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ISOLATION AND CHARACTERIZATION OF ACTINOBACTERIA FROM TWO MARINE ALGAE *ASPARAGOPSIS ARMATA* AND *ZONARIA TOURNEFORTII* WITH BIOTECHNOLOGICAL INTEREST

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Abstract

Description of the subject: The screening has always been an essential way to research microorganisms with high potential for the new molecules production. To do this, we will describe in this present work the approach followed in order to obtain a very specific marine actinobacteria.

Objective: The objective of this study is the screening of actinobacteria isolated from two marine algae *Asparagopsis armata* and *Zonaria tournefortii*, taken from the Golden “Horn” in the Tipaza region. An adaptive vision was applied for the isolation and characterization of the actinobacteria strains obtained as well as the screening of the technological properties of these strains, namely, antimicrobial activities, protease and surfactant production.

Methods: The isolation of the strains was performed basing on different specific media for the marine actinobacteria cultivation. The identification of these isolates was based on morphological characteristics (macroscopic and microscopic study); physiological and on the biochemical tests available in the laboratory. The demonstration of proteolytic activity and the identification of biosurfactant producing microorganisms were based on reference methods commonly used in the field. Antimicrobial activity was performed against three Gram-positive bacteria, two Gram-negative bacteria, one yeast and four filamentous fungi using the agar cylinder technique.

Results: The results show that all seven isolates are Gram positive; the majority has a positive catalase, positive oxidase and positive mannitol. These tests allowed us to classify the seven isolates into two different genus; six strains of the genus *Streptomyces* and one is affiliated to *Rhodococcus*. The majority of the strains are able to inhibit the growth of target microorganisms. These strains are endowed with very important technological properties (proteolytic and biosurfactants production and antimicrobial activities).

Conclusion: In the present study, we concluded that the 07 isolates of marine actinobacteria represent an interesting variety of microorganisms isolated from an extreme ecological niche with a source of production of natural substances of environmental and industrial interest.

Keywords: Screening, Actinobacteria, Technological properties, *Asparagopsis armata*, *Zonaria tournefortii*

ISOLEMENT ET CARACTÉRISATION D'ACTINOBACTÉRIES À PARTIR DE DEUX ALGUES MARINES *ASPARAGOPSIS ARMATA* AND *ZONARIA TOURNEFORTII* AYANT UN INTÉRÊT BIOTECHNOLOGIQUE

Résumé

Description du sujet : Le criblage a toujours été un moyen essentiel pour rechercher des microorganismes à fort potentiel pour la production de nouvelles molécules. Pour ce faire, nous décrivons dans ce travail l'approche suivie afin d'obtenir une actinobactéries marine très spécifiques.

Objectif : L'objectif de cette étude est le criblage d'actinobactéries isolées de deux algues marines *Asparagopsis armata* et *Zonaria tournefortii*, prélevées dans la "Corne" d'Or dans la région de Tipaza. Une vision adaptative a été appliquée pour l'isolement et la caractérisation des souches d'actinobactéries obtenues ainsi que pour le criblage des propriétés technologiques de ces souches, à savoir, les activités antimicrobiennes, la production de protéases et de tensioactifs.

Méthodes : L'isolement des souches a été effectué sur différents milieux spécifiques pour la culture des actinobactéries marines. L'identification de ces isolats a été basée sur les caractéristiques morphologiques (étude macroscopique et microscopique), physiologiques et sur les tests biochimiques disponibles dans le laboratoire. La démonstration de l'activité protéolytique et l'identification des microorganismes producteurs de biosurfactants ont été basées sur des méthodes de référence couramment utilisées dans ce genre de travaux. L'activité antimicrobienne a été réalisée envers trois bactéries Gram-positives, deux bactéries Gram-négatives, une levure et quatre champignons filamenteux en utilisant la technique du cylindre sur gélose.

Résultats : Les résultats montrent que les sept isolats sont Gram positif ; la majorité a une catalase positive, une oxydase positive et un mannitol positif. Ces tests nous ont permis de classer les sept isolats dans deux genres différents ; six souches du genre *Streptomyces* et une est affiliée à *Rhodococcus*. La majorité des souches sont capables d'inhiber la croissance des microorganismes cibles. Ces souches sont dotées de propriétés technologiques très importantes (production de protéases et de biosurfactants et d’activités antimicrobiennes).

Conclusion : Dans cette étude, nous avons conclu que les 07 isolats marins d'actinobactéries représentent une variété intéressante de microorganismes isolés d'une niche écologique extrême avec une source de production de substances naturelles d'intérêt environnemental et industriel.

Mots-clés : Criblage, Actinobactéries, Propriétés technologiques, *Asparagopsis armata*, *Zonaria tournefortii.*

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INTRODUCTION

Marine microorganisms have unique properties since they have to adapt to extreme marine environment conditions such as high or low temperature, alkaline or acidic water, high pressure and limited substrate in the deep-sea water [1]. These idiosyncratic characteristics have attracted many researchers to explore in depth to obtain marine microorganisms used in industry. For this purpose, for a long time, researchers have investigated in recognizable world to identify novel microbial biocatalysts [2]. However, the marine environment has been shown as an almost entire reserve of novel microorganisms, with high potentialities [3]. Recent biotechnological progresses require that algae show an amazing richness. Marine organisms can be an adequate source for such microorganisms as marine actinobacteria which have proved useful for both process improvement and for the development of new manufacturing procedures [4]. Of particular interest, *Zonaria tournefortii* and *Asparagopsis armata*, are respectively brown and red algae widely occurring in Mediterranean waters known by microbial diversity [5]. Consequently, the present study invests in a recent research focused on the isolation and biotechnological screening of actinobacteria isolated from two marine algae *Asparagopsis armata* and *Zonaria tournfortii*. An adaptive approach was applied for the isolation and characterization of the actinobacteria strains obtained as well as the screening of the technological properties of these strains (antimicrobial activities, protease and surfactant production).

MATERIALS AND METHODS

1. Sampling

The samples were collected from two algae, a red alga (*Asparagopsis armata*) and a brown alga (*Zonaria tournefortii*) at different sites of the Mediterranean coast of Algeria on the East of Tipaza (GPS coordinates: 2°39' 00 " East, 36°37' 12 " North) at the Laboratory of "Natural Products Chemistry and Biomolecules (LCSN-BioM)" from the University of Blida1.

2. Procedure and treatment

The algae sample should be obligatory transferred into sterile bottles containing seawater and stored in the dark at the ambient seawater temperature in a cool box (from 2 to 16°C according to the month of sampling). The samples were transported to the laboratory and processed immediately after collection [6].

Within 3 h after collection, the algae specimens were initially washed 3 times with sterilized seawater (0.22µm filter) to remove microorganisms weakly attached to the algae surface and then rinsed with Phosphate Buffered Saline solution (PBS (×1)) to facilitate the elimination of seawater prior to the isolation step [6,7].

3. Isolation of actinobacteria

Several protocols for the isolation of microorganisms derived from algae were used using different media.

- The first method is based on cutting the algae into square pieces of about 1cm^2 and placing them directly on Petri dishes containing the specific isolation medium and incubated at 30°C.
- The second method consists in cutting the algae in square pieces (small pieces) and dipping them in 9 mL of filtered and sterilized sea water and strongly homogenizing them with a vortex in order to recover the maximum of bacterial strains. Dilutions from 10^{-1} to 10^{-4} were prepared and then 100 µL of each suspension-dilution were spread on the Petri dishes containing the isolation medium and incubated at 30°C [8].

Four different media supplemented with 50 µg/mL amphotericin (antifungal) and 10 µg/mL nalidixic acid (Gram-negative antibacterial) were selected to isolate actinobacteria. The first three media (M1, M2, and M3) are commonly used for selective isolation of actinobacteria [9]. Except M1, and to approximate the salinity of seawater without entering halophilic conditions, the media are supplemented with 0.5% NaCl [10]. For all four media, the pH was adjusted to 7.2 before sterilization.

The compositions of the different media are (g/L):

- M1: Agar (18), starch (10), yeast extract (4), and peptone (2).
- M2: Agar (18), starch (10), KNO_3 (2), K_2HPO_4 (2), NaCl (2), casein (0.3), $MgSO_4.7H_2O$ (0.05), $CaCO_3$ (0.02), and $FeSO_4.7H_2O$ (0.01).
- M3: Agar (18), yeast extract (0.25), and K_2HPO_4 (0.5).
- M4: Agar (18), yeast extract (3), malt extract (3), peptone (5), and glucose (10).

Media are incubated at 30°C and observed daily after one week up to one month of incubation to check the growth of actinobacteria isolates on the surface of Petri dishes.

4. Choice of isolates for determination

A total of seven actinobacteria isolates were completely determined. They were isolated from all the algal part. However, when observing the colonies,

we found that some isolates were composed of macro and micromorphologically identical isolates to each other (within the same group) and which predominated in relation to the total actinobacteria and appear to belong to the same species. To confirm these observations, a number of strains from each group (included among the 07) were identified in such a complete way. The remainder of the isolates (one isolate) present in the Petri dishes were systematically collected and studied through careful macro and microscopic observations, using specific media for the determination of actinobacteria and, in particular, in noting the production of pigment (important character).

5. Conservation of isolated actinobacteria strains

All pure bacterial isolates were coded and then preserved for short and long term. Pure actinobacteria were always grown in International *Streptomyces* Project-2 (ISP 2).

-Short-term conservation is done by inoculating the pure colonies on M2 supplemented with 0.5% NaCl, and incubating them at 30°C for one week, then storing them at +4°C. The transplantation of the strains is made every two months.

-Long-term conservation is achieved by inoculating cultures of actinobacteria in a specific liquid medium added with sterile glycerol at a concentration of 30% (v/v) in special cryotubes and immediately stored at -40°C [11].

6. Morphological and biochemical characterization of marine actinobacteria isolates

6.1. Morphological study

The study of bacterial morphology is an important attribute allowing a preliminary orientation for identification. It is based on macro- and microscopic observations.

6.1.1. Macroscopic identification

The macroscopic observation of the colonies obtained on Petri dishes allows the description of the colonies, based on: shape, size, color, surface and contour aspect. The isolated actinobacteria were characterized by inoculation on different International *Streptomyces* Project (ISP) media [12]. The media are:

-ISP2 (g/L): Yeast extract 3 g, Malt extract 5 g, Glucose 5 g, Agar 20 g.

-ISP3 (g/L): Oatmeal 15 g, Agar 20 g, $FeSO_4 \cdot 7 H_2O$ 1 g, $MnCl_2 \cdot 4H_2O$ 1 g and $ZnSO_4 7H_2O$ 1 g.

-ISP4 (g/L): Starch.10 g, Di potassium Phosphate 1g, Magnesium Sulfate USP 1g, Sodium Chloride 1 g, Ammonium Sulfate 2 g, Calcium Carbonate 2 g, Ferrous Sulfate 1mg, Manganous Chloride 1 mg, Zinc Sulfate 1mg and Agar 20 g.

The Plates were incubated at 30°C for 7, 14, and 21 days, in order to observe the evolution of the growth of mycelia (AM and SM).

6.1.2. Microscopic observations

All colonies, macroscopically similar to actinobacteria, are observed under the microscope (100×). In the fresh culture, microscopic observation allows the determination of the morphology (shape, grouping mode, density) and as an essential orientation step; the Gram staining was done according to the standard protocol. Those pertaining to actinobacteria are transplanted, by the streak method, on the same media as those used for isolation. This last manipulation is repeated until pure strains are obtained [13].

6.2. Biochemical study

6.2.1. Catalase test

The search for this enzyme was carried out by putting a bacterial colony in contact with a drop of hydrogen peroxide. A positive catalase reaction results in an abundant gaseous release in the form of oxygen bubbles [14].

6.2.2. Oxidase test

To realize this test, an oxidase disc containing *N*-dimethyl para-nitrophenylene-diamine oxalate, previously soaked with a drop of sterile distilled water, was placed on a slide and brought into contact with a freshly cultured colony. The presence of a cytochrome oxidase results in the appearance of a purple stain within 10s to 60s indicate positive reaction [13].

6.2.3. Mannitol-mobility test

The fermentation of mannitol and the mobility of the bacteria were carried out in mannitol-mobility mediums which have been realized with a central puncture, down to the bottom of the tube. The appearance of a bacterial culture along the central puncture indicates that the bacteria are immobile while the appearance of a cloud in the medium due to the horizontal diffusion of the bacteria from the seeding line indicates that the bacteria are mobile [14].

6.2.4. Determination of respiratory type

The respiratory type of the bacteria was determined on an agar reducing medium "meat liver", distributed in fine and deep test tubes. The escape of oxygen is carried out by incubation in a water bath at 100 °C for 30 min. Then seeded by central puncture with a spiral return to the bottom of the tube [14].

7. Antimicrobial screening of the marine actinobacteria isolates

7.1. Highlighting of the antibiotic activity of actinobacteria strains

In order to highlight the antimicrobial activities of the seven identified strains and to try to determine their spectrum of activity, it is necessary to cultivate them on different culture media and then to put them in contact with the different target microorganisms. The actinobacteria strains grown on M2 medium were selected for their antimicrobial activity. The antagonism test (agar cylinders) against the target germs allowed to highlight the activity of these strains. Actinobacteria strains were spread tightly on the surface of ISP2 agar medium (forming a homogeneous mat) after incubation at 30°C. Agar cylinders (ISP2 medium) of approximately 6 mm in diameter were removed with a punch [15] and then deposited on the surface of Mueller-Hinton medium for bacteria and yeast, and Sabouraud for fungi, previously inoculated with the target microorganisms (10^7 CFU/mL). Petri dishes containing agar cylinders are placed at +4°C for 4h to allow the diffusion of bioactive substances, elaborated by the control strains, and then incubated at for a specific time period for each target strain. The inhibitory activity is expressed by the appearance of an inhibition zone which is evaluated after 18 to 24 h of incubation. The strains with the largest spectrum of activity against the tested target germs were therefore selected [16].

7.2. Target microorganisms

In the context of the investigation of new strains of actenobacteria producing diffusible antimicrobial metabolites, the purified isolates were tested for their antimicrobial activity against a series of microorganisms identified by an ATCC number (American Type Culture Collection), from the laboratory of antibiotic therapy and hygiene of the Pasteur Institute of Algiers accredited by the WHO (World Health Organization) whose target microorganisms are: 02 Gram-negative bacteria: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 25843); and 03 Gram-positive bacteria: *Staphylococcus aureus* (ATCC 25923), *Listeria monocytogenes* (ATCC 49594) and *Bacillus subtilis* (ATCC 6633).

7.3. Highlighting of the anti-fungal activities

The production of antifungal metabolites by actinobacterial strains is demonstrated by the agar cylinder technique using Sabouraud medium instead of Muller-Hinton medium [16]. The fungi used are: Fusarium *culmorum*, *Aspergillus carbonarius* (ATCC 5010), *Aspergillus ochraceus* (ATCC 3150), and *Mucor ramannianus* (ATTC 9628) and *Candida Albicans* (ATCC 10231) as yeast. Incubation was performed at 28°C for filamentous fungi and 37°C for yeast.

8. Screening and selection of protease-producing strains

The demonstration of the proteolytic activity of isolated actinobacteria is performed in a selective medium containing skim milk (Milk Nutrient Agar) as a source of casein-rich nitrogen [17]. The seven strains were inoculated on a solid selective culture medium containing casein as a protein substrate which used to demonstrate the proteolytic activity. It is composed of 5 g of peptone, 3 g of yeast extract and 15 g of agar in 750 mL distilled water and the pH is adjusted to 7 with NaOH. After sterilization and cooling to 60°C, 250 mL of skim milk is added separately. After homogenization, the medium is poured into Petri dishes and incubated at 30°C for 7 days. The inoculated Petri dishes are examined every 24 h. The proteolytic activity is manifested by the diffusion of a transparent halo in the medium which indicates the secretion of proteases by the strains and the hydrolysis of milk proteins (caseins). In fact, it is a semi-qualitative test that is used as a screening tool. The ratio of halo diameter to colony diameter (dh/dc) allows a preliminary selection of isolated strains [18].

9. Screening of biosurfactant-producing strains and Hemolytic activity

The obtained isolates were tested and screened for their both hemolytic activities and the production of surface active substances. The first step is the used ISP2 medium as an overnight culture medium inoculated by a cylinder of strongly blunt mycelium. This experiment was carried out in 50 mL flasks containing 25 mL of ISP2 liquid medium which is incubated at 30°C under shaking at 150 rpm for 3 to 4 days. The second step is the preparation of surfactant production medium which is made in 250 mL flasks by filling a volume of 25 mL of MM' medium (mineral salt medium), its composition (g/L): NH_4Cl (0.4), K_2HPO_4 (0.3), NaCl (10), $MgCl_2$ (0.33), $CaCl_2$ (0.05), yeast extract (0.1) and 1 mL of a solution containing trace elements prepared in 1L of distilled water. The pH of the medium was adjusted to 7.2 and the medium was sterilized by autoclaving at 120°C for 20 min [19, 20]. Separately, adding a volume of 500 µL of carbon source (olive oil, table oil and crude oil) sterilized by membrane filtration (0.45 µm).

The surfactant production medium was inoculated with 2% (v/v) of previously prepared overnight culture medium and incubated at 30°C under agitation at 150 rpm. The culture broth was then tested for extracellular biosurfactant production by determining oil displacement. Biosurfactant production was followed by the oil displacement test, which is a rapid and reliable technique in which is based on the ability of biosurfactants to change the contact angle at the oil-water interface [21]. The test consists of filling Petri dishes with 10 mL of distilled water, into which 20 µL of crude oil was added, and then 10 µL of cell supernatant (obtained after centrifugation of the culture broth) was carefully added to the middle of the dishes. The surface activity of our producing strains was checked by measuring the diameter of the clear zone around the crude oil. This correlated with the activity of the surfactant production. The hemolytic activity was determined on gelose nutrient or LB solid medium. The composition is based on the dissolution of 28 g of nutrient agar in one liter of sterile water, melted then cooled the medium and added 5 to 10% of sterile defibrinated human blood [22]. Then shake carefully to avoid the formation of air bubbles in the agar after pouring it into Petri dishes. Colonies were pipetted onto the blood agar with a sterile pipette and incubated at 30°C for 190 h. After incubation; the presence of a transparent halo in the medium indicates the presence of biosurfactant producing organisms. And distraction of red blood cells releasing hemoglobin into the blood plasma [20].

RESULTS

1. Isolation and cultivation of actinobacteria

From all of the selective media used, seven different bacterial colonies have been chosen, which are macroscopically similar to actinobacteria. Microscopic observation, in fresh state and after Gram staining, revealed that all seven colonies are Gram positive. Six strains (AZ3, AR2, AR1, AZ10, K1 and AZ4) present a filamentous aspect, sometimes fragmented, which makes them similar to filamentous actinobacteria and one strain (AZ9) belongs to the *Rodochoccus* as non-filamentous actinobacteria.

2. Macroscopic and microscopic observation

Isolates AZ3, AR2, AR1, AZ10, AZ9, K1 and AZ4 have very good growth on ISP2, ISP4 and ISP3 (moderate for AR1 and AZ10) as well as on Bennett. Growth is variable (low, moderate or good) on nutrient agar medium and however on ISP2, the sporulation is better. As shown in and (Table.1), strains AZ3 and AR2 have serrated form and big spore with powder surface and they develop as detachable colonies. Substrate mycelium is abundant, well developed with white centers and gray sides. For AZ4 has a spore average size with surface smooth and powdery. Colonies of the isolates AR1 and K1 are round form, with smooth surface (AR1) and powder (K1) and the size of the colonies are small. The colonies of the isolate AZ10 are irregular in shape; the surface is milky cream with the small size of spore colonies. And AZ9 have as non-filamentous actinobacteria is characterized by spore average sizes with surface smooth and powdery morphologically resembles the strain AZ4. Six strains having the typical micromorphology of *Streptomyces* genus and one strain is looks like of *Rhodococcu*s. Strain AR2 is similar to strain AZ3 (*Streptomyces* grey series, type S="*Spira*" with spiral spore chains). Concerning the MS, strain K1 is very similar to strain AZ9 and a little bit also to strain AZ4 (*Streptomyces* of the yellow series, type RF="*Rectus Flexibilis*", with straight to flexible spore chains). For strain AR1 has an AM with branched filaments that fragment totally into a long Chain of rod-shaped spores (*Streptomyces* of the white series, type S="*Spira*" with spore chains in the form of extended, long and open coils spores.

Table 1: Morphological characteristic of actinobacteria isolates on ISP2 medium

Isolates	Substrate Mycelium	Aerial Mycelium	Contour	Form	Spore size	Surface aspect
AR1	White	Yellowish brown	Flattened	Round	Small	Smooth
AR2	Grey	Reddish brown	Domed	Serrated	Big	Powder
AZ3	Grey	Pinkish brown	Domed	Serrated	Big	Powder
AZ4	Yellowish white	Yellowish brown	Domed	Irregular	Average	Smooth, powdery
AZ10	Pink	pink	Domed	Irregular	Small	Milky cream
K1	Yellowish white	Yellow	Flattened	Round	Small	Powder
AZ9	Yellowish white	Brown	Domed	Irregular	Average	Smooth, powdery

3. Biochemical study

With regard to biochemical characteristics, except of AZ10, all strains are oxidase-positive. The strains AR1, AZ3, AZ9, AZ4, and AZ10 are catalase-positive while AR2 and K1 are catalase-negative. The fermentation of mannitol appearing on the K1 culture indicates that the strain is immobile, whereas the other strains are mobile. The study of the respiratory type on meat-liver agar revealed that all the strains are strict aerobics.

4. Antimicrobial activities

The actinobacteria belonging to the genus *Streptomyces* are known to be major antibiotic producers. Thus, strategies for the search for new antibiotics are first and foremost based on the identification of the different activities on a solid medium. The screening has always been the essential way to obtain new antimicrobial molecules; it is always practiced by many laboratories [23]. The presence of inhibition zone around the disk of the producing strain allows confirming the presence of an antagonist activity which results in the production and secretion in the culture medium of an antimicrobial molecule. However, the absence of the area around the disc indicates an inhibition of growth of the test strain [24].

4.1. Antibacterial activities

Our results show that all isolated strains have an antibacterial activity and four of them have an antifungal activity. The higher inhibitory activity was obtained by the strain AZ3 compared to other isolates towards *Staphylococcus aureus*, *Bacillus subtilis* and *Listeria monocytogenes* with an inhibition zone of 40, 35 and 28 mm, respectively. Followed by AR2 against *Staphylococcus aureus*, *Bacillus subtilis*, and *Listeria monocytogenes* with inhibition zone of 28, 26, and 21 mm, respectively. With a lesser degree we find K1 against *Staphylococcus aureus, Bacillus subtilus* and *Pseudomonas aeruginosa* with inhibition zone of 15, 14, and 14 mm, respectively. As for the strain AZ4, the greatest diameter (17 mm) of the inhibition zone was obtained against *Staphylococcus aureus* and 14 mm against *Bacillus subtilus.* Less activity was observed for AZ9, it was induced against *Bacillus subtilis* and *Staphylococcus aureus* with inhibition zones of 14 and 12 mm, respectively. The smallest diameters of the inhibition zones (13 and 12 mm) were obtained with the AR1 and AZ10 against *Staphylococcus aureus* and *Pseudomonas aeruginosa.*

4.2. Antifungal activities

The inhibition zones against fungi are less compared to those obtained against bacteria. Four strains have significant activities towards four filamentous fungi. Among the target germs strongly inhibited, we found *Mucor ramannianus* (25 mm), *Aspergillus ochraceus* (17 mm), *Aspergillus carbonarius* (14 mm) inhibited by AZ4. The AZ9 has a large spectrum of inhibitory activity against *Mucor ramannianus* (25 mm) and a moderate activity against *Aspergillus ochraceus* (17 mm). With almost the same spectrum we record 23 mm against *Mucor ramannianus* and 17 mm against *Aspergillus carbonarius* by K1. The lowest inhibition zone was obtained by AZ10 against *Mucor ramannianus* (12 mm).

5. Screening and selection of protease-producing strains

The results obtained (Table 2) after incubation on the selective medium indicated that most strains had proteolytic activity. This observation clearly demonstrates that, on the one hand, these strains are capable to hydrolyze the casein (protein) which gives usable soluble amino acids and on the other hand, that the enzymes which are responsible for this hydrolysis are secreted into the external medium [13]. These results make it possible to consider these strains as strains producing extracellular proteases, which represent a very positive point for their production and characterization. The diameter of the halo is proportional either to the quantity of proteases released by the colonies, or to the diffusion of the enzymes secreted in the medium during the incubation period.

Table 2: Demonstration of the proteolytic activity on milk agar medium

Strains	Diameter of the hydrolysis zone (mm) after 7 days of incubation	Result
K1	10	+++
AZ3	9	++
AZ4	7	++
AR2	5	++
AZ9	4	++
AR1	3	+
AZ10	0	-

6. Screening of hemolytic activity

The retained isolates for the hemolysis test revealed that five are positive hemolysis by creation of clear or colorless areas around the colonies. The positive results (Table 3) suggested that these strains are biosurfactant producers and hemolysis enzyme producers [22].

Table 3: Demonstration of hemolytic activity on blood agar medium by the appearance of clear zones around the colonies

Strains	Diameter (mm)	Color	Result
K1	23	Transparent	++
AR1	15	Transparent	+
AR2	12	Greenish	+
AZ9	14	Transparent	+
AZ3	3	Greenish	++
AZ4	2	Transparent	++
AZ10	0	Absence	-

7. Screening of biosurfactant-producing strains

The obtained results showed that the six strains AR1, AZ3, AZ4, AZ9, K1, and AR2 have a very interesting biosurfactant producing potential with large clear zones.

Experimental results show that the production of biosurfactants using olive oil as carbon source increased from the first day of incubation until the fifth day for strains AR1, AZ4, AZ9 and AZ3, showing a maximum production (PDD= 6.5 cm, 7 cm, 7 cm, and 7.5 cm, respectively), and decreased for eighth day. The K1 strain has the potential to produce biosurfactant with table oil; it is expected to reach maximum production with a PDD of 8 cm. Petroleum as a source of carbon does not give an interesting result. In fact, the AR2 strain is the only one that gives a high PDD (5 cm) with Petroleum. Strain K1 can be chosen as the highest biosurfactant producing strain with table oil as carbon source (Fig. 2)

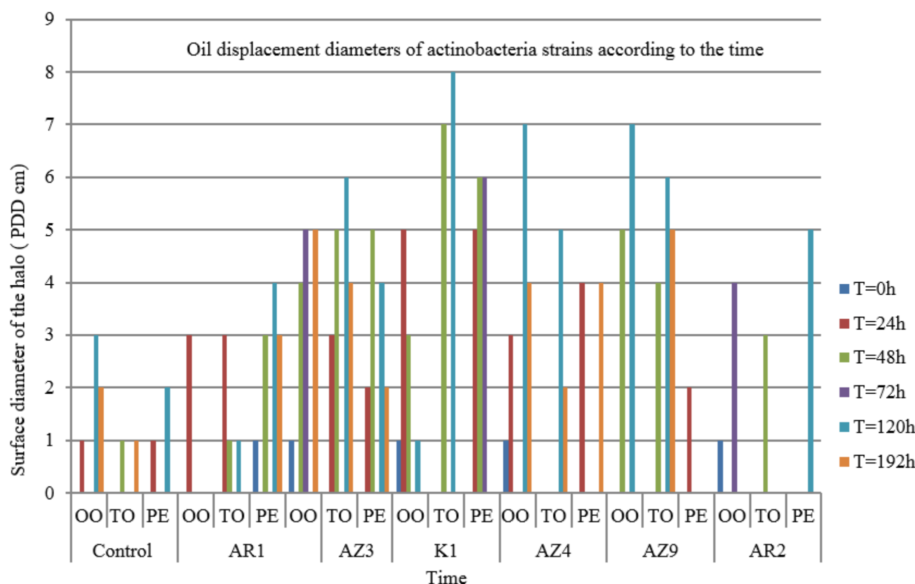

Figure 2: Selection of biosurfactant producing strains based on the oil displacement test with different carbon sources as a function of time

OO: olive oil, TO: Table oil, PE: Petroleum.

DISCUSSION

The group of actinobacteria, formerly called actinomycetes, is still considered to be the most important bacteria due to its production of more than 70% of the antibiotics marketed in the world [25]. The best known actinobacteria species are of terrestrial origin. To this end, the isolation of this group in the marine environment is strongly sought. Phenotypic taxonomy still remains among the steps necessary for the identification at the genus stage of actinobacteria, in our case, which has enabled us to differentiate two genera, *Streptomyces* and *Rhodococcus*. A totally, seven strains of actinobacteria were obtained using specific media in order to isolate these particular microorganisms. To our knowledge, rare work has been carried out on the isolation of actinobacteria of marine origin, in particular from marine algae. This environment has been focused for the discovery of novel natural products with biological potency. Among the work on the isolation of this type of bacteria of marine origin we can cite this review which summarizes the latest studies on marine rare actinobacteria and their natural products: Marine Rare Actinobacteria: Isolation, Characterization, and Strategies for Harnessing Bioactive Compounds [26]. Locally, Habibeche [27] proposed to study the morphological, physiological aspects of six actinobacteria of marine origin using different media and the highlighting of the antimicrobial activity of these strains by two different methods. Also, Boudrahem [28], for the research purpose of active secondary metabolites produced by marine actinobacteria, based on a morphological characterization (macro and micromorphology), allowed to attach the selected 14 isolates to *Streptomyces* genus and to put in evidence the antibacterial and / or antifungal activity of these isolates.

At our level, our study was carried out following the request of the production sectors seeking to invest in the field of biotechnology by using our local strains, in particular those isolated from marine environments. To do this, our research is situated within the framework of the objectives of our laboratory on biomolecules of microbial origin. To this end, we wish to make a collection of strains of actinobacteria isolated from marine algae. This first phase resulted in a very large collection of strains of actinobacteria with biotechnological properties.

CONCLUSION

In the present study, the screening of actinobacteria as main objective allowed us to obtain very interesting strains from a taxonomic and technological point of view which were isolated from two species of two marine algae *Asparagopsis armata* and *Zonaria tournefortii* in "Tipaza" region. Phenotypic taxonomy has enabled us to differentiate two genera, *Streptomyces* and *Rhodococcus*. The study of the microbial activity of our isolates has determined that the majority of the strains have the ability to produce active biomolecules having an inhibitory activity against pathogenic organisms such as *Staphylococcus aureus*, *Bacillus subtilis*, *Listeria monocytogenes*, *Mucor ramannianus*, *Aspergillus ochraceus* and *Aspergillus carbonarius*. The seven actinobacteria obtained in our case are of great biotechnological importance (producers of protease and biosurfactants) and which are to be better exploited in the various environmental and industrial fields.

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