranging between white, yellow and brown. Some bacteria produced diffuse pigments that spread over the culture medium which were found in six isolates with a predominance of carotenoid pigments (yellowish) in fourplates and melanoid pigments (brown) in twoplates. Regarding the aspect, colonies which presented a mucoid aspect (n=12) predominated over the colonies that presented a dry aspect (n=04) (Table 1). The isolated strains showed aninteresting chromogenic diversity with predominance of undetermined or transparent (31.25%), brown (18.75%) and yellow (18.75%) mycelia colors (Figure 2). The isolated actinomycetes strains showed micromorphological characteristics that were evaluated by Gram and microculture methods (Plate 2). All isolated strains are Gram-positive. Out of the 16 isolates, 15 showed in bacillary forms. Only one isolate displayed coccoid form. Filamentous structures forming branched pseudohyphae were also seen. From these observations, it was possible to deduce the probable generato which isolates belong (Table 2). The actinomycetes pigmentation could support the genera identification and contribute to taxonomic classification (Barka *et al.,* 2016; Goodfellow and Haynes, 1984). The colors and tones seen in aerial mycelia (front side) and vegetative mycelia (reverse side) consisted of white, grayish white, gray, cream, pale yellow, yellow, brown, yellowish black and undetermined (transparent) colors.

According to Shouce and Bhati (2019), white, cream, limestone, brown, grey, pink and violet colors could be seen in aerial myceliumwhereas brown, yellow and orange could be seen in vegetative mycelium of actinomycetes. These patterns were described by Nivetha *et al* . (2021) and Singh and Singh (2021), supporting our characterization descriptions which found predominance of these patterns already evaluated previously. However, it is worth mentioning the pigmentation diversification identified in our actinomycetes strains which suggests that new characteristics could be originated according to the habitat wherein the microorganism is once that in controlled environments or in their natural environment actinomycetes rely on their secondary metabolism to produce diversified substances that sustain their survival and maintenance (Sharma *et al.,* 2014; Ramos *et al.,* 2015; Yadav *et al.,* 2018; Xu *et al.,* 2014).On this point, some of our isolated actinomycetes strains had their pigmentation ranging from darker to lighter in their aerial mycelia(SJP 28-A, SJP 11-B, SJP 21-C and SJP 25-A).These findings were also described by several works (Kurnianto *et al.,* 2020; León *et al.,* 2011; Muleta and Assefa, 2018; Ramos *et al.,* 2015)which isolated different actinomycetes strains in different culture media, and these microorganisms also showed aerial mycelia whose tones ranged in a brown scale and vegetative mycelia presenting lighter colors.

Actinomycetes colonies tend to change their pigmentation over time, this was seen in SJP 21-C strain whose color changed from cream to yellow after 72 hours incubation. This color change was also observed in the study of Lwin *et al* . (2020) in which three of their actinomycetes strains presented color variation after 5-6 days. Thus, we can notice that coloration as sole criterion for taxonomic classification is insufficient nowadays and needs to be supported by genetic analysis (Barka *et al.,* 2016) since the dye characteristics can be modulated according to the culture medium composition they are in and its incubation time. Culture media containing carbon sources such as starch, glycerol and fructose promote melanin production (Dastager *et al.,* 2006), however, these substances are not present in the medium employed in the present study: the Czapek Dox agar medium which is a rich source for sodium nitrate instead. Still, some isolated actinomycetes strains were capable of producing mycelium brown color in this culture medium.

Even so, the characteristics displayed can support the presumptive identification since some actinomycetes genera have well-defined morphological features, for example coccoid forms such as *Micrococcus*, coccobacillary forms such as *Arthrobacter*, fragmented filaments such as *Norcadia* and filamentous hyphae such as *Streptomyces* (Raju *et al.,* 2010; Santos *et al.,* 2019). It is worth emphasizing that there are works that utilized the chromogenic characteristics as a baseline for taxonomic framingon actinomycetes such as Romero *et al*. (2012).

Table 1. Macromorphological characterization of the isolates after 72 hours incubation.

Strains	Mycelium color		Aspect	Diffusepigments	
	aerial	vegetative		carotenoids	melanoids
SJP 2-A	White	Brown	Mucoid	-	-
SJP 7-A	-	-	Mucoid	Yellow	-
SJP 11-B	Cream	Cream	Mucoid	-	-
SJP 12-A	-	-	Mucoid	Yellow	-
SJP 13-A	-	-	Mucoid	-	-
SJP 18-A	Yellow	Yellow	Mucoid	-	-
SJP 21-C	Cream	Cream	Mucoid	-	-
SJP 22-B	Yellow	Yellow	Mucoid	-	-
SJP 23-A	Yellow	Yellow	Mucoid	-	-
SJP 24-B	-	-	Mucoid	Yellow	-
SJP 25-A	White	Gray	Dry	-	Brown
SJP 27-A	White	Brown	Dry	-	Brown
SJP 28-A	Yellowish black	Yellowish black	Dry	-	-
SJP 28B	Grayish white	Brown	Dry	Yellow	-
SJP 33-A	-	-	Mucoid	-	-
SJP 40-B	Pale yellow	Pale yellow	Mucoid	-	-

Subtitle: (-) represents absent or undetermined color, consisting of transparent colonies Source: authors

Table 2. Micromorphological characterization of *the i*solate*s* after 72 hours incubation.

Strains	Gram method descriptions	Microculture description	Probable genus
SJP 2-A	Gram+ verod	Branched pseudohyphae	*Streptomyces* sp.
SJP 7-A	Gram+ verod	Free bacilli	*Bacillus* sp.
SJP 11-B	Gram+ verod	Pseudohyphae and free bacilli	*Streptomyces* sp.
SJP 12-A	Gram+ verod	Tangle of pseudohyphae and free bacilli	*Streptomyces* sp.
SJP 13-A	Gram+ verod	Branched pseudohyphae	*Streptomyces* sp.
SJP 18-A	Gram+ verod	Free and in short chains bacilli	*Bacillus* sp.
SJP 21-C	Gram+ verod	Free and in short chains bacilli	*Bacillus* sp.
SJP 22-B	Gram+ verod	Free bacilli	*Bacillus* sp.
SJP 23-A	Gram+vecocci	Pseudohyphae e cocobacilos	*Frankia*sp.
SJP 24-B	Gram+ verod	Tangle of pseudohyphae	*Streptomyces* sp.
SJP 25-A	Gram+ verod	Branched pseudohyphae and bacillary forms	*Streptomyces* sp.
SJP 27-A	Gram+ verod	Pseudohyphae and free bacilli	*Streptomyces* sp.
SJP 28-A	Gram+ verod	Pseudohyphae and free bacilli	*Streptomyces* sp.
SJP 28-B	Gram+ verod	Tangle of filamentous pseudohyphae	*Streptomyces* sp.
SJP 33-A	Gram+ bacillus	Tangle of pseudohyphae and free bacilli	*Streptomyces* sp.
SJP 40-B	Gram+ bacillus	Tangle of pseudohyphae and short chains of bacilli	*Streptomyces* sp.

Source: authors.

Table 3. Biochemical characterization of the isolates

Strains	Catalase	Simmons citrate	Phenylalanine	TSA (motility)	TSI	Urea
SJP 2-A	+	-	-	+	AC/AL	-
SJP 7-A	+	-	-	-	AC/AL	-
SJP 11-B	+	-	-	-	AC/AL	-
SJP 12-A	+	-	-	-	AC/AL	-
SJP 13-A	+	+	-	+	AC/AL	-
SJP 18-A	+	-	-	-	AC/AL	-
SJP 21-C	+	-	-	-	AC/AL	-
SJP 22-B	-	-	-	+	AC/AL	-
SJP 23-A	+	-	-	-	AC/AL	-
SJP 24-B	+	-	-	-	AC/AL H_2S+	-
SJP 25-A	-	-	-	-	AC/AC H_2S+	-
SJP 27-A	+	-	-	-	AC/AL	-
SJP 28-A	-	-	-	-	AL/AL	-
SJP 28-B	-	-	-	-	AC/AL H_2S+	-
SJP 33-A	-	-	-	+	AC/AL H_2S+	-
SJP 40-B	-	-	-	-	AC/AL	-

Subtitle: Base/Apex; AC = acid; AL = alkaline; Source: authors.

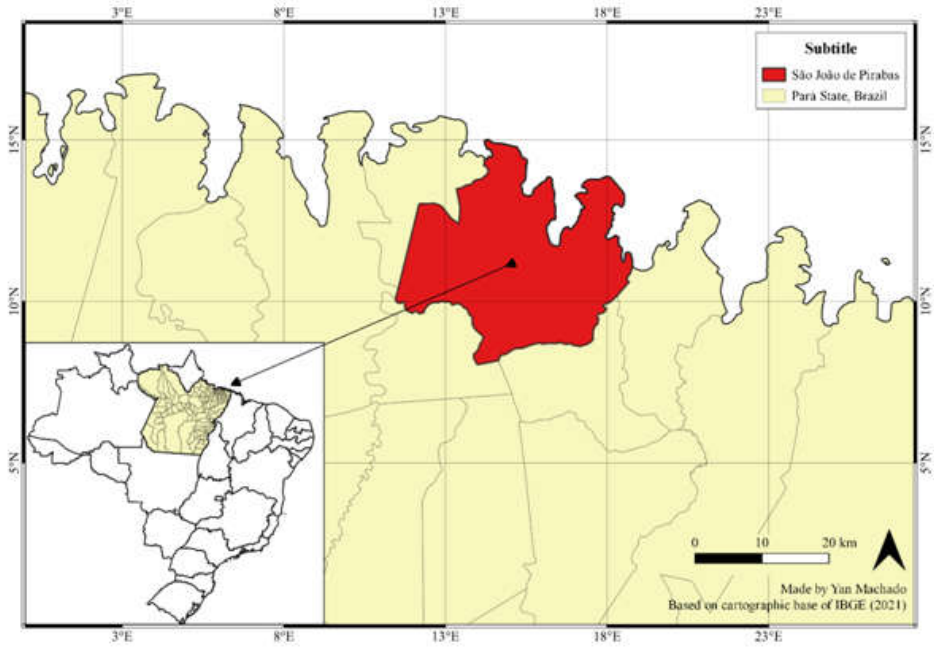
Furthermore, the capability of producing diffuse pigments is a relevant actinomycetes feature. The synthesis of diffuse pigments such as purple, yellow, blue and green is related to the antibiotic production (Abdin *et al.,* 2018). SJP 7-A, SJP 12-A, SJP 24-B and SJP 28-B isolates produced yellow diffuse pigments and these strains had interesting actions against the test pathogens afterwards: (i) SJP 7-A, SJP 12-A and SJP 24-B presented the widest inhibition zones, and (ii) SJP 28-B showed a broad spectrum action. These findings corroborate with Abdin *et al* . (2018) regarding the diffuse pigments synthesis which demonstrate a relationship with antibiotics production.

Biochemical Characterization: All isolated actinomycetes strains had their metabolism evaluated by means of growth in culture media taking in concern the production of specific biochemical substrates in order to classify the strains from a metabolic and physiological point of view and support their presumptive identification. Out of 16 isolates, it was shown that 10 strains synthesize the catalase enzyme, 15 strains do not use citrate as a carbon source. In the TSI test which reveals the capacity and metabolism by which carbohydrates are metabolized by bacteria it was noticed that acid metabolism is the most used, 11 strains utilized glucose by this pathway.

Table 4. Sensitivity test of the isolates against resistant bacteria.

Strains	Confrontationmethod	Zone ofinhibition (halos)			
		*Klebsiella*sp.	*E. coli*	*S. aureus* ATCC® 25923	*E. faecalis* ATCC® 29212
SJP 2-A	Direct	-	-	18 mm	-
	Indirect	-	-	11 mm	-
SJP 7-A	Direct	-	36 mm	36 mm	-
	Indirect	-	-	-	-
SJP 11-B	Direct	-	-	-	-
	Indirect	-	-	-	-
SJP 12-A	Direct	-	-	34 mm	-
	Indirect	-	-	-	-
SJP 13-A	Direct	-	-	20 mm	-
	Indirect	-	-	-	-
SJP 18-A	Direct	-	-	18 mm	-
	Indirect	-	-	11 mm	-
SJP 21-C	Direct	-	-	39 mm	-
	Indirect	-	-	11 mm	-
SJP 22-B	Direct	-	-	-	-
	Indirect	-	-	13 mm	-
SJP 23-A	Direct	-	16 mm	19 mm	-
	Indirect	-	-	10 mm	-
SJP 24-B	Direct	-	-	33 mm	-
	Indirect	-	-	11 mm	-
SJP 25-A	Direct	-	-	-	-
	Indirect	-	-	9 mm	-
SJP 27-A	Direct	-	-	18 mm	-
	Indirect	-	-	-	-
SJP 28-A	Direct	-	-	17 mm	-
	Indirect	-	-	-	-
SJP 28-B	Direct	-	16 mm	-	-
	Indirect	10 mm	-	11 mm	-
SJP 33-A	Direct	16 mm	18 mm	27 mm	-
	Indirect	-	-	-	-
SJP 40-B	Direct	-	-	19 mm	-
	Indirect	-	-	-	-

Subtitle: (-) represents absent or undetermined antimicrobial activity. Source: authors.

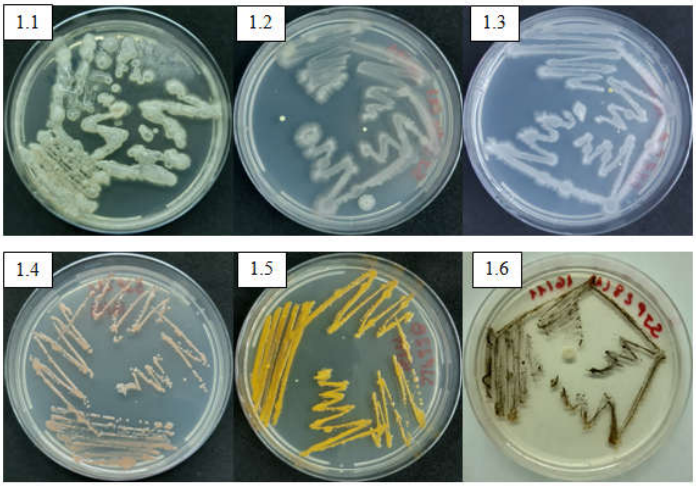


Source: created by authors. Based on cartographic data of Brazilian Institute of Geography and Statistics Foundation (IBGE, 2021).

Figure 1. Locationof São João de Pirabas, Pará, Brazil

None of the actinomycetes isolates showed degradation of urea which indicates they are not urease producers (Table 3). The biochemical profiles of actinomycetes strains showed few differences, but it was possible to assess metabolic differences between them despite being isolated from the same zone. These findings reflect the importance of carrying out more accurate research in the mangrove ecosystem which can be of great importance as a prospective source for new actinomycetes strains (Lee *et al.,* 2014).

The biochemical characteristics of the isolated actinomycetes strains in our study showed a resemblance with other actinomycetes strains from other reports carried out in different countries. Similar to our findings, Lwin *et al* .(2020), Nivetha *et al* . (2021) and Palla *et al* . (2018) also evaluated their isolates by means of several biochemical tests and it was found that they were all positive in catalase test. The other tests performed in these studies such as the tests employed in our study showed heterogeneous results for biochemical profile, and



Subtitle: 1.1) SJP 33-A Colony; 1.2) SJP 24-B colony; 1.3) SJP 7-A colony; 1.4) SJP 21-C colony; 1.5) SJP 22-B colony; 1.6) SJP 28-A colony. Macroscopic view of the front side of plates with colonies of actinomycete isolates Source: authors.

Plate 1. Macroscopic characteristics and pigment production of the isolates

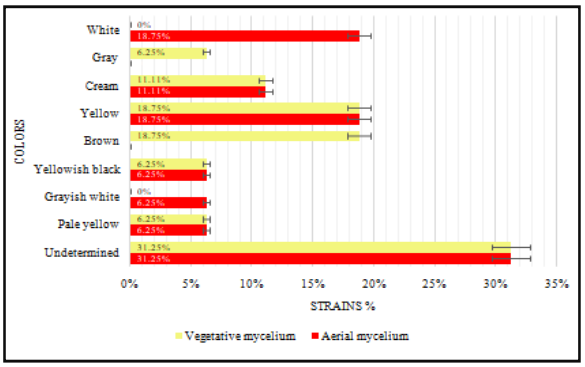
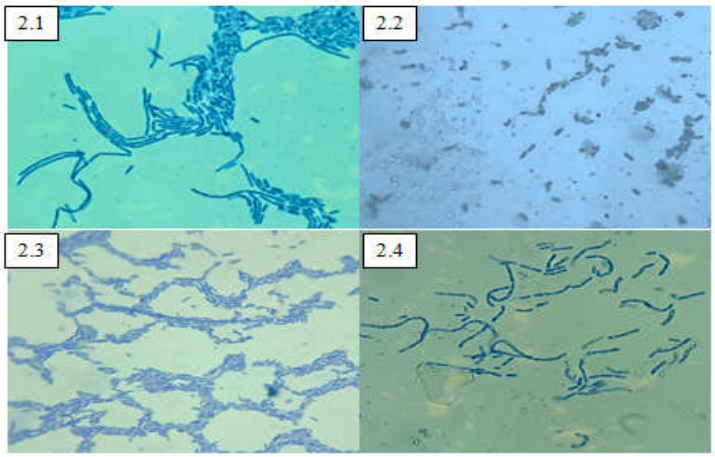


Figure 2. Mycelial colors of the isolates



Subtitle: 2.1) SJP 12-A micromorphology; 2.2) SJP 23-A micromorphology; 2.3) SJP 25-A micromorphology; 2.4) 28-B micromorphology. These structures were analyzed by the coverslip method under 1000x magnification in immersion oil **Source:** authors.

Plate 2. Micromorphology characteristics of the isolates by microculture method

Source: authors.

Plate 3. Antimicrobial activity against *Staphylococcus aureus* ATCC® 25923.

it does not seem to have a direct relationship with antimicrobial compounds production, because actinomycetes strainsin the current study were able to inhibit the pathogenic test strains, although presenting different biochemical profiles. It shows that these biochemical characteristics have a link closer to metabolic and nutritional dissimilarity of actinomycetes. It is important to highlight that the biochemical profile, which is related to the action of proteins and enzymes expressed by organisms, can be seen as an indirect portrait of the microbial genome since these substances are products from genes. Thus, biochemical characterization can be understood as an important step for actinomycetes classification (Li *et al.,* 2016) along with morphological and molecular characterization since it is an arduous taskto group them taxonomically (Oliveira *et al.,* 2014; Barka *et al.,* 2016).

Antimicrobial Activity Against Test Strains: The antimicrobial potential of 16 isolates was evaluated. Significant inhibition halos were found which ranged from 20 to 39 mm by some isolated actinomycetes strains (SJP 7-A, SJP 12-A, SJP 21-C, SJP 24-B and SJP 33-A) (Table 4). The pathogenic bacteria (test strains) that showed relevant sensitivity to the actinomycetes strains were *Staphylococcus aureus* ATCC® 25923, *Escherichia coli* and *Klebsiella* sp. The *S. aureus* pathogen was the most susceptible test strain (Plate 3) since it showed sensitivity to 15 isolated actinomycetes strains, except to the SJP 11-B strain. The *E. coli* pathogenwas sensitive to the SJP 7-A, SJP 23-A, SJP 28-B and SJP 33-A strains, and the *Klebsiella* sp. pathogen was inhibited by two isolated actinomycetes strains (SJP 28-B and SJP 33-A). Quite the opposite, none of the actinomycetes showed activity against *Enterococcus faecalis* ATCC® 29212 under the work conditions. The SJP 28-B and SJP 33-A actinomycetes strains showed higher action potential being able to inhibit three out of four test bacteria employed. The method that presented the better results for inhibition in which more test strains were inhibited by the actinomycetes under the work conditions was the direct confrontation (Table 4). Superbug infections have increased significantly over the past few years (Murray *et al.,* 2022) due to the increase in antimicrobial resistance in association with limited availability of antibiotics for clinical use, and the lack of development of new low side effects antibacterial drugs. Antimicrobial activity evaluation of the actinomycetes isolates in the present study was carried out against four pathogenic bacteria. The results of the susceptibility tests revealed extraordinary antibacterial activity against *Klebsiella* sp., *E. coli* and *S. aureus* which are responsible for the highest incidence in deaths from nosocomial infections nowadays (Murray *et al.,* 2022). The bacterial cell wall is considered an important factor in determining the pathogen sensitivity to antibacterial substances (Kurnianto *et al.,* 2020) because it could preventcertain drugs action. In this study, nevertheless, expressive broad-spectrum antimicrobial activity was observed by the isolated actinomycetes strains which were able to act on both Gram-positive and Gram-negative pathogens. Four mangrove-derived strains selected in the study of Das *et al*. (2014) were also capable of inhibiting Gram-positive and Gram-negative bacteria, exhibiting broad-spectrum action. However, all those strains had greater antagonistic effects against Gram-positives. In contrast, two of our actinomycetes strains, SJP 7-A and SJP 28-B,showed equal and greater inhibition, respectively, in Gram-negative compared to Gram-positive, although it is known that Gram-negatives are considered naturally more resistant due to the presence of an outer membrane consisting of lipopolysaccharide (Das *et al.,* 2014; Parunago *et al.,* 2007; Sangkanu *et al.,* 2017). The results described in the current research are in accordance with Das *et al*. (2014) whom affirm that the mangrove harbors actinomycetes owing important and diverse physiological properties which could be potential sources for antimicrobial products, reinforcing the importance of the current research as a baseline information for the advance on the investigation of mangrove-derived actinomycetes from Amazonian tropical soils off the coast of Pará, Brazil.

Actinomycete colonies color is an attribute that could not be directly linked to antimicrobial compounds production, since our work showed intriguing broad antagonistic effects against test strains being triggered by transparent colonies isolates (SJP 7-A, SJP 12-A, SJP 24-B and SJP 33-A) and cream colored (SJP 21-C). On the other hand, some studies stand up for the correlation between actinomycetes colony growth and increased antimicrobialsproduction (Dholakiya *et al.,* 2017; Singh *et al.,* 2014). Maximum growth of cell biomass when well-developed colonies reach a stationary phase between 7 and 11 days (Kurnianto *et al.,* 2020) could determine a greater production of antibacterials because thedevelopment of spores occurs at that stage and the actinomycetes, in order to ensure its continuation, produce antagonist compounds against other deteriorating microorganisms (Barka *et al.,* 2016). Even so, the actinomycetes in the current study displayed high inhibitory activity against resistant pathogens in about three days of growth as did the isolates from Singhand Singh (2020)whose better inhibition results were observed on the third incubation day against uropathogens. Both the present study and Singh and Singh's (2020) evidencedthat the production of important bioactive compounds does not happen only in a high rate of colony development but also throughout several stages of actinomycetes proliferation from hyphae, mycelia, spores up to the spore chain. The strong antimicrobial activity by the isolated actinomycetes strains in the present research towards nosocomial pathogens are good indicators that these strains are potential candidates for the development of new valuable bioactive compounds with clinical innovation capability since mangrove-derived actinomycetes strains own specificities determined by the conditions in which they survive in that harsh environment (Xu *et al.,* 2014).Therefore, it is essential to apply these actinomycetes strains in further studies in the biotechnology fieldproviding genetic studies,purification, minimum inhibitory concentration and chemical analysis of their antimicrobial bioactive compounds in order to develop new drugs toward resistant pathogens of clinical importance.

CONCLUSION

São João de Pirabas mangrove, located off the coast of Pará State, Brazil, is a rich and prospective source for actinomycetes owning antimicrobial potential against pathogenic bacteria of sanitary contingency. The most frequent presumptive genera that inhibited test strains in the present work were *Streptomyces* sp., *Bacillus* sp. and *Frankia* sp. There is a lack of characterization works on mangrove-derived actinomycetes in the federative unit pointed, and the present study is a pioneer on isolating actinomycetes owning antimicrobial potential in the region. We hope to provide a baseline information for further local works on mangrove-derived actinomycetes. The isolates from the current study need further characterization towards drugs development enabling the writing of new chapters in the urgent combat against superbugs.

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