

Antimicrobial Activities of Four Strains of *Streptomyces sp* Isolated from the Pond of the Village of Demba Tiarki Tara in Mali

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Abstract: The search for new molecules is needed to cope with the recrudescence of bacterial resistance. Actinomycetes, especially the genus *Streptomyces* remains the most requested for the production of bioactive substances. Nineteen samples of soil and mud from the pond of Demba Tiarki Tara were collected and treated. In total, 7 strains of *Streptomyces* were isolated by the calcium carbonate enrichment technique for the isolation of *Streptomyces*. The antimicrobial potency of isolated *Streptomyces* was assessed on human and plants pathogens (*Staphylococcus aureus*, *Aspergillus flavus*, *Penicillium sp*, *Xanthomonas sp*, *Salmonella typhi*, *Helminthos sp*). Four of the tested actinomycetes strains showed antimicrobial activities against the pathogens used in the in vitro test. But only one strain showed high antagonist activity against *Staphylococcus aureus* and has a strong ability to produce melanin. Three strains were weakly active on *Salmonella typhi*. This result showed, for the first time, that the pond of Demba Tiarki Tara (DTT) contains bacteria producing bioactive compounds indicating the medical interest that the local population has in this pond. Secondary metabolite production by one strain may be an effective antibiotic for the management of resistant *Staphylococcus aureus* strains.

Keywords: Antimicrobial Activities, *Streptomyces*, Pond

1. Introduction

Actinomycetes are microorganisms of nature, of various habitats (soil, water, tree, etc.) which are very much in demand for their power of production of bioactive substances, 70-80% of their metabolites are marketed around the world [1]. These bioactive substances are generally used in agronomy, medicine and biotechnology. In the pharmaceutical field, 40% of the metabolites produced by actinomycetes are used as antibiotics or anticancer drugs [2, 3, 4]. Bacterial resistance is a very common phenomenon in

therapeutic care. Bacteria are becoming more resistant to available antibiotics [5] due to misuse and poor control. In the face of the recrudescence of bacterial resistance, nature, through Actinomycetes, can be an alternative source of antibacterial substances. In recent decades, researchers have been searching for new native strains of microorganisms capable of producing active secondary metabolites [6]. Mangroves, rivers and ponds are favorable environments for the proliferation of Actinomycetes, especially the *Streptomyces* genus producing bioactive substances [6, 7, 8], and such phytohormones [9]. In particular, mangroves, with fluctuating salinity, are an ideal environment for new species

of *Streptomyces* producing secondary metabolites [10, 11]. In some localities in Mali, the local population uses water or sludge for treatment. This is the case of the water of the pond of Demba Tiarki Tara (DTT) which is used for therapeutic care especially the affections of the skin. However, this study is initiated to search for the presence of microorganisms producing antibacterial substances.

2. Materials and Methods

2.1. Site and Sampling

The study took place in the village of DTT is about 290 km from Bamako; Latitude 15 and Longitude -7.70; in the circle of Nara, region of Koulikoro. The pond is at latitude 14.914-14.917, and longitude is -7.728 and -7.725. Soil and sludge samples (n=19) were collected in 50 ml sterile flasks, 10 to 15 cm deep at the pool level. Soil samples were identified by a combination of letter and number from S11 to S29. The *Actinomyces* colonies thus isolated were numbered by adding a third digit to the soil sample coding. These collected samples were tagged, stored and sent to the laboratory prior to analysis.

2.2. Characterization Physicochemical of Soil

Each soil sample was characterized by measuring pH and moisture content [12].

2.3. Isolation of Actinomycetes

The calcium carbonate enrichment technique was used as it allows a 100-fold increases in *Streptomyces* colonie count [13]. 1 g of soil sample was dried in the open dust-free, mixed with 0.1 g of CaCO₃ and incubated at room temperature for 7 days. The samples were crushed, transferred to tubes. The enriched soil samples were mixed in 10 ml of sterile physiological saline and then heated at 55 °C for 1 hour in order to select *Streptomyces*. After cooling and sedimentation, with the supernatant, dilutions were made up to 10⁻⁴. A volume of 1000 µl of suspension of each dilution is spread in Petri dishes, on medium Küster (Glycerol) whose composition for 1 liter is as follows: Starch 5g, Glycerol 1ml, KNO₃ 2g, Peptone 0.3g, NaCl 2g, K₂HPO₄ 2g, Micronutrients 1ml, Agar 18g, Distilled water 1000ml, and the pH was adjusted to 7. The composition of the micronutrient solution per 100ml is as follows: Mg²⁺ sulfate, 5g; Ca²⁺ carbonate, 2g; ferrous sulphate 1g and distilled water 100 ml. The samples were incubated at room temperature for 1 to 2 weeks. Typical colonies of actinomycetes are selected and purified on ISP2 medium.

2.4. Characterization of Strains

Strains were characterized microscopically, macroscopically and biochemically. Microscopic characterization focused on Gram, sporulation, and aerial mycelium. The macroscopic aspects concerned the shape, the

consistency, the size and the color of the colonies. Biochemical characteristics included catalase reactions, oxidase, and melanin production. The behavior of the strains was examined on different culture media (ISP2, ISP3, ISP4, ISP5, and Tyrosine Agar), pH and NaCl concentration. The agar-tyrosine medium (Glycerol 15g, L-Tyrosine 0.5g, L-Asparagine 1g, K₂HPO₄ 0.5g, MgSO₄ 7H₂O 0.5g, NaCl 0.5g, Fe₂O₄ 7H₂O 0.01g, Micronutrients 1ml [FeSO₄ 7H₂O = 0.1g, MnCl₂ 4H₂O = 0.1g, ZnSO₄ 7H₂O = 0.1g, 100ml distilled water; Agar 20g, distilled water 1000ml, pH 7.2) is used to perform the test. the production of melanin.

2.5. Antimicrobial Activity

The antimicrobial activities were evaluated by the piece-work technique and diffusion. For the slicing technique, the portions of the selected strains were knocked down on the pathogens (*Staphylococcus aureus*, *Xanthomonas sp*, *Aspergillus flavus*, *Penicillium sp*, *Salmonella typhi* and *Helminthos sp*) in culture. For the diffusion method, the strains were cultured in Bennett liquid medium (Glucose 10g, Extract Yeast 2g, Meat Extract 1g, Peptone 2g, Distilled water 1000 ml, pH = 6.8) for 10 days. The Bennett culture medium is likely to contain substances produced by the bacteria [14]. The cultures were centrifuged, the supernatants were recovered by filtration and contacted pathogens in depressions formed on the solid culture medium (Nutrient Agar enriched with 1% glucose) in the Petri dishes. Incubate for 1 week, antibacterial activity was evaluated for each strain of bacteria. These bio assay pathogens have been isolated from human samples (*S.aureus* and *S. typhi*), and plants (*Xanthomonas sp*, *A. flavus*, *Penicillium sp* and *Helminthos sp*). These strain have been collected and keep at the LaboRem-BioTech.

3. Results

The studied soils samples, their physico-chemical characteristics (pH and humidity) and their content in bacteria and actinomycetes are presented in table 1.

The analysis of data in table 1 showed that the soil samples have pH ranging from 5.64 to 9.100, with a majority of soils with basic pH. Actinomycetes were isolated in only six soils, out of nineteen samples in this study. Apart 6 Actinomycetes isolated from S11 and S18 with an acidic pH, all the other Actinomycetes were isolated in soils samples with a neutral pH or basic (case of S22). The macroscopic, microscopic and biochemical characteristics of the isolated Actinomycetes strains are presented in table 2. All strains of Actinomycetes isolated are gram +, catalase positive 50% of strains, producing spores and Rigeuse-down in culture medium. These characteristics are specifics of actinomycetes [15]. The International *Streptomyces* Projet (ISP2, ISP3 and ISP5), Agar-tyrosin and Bennett were a bests medium for culture and they are store during for month on Bennett.

Table 1. Soil samples, pH, humidity level, colony number and Actinomycetes colonies isolated.

Soil sample	pH	Humidity level (%)	colonies Number UFC/ml	Actinomycetes Colonies Isolated
S11	6.10	6.60	33	3
S12	5.80	15.21	10	0
S13	6.25	14.50	0	0
S14	7.10	3.40	15	2
S15	6.95	17.40	23	1
S16	6.70	6.12	50	0
S17	8.24	11.30	3	0
S18	5.64	15.00	3	3
S19	7.60	11.30	49	0
S20	7.90	10.21	123	0
S21	8.60	2.30	12	0
S22	9.10	8.10	234	7
S23	8.80	18.64	11	0
S24	7.95	5.20	6	0
S25	6.85	19.54	21	0
S26	9.60	22.65	0	0
S27	6.85	9.54	1	0
S28	6.90	8.12	7	0
S29	7.45	16.48	18	6
Total			619	22

The *Streptomyces* index is 3.55%.

On Bennett medium liquid, only the strain S296 produce a substance with a specific coloration rose. For certain researchers these production of bio actives substances is correlated with production of pigment (table 3) [14, 16].

Strain S296 has a different color and macroscopic characteristics on different culture medium ISP (International *Streptomyces* Projet) and Agar-tyrosine (Figure 1).

Table 2. Macroscopic, microscopic and biochemical characteristics of isolated Actinomycetes strains.

Isolated strains	Gram	Oxydase	Catalase	Spore	Appearance of colonies on ISP2	Color of the aerial mycelium on ISP2
S221	+	-	+	+	Rigeuse-down	Brown
S222	+	+	+	+	Rigeuse-down	Yellow orange
S226	+	+	+	+	Rigeuse-down	Yellow
S296	+	-	+	+	Rigeuse-down	Bronze

The table 3 show the production capacity of melanin. Strain S296 has great potential to produce melanin. In culture on tyrosine medium, S296 produces a dark-brown colored aerial mycelium and a substrate. There is a strong color variation of aerial mycelium and substrate of else strain.

Table 3. Aspect of strains and production capacity of melanin on agar-Tyrosine medium.

Strains	Appearance	Growth	Aerial Mycelium	Substrat	Production capacity of melanin
S221	Rigeuse	+++	Yellow-gray	Yellow orange	-
S222	Rigeuse	+++	Waxy gray	Yellow orange	-
S226	Rigeuse	+++	Yellow-green	Pale yellow	-
S296	Rigeuse	+++	Dark-brown	Dark-brown	+++

Agar-tyrosine medium is best for growth these strains isolated. Only strain S296 had production capacity of melanin (Figure 1).

Table 4. Antimicrobial Testing of Selected Strains.

Selected Strains	<i>S. aureus</i>	<i>A. flavus</i>	<i>S. typhi</i>	<i>Xanthomonas</i> 20Xoo	<i>Penicillium</i> sp	<i>Helminthos</i> sp
S296	+++	-	-	-	-	-
S226	-	-	+	-	-	-
S222	-	-	+	-	-	-
S221	-	-	+	-	-	-

+++= \varnothing >8mm, += \varnothing <1mm, \varnothing =inhibition area. Note: Absence of antifungal reaction of the strains on the pathogens used.

This table show that none strain has not an antifungal reaction but present antimicrobial reaction. Strain S296 has antimicrobial reaction on *Staphylococcus aureus* (gram +) and others S226, S222, S221 have a weak reaction against *Salmonella typhi* (gram -) (Figure 3). The inhibition zone created by S296 on *S. aureus* starts from 24 hours and grows the following days, at day 4 the expansion stops with a diameter of 10mm. At day 15 the diameter not change.

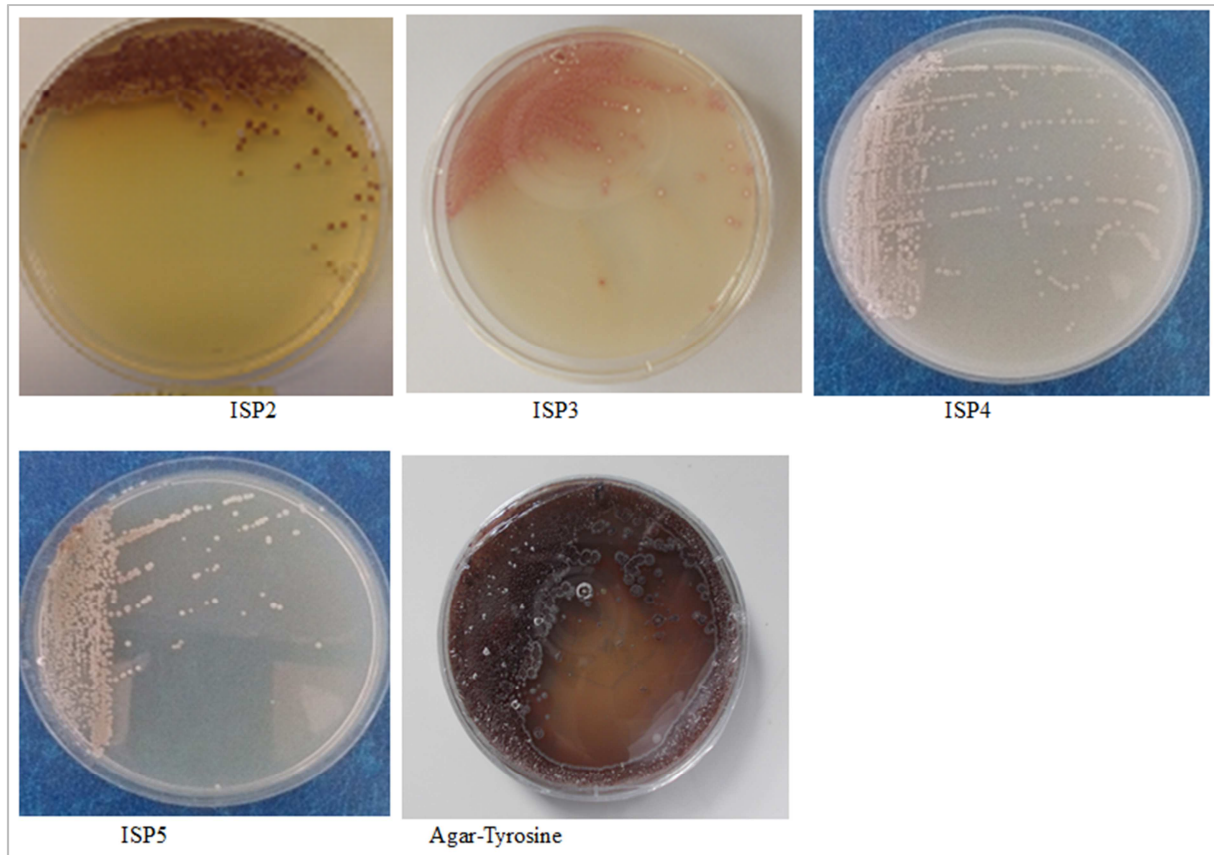


Figure 1. Appearance of S296 colonies in different ISP medium.

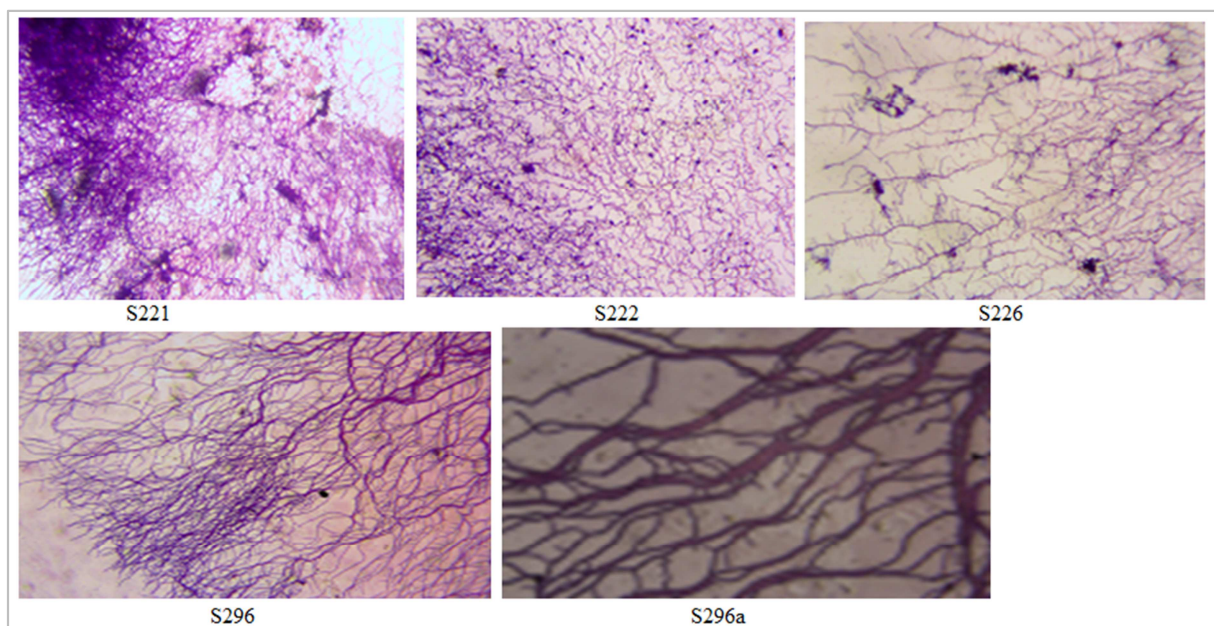


Figure 2. Appearance of aerial mycelia of some isolated *Streptomyces* strains observed at 400X and (1000X for S296a), Nikon microscope.

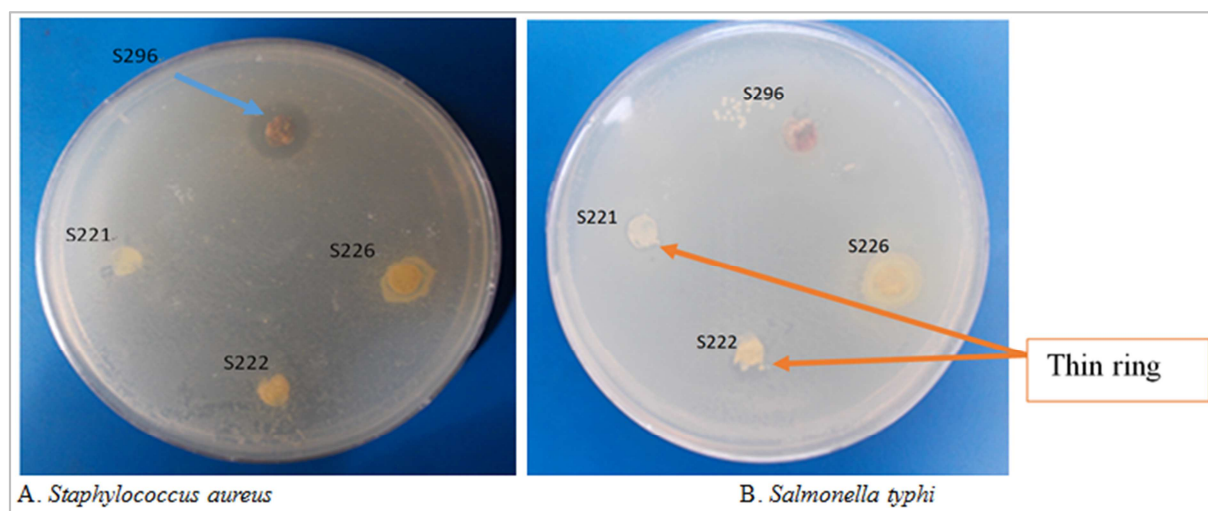


Figure 3. Test antimicrobial activities of strains S296, S226, S222 and S221 on pathogens *S. aureus* and *S. typhi*.

The antimicrobial activity of strain S296 is very visible and clear on *S. aureus* forming a good zone of inhibition. S226 has no antimicrobial activity on *S. aureus* and *S. typhi*, but shows a reaction that probably produces an invasive yellow-gray-colored substance. This sticky substance can probably be a defense of the strain itself. Strains S221 and S222 do not react with *S. aureus*, but exhibit low antimicrobial activity of less than 1 mm inhibition zone with *S. typhi*.

4. Discussion

The CaCO₃ enrichment technique [13] selectively isolated actinomycetes strains of the *Streptomyces* genus from the mud and soil samples from the Demba Tiarki Tara pond. All isolated strains are Gram-positive bacteria, producing spores from 10 to 15 days of culture (Figure 2). They all have a positive catalase reaction, whereas 50% (2/4) had a positive oxidase. The strains grow well on the different culture media ISP (International *Streptomyces* Project) of Actinomycetes (ISP2, ISP3, ISP4, ISP5, ISP7) at pH=7.2; but the culture is so fast and extraordinary on agar-tyrosine medium (ISP7). The agar-tyrosine medium is the most favorable for assessing the production of melanin by bacteria. The aerial mycelium of isolated bacteria varies in color depending on the culture media. The aerial mycelium of S296 varies to bronze on ISP2, purple on ISP3, brown yellow on ISP4 and ISP5, and dark-brown on agar-tyrosine (Figure 1). Isolated strains grew faster on ISP5 (24 hours) than on ISP2 (72 hours on average). The colonies have a much rougher appearance and depressed on the medium ISP2, by cons they are of powdery appearance on ISP5. Strain S296 has a more typical morphology, well depressed, cluster colony, dark pink color on ISP2 medium (Figure 1). Of the seven strains isolated, four of them; S296, S226, S222, and S221 are capable of producing bioactive substances in varying degrees. This result is explained by the alkalinity of the water in the pond. The alkaline or acidic medium is favorable for the proliferation of actinomycetes producing bioactive

substances [17]. Strain S296 showed particular interesting behaviors: production of purple dye pigments in culture on Bennett's liquid medium, production of melanin pigments in tyrosine medium (Figure 1), interesting antimicrobial reaction with *Staphylococcus aureus* (Figure 3). Many scientists showed that Bennett liquid medium is better for bacteria to product antimicrobial bioactive substances [14]. Some authors, like Charu [18] find that carbon sources like Peptone and potassium nitrate are favorable for a large production of antifungal metabolites. These particularities of the strain S296 to product dye pigments and melanin are a good characteristics and used of the taxonomy of genus [19], and therefore, these strains have more potentiality to producing bioactive substances [15]. Indeed, the great production of melanin have been described in certain *Streptomyces* genus, alike *Streptomyces antibioticus* [20] and *Streptomyces glaucescens* [21]. The antibacterial response of S296 against *S. aureus* can justify these melanin production. The antibacterial response of strain S296 appears 24 hours of contact with *S. aureus* measuring approximately 8 mm in diameter and reaching 10 mm in 72 hours. Strain S226 reacted with *S. aureus* and *S. typhi*, but no growth inhibition ring of these bacteria. On the other hand strains S222 and S221 had reactions on *S. typhi* with very thin ring formation, which did not evolve until 10 days of incubation. This weak reaction deserves more study in order to find the optimal conditions [22] for the production of active metabolites. Because some authors believe that the production of active secondary metabolites is often influenced by carbon sources and crop conditions such as pH, temperature and incubation time [18, 23]. The diffusion technique from the culture solution (Bennett Medium) did not give any reactions. This can be explained by the fact that the bioactive substances produced by these bacteria are not diffusible or are not enzymes. However, it should be noted that enzymes are more produced by actinomycetes [24], such as *Streptomyces parvulus* strain sankarensis-A10 [25] which has a strong ability to produce alpha amylase, lipase, cellulase and protease. So for this study probably the bioactive substances of these strains

would be endogenous. In this study we find that isolated Actinomycetes do not have antimicrobial reactions on fungi. On the other hand, the isolated bacteria were active on those with gram positive (*S. aureus*) and gram negative (*S. typhi*). The antibacterial response to the bacteria tested is stronger on gram + bacteria than on gram -, and this has been described by some researchers [26, 27]. The low isolation of actinomycetes producing active metabolites may be explained by the absence of vegetation in the water or the watershed of the pond. Oskay [4] states that the diversity of actinomycetes in a medium is influenced by plant flora. Plants produce beneficial chemicals for actinomycetes and vice versa. But the pond of DDT has a very low vegetation or even zero in its nest.

5. Conclusion

The results of this study show that the pond of Demba Tiarki Tara contains *Streptomyces* producing secondary metabolites having antimicrobial properties. In particular, strain S296 has good characteristics such as the production of melanin and coloring pigments, which can potentiate its antimicrobial activities. Probably this production of pigments by strain S296 could have other properties that we will have to study better. It will be necessary to identify all the compounds secreted by these strains, and to know all the conditions favorable to a better production of secondary metabolites. The continuation of this work may potentially provide in the future effective molecules to fight against bacterial infections.

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