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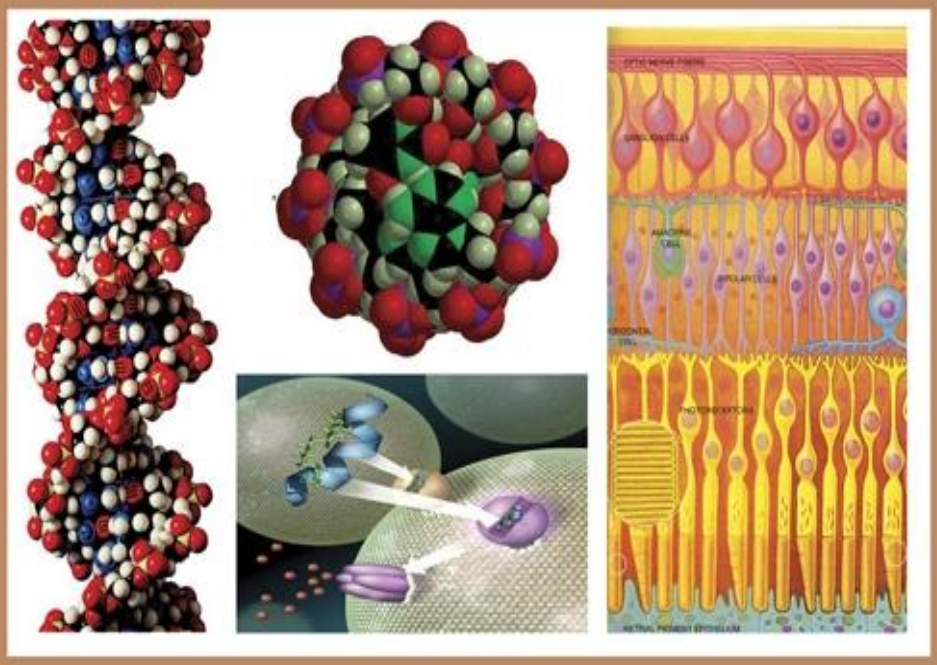

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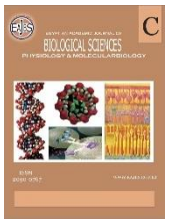

Isolation, Screening and Characterization of an Antagonistic *Actinobacteria* from Algerian Semi-Arid Region.

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ABSTRACT

Most antibiotics have been isolated from *Actinobacteria*, with contributions from the *Streptomyces* and *Nocardia* genera. Recently, *Rhodococci* are known to produce a variety of antimicrobial substances. Soil samples were collected from the Bougtob region in El-Bayadh city (34°02'32.7"N 0°05'21.4"E), using the classic method of Pochon and Tardieux 1962. Thirty isolates with the same *Nocardiopsis* morphology were isolated on 65. GYM *Streptomyces* medium using dilution techniques, and tested for their ability to produce antimicrobial agents. The morphological and biochemical tests were used to identify thirty isolates of the *Nocardiopsis* genus. The antimicrobial activity of the compound produced by the *Rhodococci* strain was tested by the cross-streak assay method, against the Gram-positive bacteria (*Bacillus subtilis, Micrococcus luteus* and *Staphylococcus aureus*) and the Gram-negative bacteria (*Escherichia, coli).* Out of thirty strains, two showed broad-spectrum activity. The strains were identified using various biochemical tests. These results indicate that the Algerian Sahara soil microorganisms could be an interesting source of antibacterial and antifungal bioactive substances.

INTRODUCTION

While the appearance of antibiotics represented a new step in the improvement of the quality and lifespan, the constant evolution of resistant bacteria limits their use.

Antibiotics reduced the mortality caused by infectious diseases during the 20th century. However, their massive and frequently irrational use in both humans and animals has led to the accelerated emergence of antibiotic-resistant bacteria. In view of the increasing frequency of antibiotic-resistant pathogens, the search for a new one has thus become crucial.

Rhodococcus, a member of the phylum *Actinobacteria*, was initially described by Zopf in 1891, then it was reevaluated in 1977. It was defined as a Gram-positive, non-motile, and aerobic strain.

Rhodococcus is distributed in a variety of aquatic, terrestrial and marine habitats, including hostile ecological niches such as deserts and highly contaminated sites (Bell *et al.*, 1998).

MATERIALS AND METHODS

Soil Samples:

Two Saharan soils samples were collected according to the Pochon and Tardieux method, (1962), from the Bougtob region in El- Bayadh city situated in western Algeria (34°02'32.7"N 0°05'21.4"E).

Samples were collected under aseptic conditions using a large sterile spatula at a depth of 15 cm, after removing the first 5 cm of the soil surface, and aseptically transported in a sterile small plastic bag to the laboratory at room temperature and were air-dried for one week (Pochon and Tardieux, 1962).

Isolation and Cultural Conditions:

For each collected sample, 1g of the soil was suspended in 100 ml of physiological water, and then serial dilutions up to 10^{-4} were prepared using sterile physiological water and vortexed at maximum speed.

Bacterial strains were isolated on 65.GYM *Streptomyces* medium containing in (g/L): Yeast extract, 4.0; Malt extract, 10.0; Glucose, 4.0; $CaCo_3$, 2.0; Agar, 12.0, this medium is adjusted to pH 7.2 (± 0.2). The Petri dishes were incubated at 30°C, for 72h. The colonies of *Rhodococci* were purified and preserved at +4 °C.

Rhodococcus characterization:

The isolates were identified by analyzing their morphological characteristics and by biochemical tests.

Macroscopic Characteristics:

The purified colonies' cultural characteristics are determined following the method described by Shirling and Gottlieb 1966. The colonies' observation with the naked eye, concerns the colonies' aspect, color, shape and size.

Microscopic Characteristics:

The microscopic examination was performed on a bacterial smear, prepared from suspect colonies in pure cultures; then, fixed and stained by the Gram's method (it's a double coloring that provides information on the shape, arrangement, and purity, as well as the wall biochemical nature of the purified cells) (Thairu *et al.*, 2014).

Biochemical Characteristics:

Several biochemical tests were used to characterize the collection of the obtained strains and to confirm the identification of the isolates belonging to the genus *Rhodococcus*.

The EC_1S30 and EC_1S33 were tested for their ability to develop in different concentrations of sodium chloride, temperature and their ability to produce enzymes (Catalase, oxidase and citrate permease).

Bacterial Growth Kinetics:

The selected strains were inoculated into 500 mL Erlenmeyers flasks, which contained 200 mL of medium (65.GYM *Streptomyces* medium), with an initial density of 0.2 and incubated at ambient temperature with an agitation of 100 rpm for 96 hours.

The growth kinetics were evaluated at an interval of 4 days, by measuring the optical density using a spectrophotometer UV-VIS (Specord® 200 Plus) at a wavelength of 550 nm (Boutrou *et al.*, 1998).

Antimicrobial Activity:

The *Rhodococci* strains antimicrobial activity was evaluated on 65.GYM *Streptomyces* medium. After 72h of incubation at 30°C, Agar cylinders were removed with a sterile Pasteur pipette and deposited on the Mueller Hinton medium surface containing (g/L): Beef extract, 2.0; Acid casein hydrolysate, 17.5; Starch, 1.5; Agar, 17.0, this medium is adjusted to final

pH 7.3 (± 0.2) at 25ºC, which was previously inoculated with each test bacteria.

The test strains (*Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923 and *Micrococcus luteus* 4698) and Gram-negative bacteria (*Escherichia coli* ATCC 25922*)*, used in this study were from American Type Culture Collection.

The plates were kept at +4 °C for 4 h, to promote the diffusion of the bioactive molecules, then incubated at 37 °C for 18–24 h. The inhibition diameters were then measured (Lemriss *et al.*, 2003).

RESULTS AND DISCUSSION

Rhodococci Isolation:

Rhodococci presents various pigmentations and demonstrated very good growth on 65.GYM *Streptomyces* medium, after 72h of incubation at 30°C. For this, a total of 30 strains were isolated in this study.

The selected strains were purified by streaking on a 65.GYM *Streptomyces* medium. (By continuing subculturing of the strains on the same type of medium, until a pure isolate was obtained with the same characteristics, as those obtained in the first isolation (color, shape, diameter, opacity…etc.). The purified strains cultures were maintained on plates and preserved in the glycerol at +4°C, for short-term storage.

Rhodococcus Characterization:

Macroscopic Characteristics:

The selected strains differ in color, aspect and size (Table. 1). (Fig. 1).

Table 1: Bacterial colonies description on 65.GYM *Streptomyces* medium.

Strain	Aspect	Size	Color
EC_1S30	Smooth	Small circular colony	Pale cream
EC_1S33	Smooth	Medium, circular colony	Glistening cream

Fig. 1: Strain growth aspect on GYM.65, after 72h of incubation at 30°C.

According to Shirling and Gottlieb, 1966, *Actinobacterials* identification was largely based on morphological characteristics. Colonies appear dry, rough, colored or not, medium-sized, powdery, regular or not, flattened or bulging, with an aerial and vegetative mycelium in general, some of them have just a substrate mycelium (Boudemagh *et al.*, 2005).

Microscopic Characteristics:

Microscopic observation by Gram staining revealed that the isolates are Gram-positive *Bacillus* (Fig. 2).

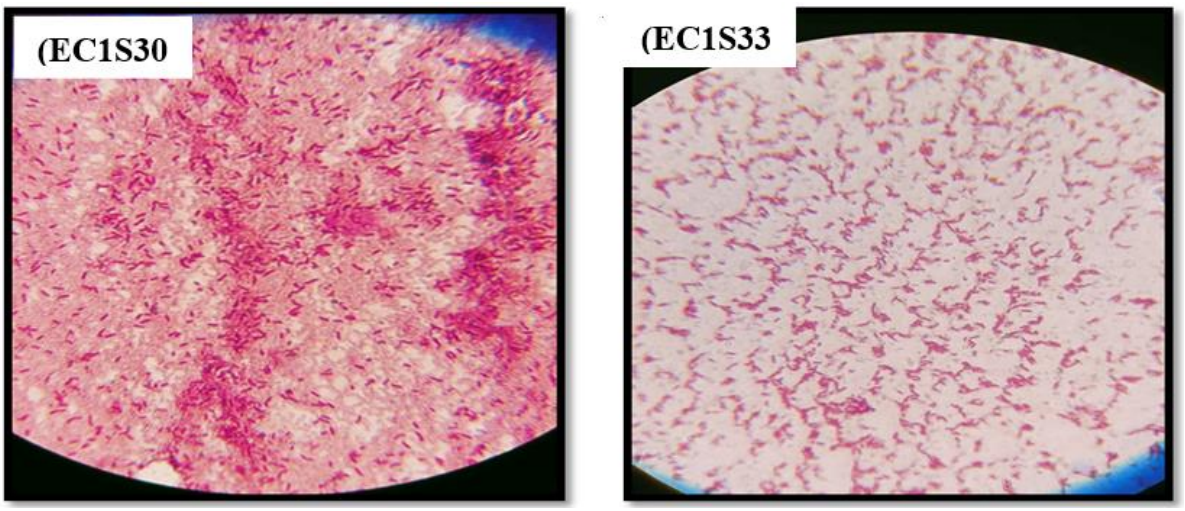

Fig. 2: *Actinobacterial* strains microscopic observation of (EC_1S30, EC_1S33), after the Gram staining, at magnification x100.

Actinobacterials include a heterogeneous group of Gram-positive and Gram-variable bacteria. Phylum also includes some Gram-negative species such as *Thermoleophilum sp.* (Zarilla and Perry, 1986), *Gardenerella vaginalis* (Gardner and Dukes, 1955), *Saccharomonospora viridis* P101T (Pati *et al.,* 2009), *Ferrimicrobium acidiphilum*, and *Ferrithrix thermotolerans* (Johnson *et al.*, 2009).

Biochemical Characteristics:

The Algerian Saharan soils represent extreme and particular ecosystems, this study allowed us to evaluate the potential of isolates to develop at different temperatures, and at different concentrations of NaCl. The study of the physical parameters provided the following results in (Fig. 3 and Fig. 4).

Effect of Sodium Chloride on the *Actinobacterial* Growth:

This study demonstrated that our isolates exhibited moderate growth at various concentrations of NaCl (4-10%), but it was observed that optimal growth was reported in the absence of NaCl (0%); our results were comparable to the literature; (Tresner *et al.,* 1968; Gottlieb, 1973), which indicated that *Actinobacteria* tolerated high NaCl concentrations; up to 10% (Fig. 3). The bacteria's ability to develop in the presence of a variable sodium chloride (NaCl). The quantity has been used to characterize several bacteria. It considers the microorganism’s ability to tolerate different osmotic concentrations.

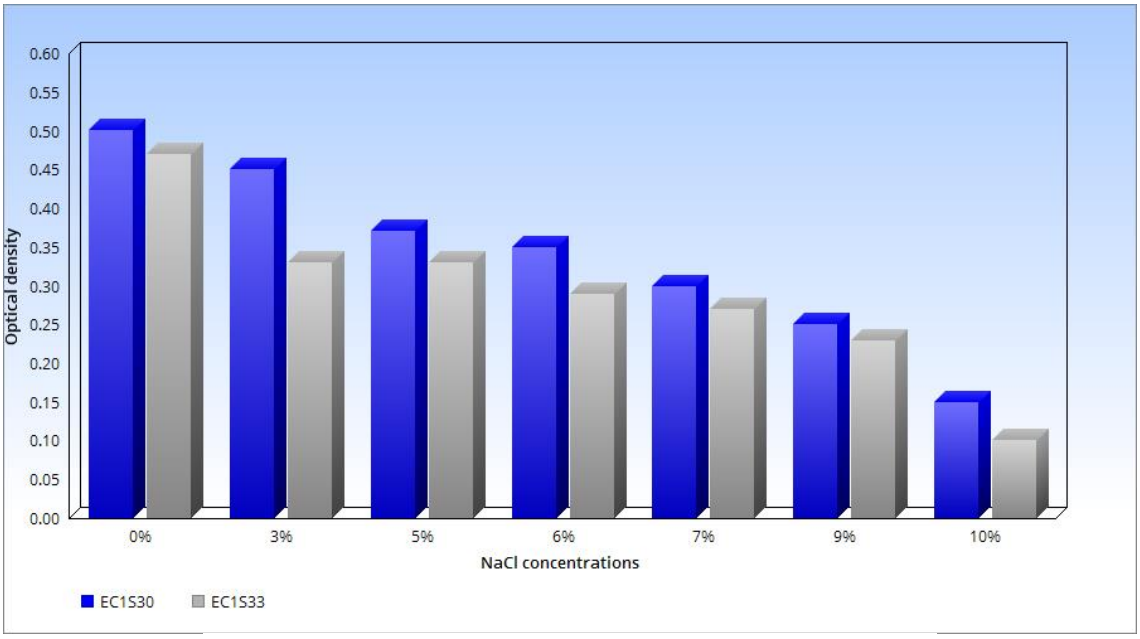

Fig. 3: Sodium chloride effect on *Actinobacteria* growth.

Temperature Effect on *Actinobacterial* Growth:

These results indicate that all isolates develop at different temperatures: 4°, 30°, 37°, and 55°C (Fig. 4), but the growth optimum temperature is 30°C. These results are consistent with those of (Rangaswami *et al.*, 2004), who demonstrated that their isolates belonging to the *Actinobacteria* can be psychrophilic or thermophilic.

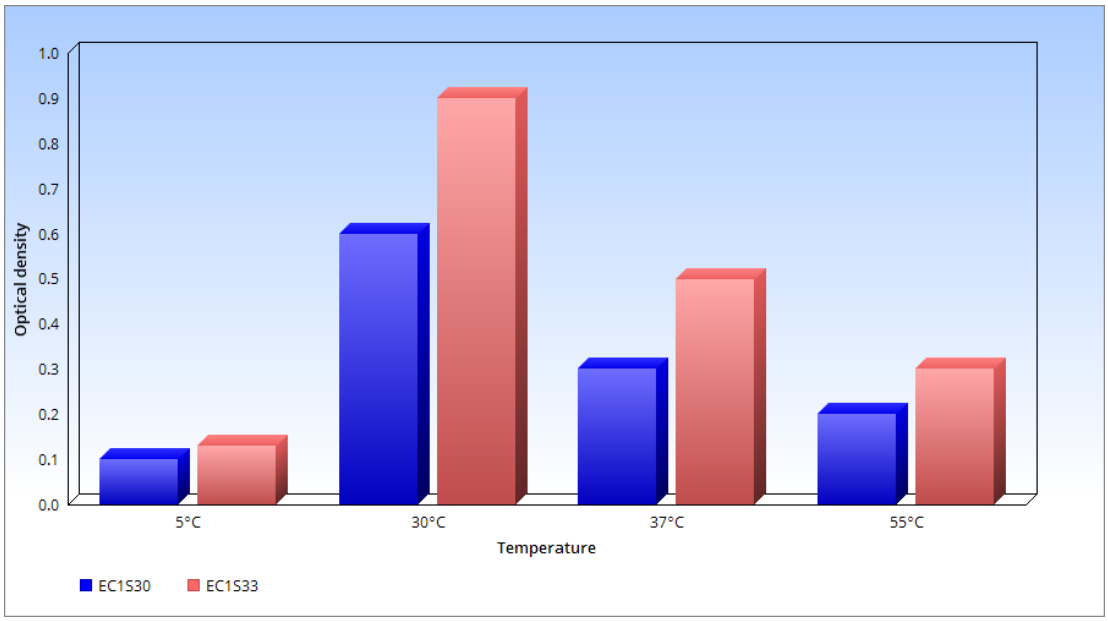

Fig. 4: Temperature effect on *Actinobacterial* growth

Catalase Test:

The strains reacted to hydrogen peroxide contact with an effervescence due to the O_2 release (Fig. 5); they are positive in catalase.

Fig. 5 : Catalase test results

In accordance with the works of (Shirling and Gotlieb, 1966; Holt *et al.*, 1994; Kim *et al.*, 1999; Petrosyan *et al.*, 2003), *Actinobacterials* are aerobic germs, which are characterized by positive catalase and oxidative metabolism. Our results are in agreement with the work of these researchers. Also according to (Delarras, 2014), the enzyme catalase presence is characteristic of the optional aerobic and aero-anaerobic facultative bacteria, which is confirmed by the Meat-Liver agar test, in which isolates were developed throughout the medium contained in the tubes.

Oxidase test

For the results of the free oxygen (O_2), utilization as the final electron acceptor in the respiratory chain, all discs persist uncolored (negative result) for all strains (Fig. 6); these results are consistent with the literature (Meliani *et al.*, 2022).

Fig. 6: Oxidase test results

Citrate Permease Test:

The Simmons citrate medium is a synthetic medium in which the only carbon source is citrate. Our research revealed that the isolates used the citrate medium as a carbon source, and the medium color changed to blue (Fig. 7).

The obtained results with our analyzed bacterial species are corroborated with those obtained by (Goodfellow, 1989; Goodfellow et Maldonado, 2006; Goodfellow and Jones, 2012).

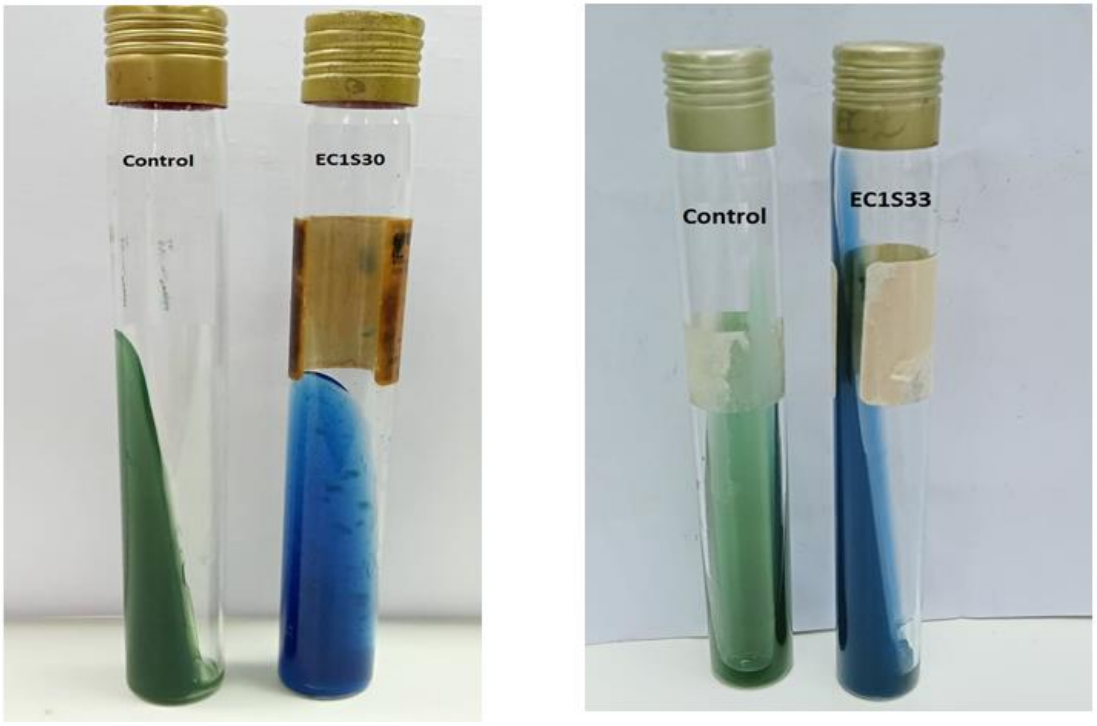

Fig. 7: Citrate permease results.

Bacterial Growth Kinetics:

The growth kinetics of the isolated strains on the liquid 65.GYM *Streptomyces* medium followed by measuring the optical density as a function of time (96h); this allowed us to establish the curves represented by the (Fig. 8).

Based on the obtained results, we have remarked the absence of the latency phase, both curves start directly with an exponential phase, in which the biomass increases progressively to reach a maximum of (0h to 24h).

Then, a stationary phase persisted from (24h to 72h), in which the production of the antibiotic was maximum. Then a deceleration phase from (24h to 48h) which was only observed only for the EC_1S30 strain immediately succeeded by a decline phase from 72h.

The first growth phase called Trophophase was characterized by rapid biomass production while the second phase which is called Idiophase and characterized by a low growth rate and maximum antibiotics production (Pirt and Righelato, 1967; Lurie *et al.*, 1975; Sejiny, 1991).

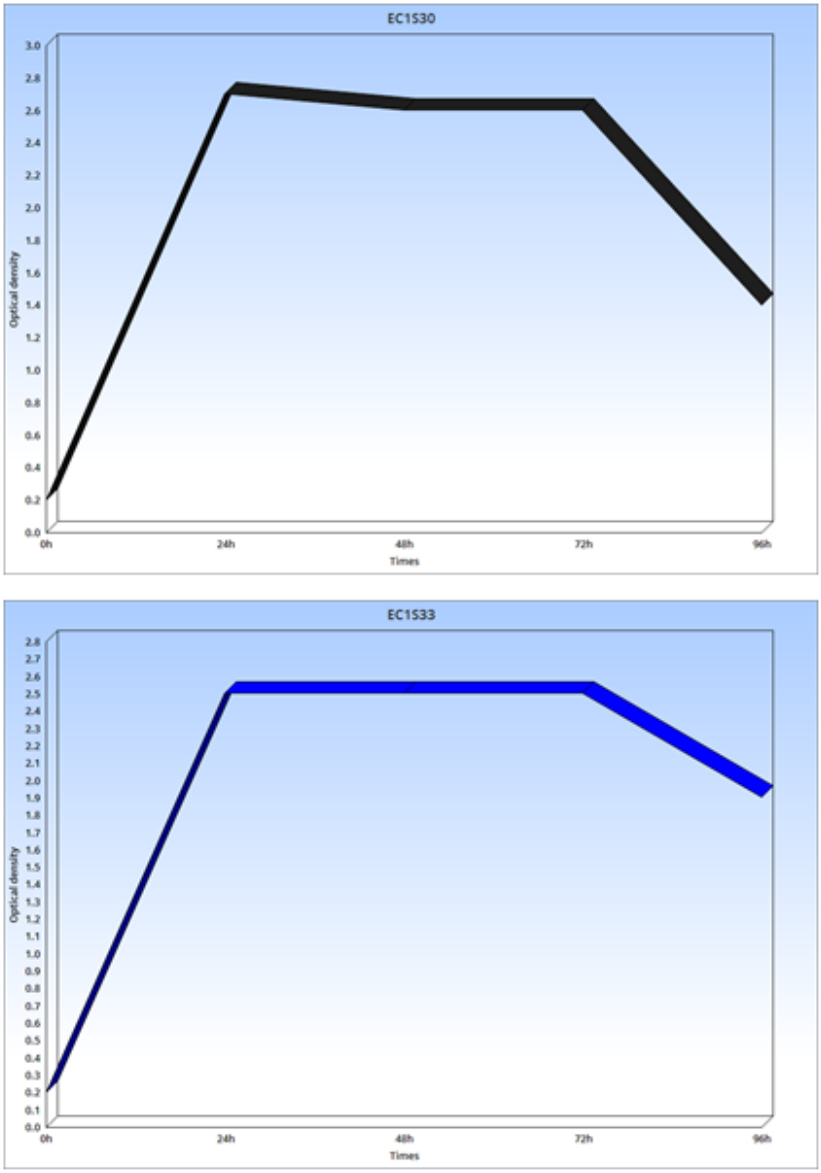

Fig. 8: Growth curves of EC1S30 and EC1S33 strains.

Antimicrobial activity

Two *Actinobacterial* isolates were subjected to preliminary screening in a 65.GYM *Streptomyces* medium solid by the agar cylinder technique, in order to determine their antibacterial activity against target bacteria: *Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus luteus* and *Escherichia coli.*

The antibacterial activity results, evaluated by the agar cylinder method, are presented in (Table. 2).

Table 2: Antibacterial activity results, evaluated by the agar cylinder method**.**

Target Bacteria	Inhibition zones diameters obtained by agar cylinders method	
	EC1S30	EC1S33
B. subtilis	15 mm	19 mm
M. luteus	16 mm	20mm
E. coli	15 mm	13 mm
S. aureus	23 mm	21 mm

The strains exhibited significant antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus luteus* and *Escherichia coli*, with inhibition zones ranging from 13 mm and 23 mm (Fig. 9).

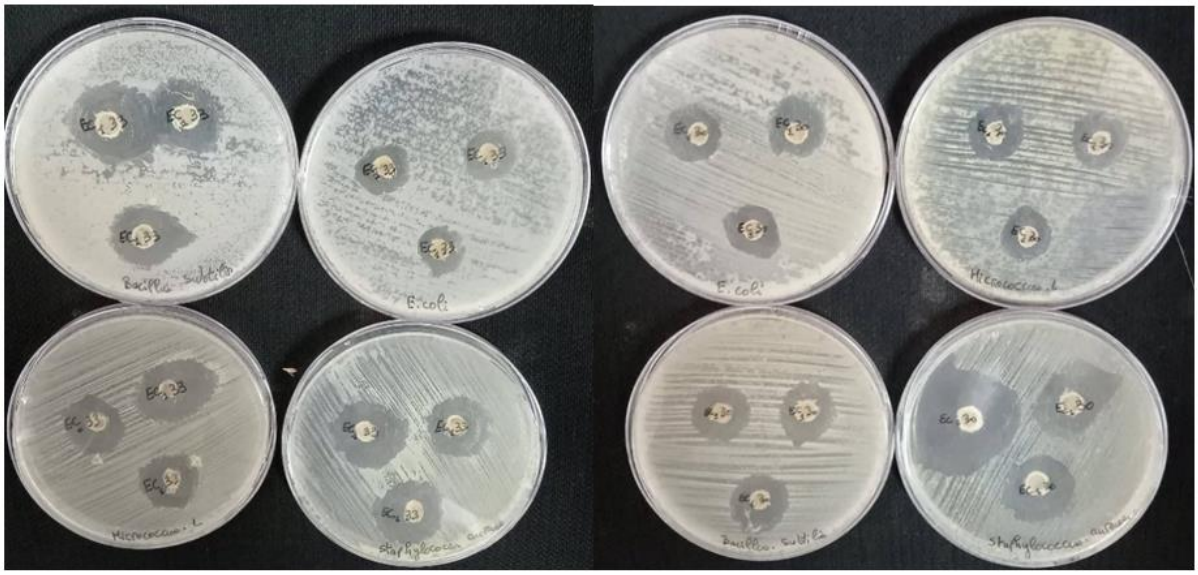

Fig. 9 : Antimicrobial activity of *Actinobacterial* strains (EC_1S30, EC_1S33) by the agar cylinders method against (*Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Micrococcus luteus).*

Our results are coherent with the obtained results by other researchers, who discussed the Gram-positive bacteria's sensitivity to *Actinobacterial* secretions compared to Gram-negative bacteria (Sabaou *et al.*, 1998).

In Algeria, several strains of *Streptomyces* isolated from the soil, water and tree bark have demonstrated beneficial antibacterial and antifungal activities against various pathogenic microorganisms (Boudemagh *et al*., 2005; Kitouni *et al*., 2005).

Conclusion

The Saharan soil's diversity of cultivable *Actinobacterias*, and the search for new antibiotics have been the subject of several studies.

These results have led to the objective of this work, which is the screening of *Actinobacterial* strains with antibacterial activity, from an isolates collection. We have isolated, identified and evaluated the antibacterial activity of the strains selected against the target bacteria by the agar cylinder method.

However, this study is the starting point to investigate and characterize the Actinobacterials properties with antimicrobial capacities; in addition, this information could be used in the research program development to identify and select effective isolates for therapeutic purposes, In the future, it will be possible to exploit with profit in a developing field, which has a significance in the pharmaceutical sector.

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