Revue Agrobiologia
www.agrobiologia.net
ISSN (Print): 2170-1652
e-ISSN (Online): 2507-7627

INVENTORY FOR BIOLOGICALLY-ACTIVES SUBSTANCES (ANTIMICROBIAL, PROTEOLYTIC, HEMOLYTIC, AND BIOSURFACTANTS) OF MARINE BACTERIA ISOLATED FROM THE RED ALGAE "*ASPARAGOPSIS ARMATA*"

SAIDANI Fatiha[1], BEICHI Madjeda[1], FERRADJI Fatma-Zohra[1], EL HATTAB Mohamed[1], BADIS Abdelmalek[1*]

1. Laboratory of Natural Products Chemistry and Biomolecules, Faculty of Sciences - University Blida 1, Road of Soumaa - BP 270- Blida 09000, Algeria.

Reçu le 16/10/2021, Révisé le 15/12/2021, Accepté le 18/12/2021

Abstract

Description of the subject: Screening has always been the essential way to obtain new microorganisms with new molecules. This screening was carried out based on qualitative measurements followed by a quantitative study of all the activities related to industrial and environmental interests, namely, the biosurfactant power, proteolytic, antimicrobial, and hemolytic activity.
Objective: The objective of this work was to isolate, identify and evaluate the biotechnological properties of bacterial strains newly isolated from the red alga *Asparagopsis armata* collected from the Mediterranean coast of Algeria. **Methods:** Twenty-five marine bacteria isolated from the red algae *Asparagopsis armata* were obtained using a specific medium (Väätänen Nine Salt Solution). The identification of these isolates was based on morphological study (macroscopic and microscopic characteristics) and the different biochemical tests (Catalase, Oxidase, Mannitol-Mobility, and the Respiratory Type). The isolates obtained were screened to produce metabolites with antimicrobial, proteolytic, hemolytic, and surface activities. The study of the antimicrobial activity of the bacterial strains was carried out by the agar- diffusion method, and it was performed against nine bacteria, five Gram-positive and four Gram-negative, two yeasts, and one fungus. The evaluation of the proteolytic activity in a solid medium of bacterial strains was carried out using a simple and practical method, widely used in screening programs for enzyme-producing microbial strains. **Results:** The results obtained allowed us to classify the twenty-five isolates in two different micromorphological genres, 9 strains of the genus *Staphylococcus* (Gram-positive, Coccis, Immobile bacteria) and 16 strains of the genus *Bacillus* (Gram-positive bacteria, Bacilli, and Mobile). Regarding the biochemical and metabolic characteristics, all the isolated strains are catalase-positive, 16 strains are oxidase-positive while the other strains are oxidase-negative and all strains are facultative aero-anaerobes. In total, 13 isolates from 25 bacterial strains screened for their potential to produce antimicrobial molecules. Seventeen strains have hemolytic-positive activity between them 13 isolates showed a considerable biosurfactant production (6 and 8.5cm of DDP was noticed at 48 to 72h of incubation). **Conclusion:** These microorganisms can be used as a biotechnological alternative for various environmental and industrial applications. This study will be followed by the selection of the most efficient strains for production studies and optimization of these activities.
Keywords: Marine bacteria; *Asparagopsis armata;* antimicrobial activity; proteolytic activity; hemolytic; biosurfactants.

INVENTAIRE DES SUBSTANCES BIOLOGIQUEMENT ACTIVES (ANTIMICROBIENNES, PROTÉOLYTIQUES, HEMOLYTIQUES ET BIOSURFACTANTS) DES BACTÉRIES MARINES ISOLÉES DE L'ALGUE ROUGE "*ASPARAGOPSIS ARMATA*"

Résumé

Description du sujet : Le criblage a toujours été le moyen essentiel pour obtenir de nouveaux microorganismes avec de nouvelles molécules. Ce criblage a été effectué en se basant sur des mesures qualitatives, suivi, d'une étude quantitative de toutes les activités d'intérêt industriel et environnemental, à savoir, le pouvoir biosurfactant, protéolytique, antimicrobien et hémolytique. **Objectif :** L'objectif de ce travail était d'isoler, d'identifier et d'évaluer les propriétés biotechnologiques des souches bactériennes nouvellement isolées de l'algue rouge *Asparagopsis armata* collectée sur la côte méditerranéenne Algérienne. **Méthodes :** Vingt-cinq bactéries marines isolées de l'algue rouge *Asparagopsis armata* ont été obtenues en utilisant un milieu spécifique Väätänen Nine Salt Solution (VNSS). L'identification de ces isolats a été basée sur l'étude morphologique (caractéristiques macroscopiques et microscopiques) et les différents tests biochimiques (Catalase, Oxidase, Mannitol-Mobilité, et le Type Respiratoire). Les isolats obtenus ont été criblés pour produire des métabolites ayant des activités antimicrobiennes, protéolytiques, hémolytiques et de surface. L'étude de l'activité antimicrobienne des souches bactériennes a été réalisée par la méthode de diffusion en gélose, contre neuf bactéries, cinq Gram-positives et quatre Gram-négatives, deux levures et un champignon. L'évaluation de l'activité protéolytique dans un milieu solide des souches bactériennes a été réalisée à l'aide d'une méthode simple et pratique, largement utilisée dans les programmes de criblage des souches microbiennes productrices d'enzymes. **Résultats :** Les résultats obtenus nous ont permis de classer les vingt-cinq isolats en deux genres micromorphologiques différents, 9 souches du genre *Staphylococcus* (bactéries Gram-positives, Coccis, Immobiles) et 16 souches du genre *Bacillus* (bactéries Gram-positives, Bacilles, et Mobiles). En ce qui concerne les caractéristiques biochimiques et métaboliques, toutes les souches isolées sont catalases positives, 16 souches sont oxydases positives alors que les autres souches sont oxydases négatives et toutes les souches sont aéro-anaérobies facultatives. Au total, 13 isolats de 25 souches bactériennes ont été criblés pour leur potentiel de production de molécules antimicrobiennes. Dix-sept souches ont une activité hémolytique positive, parmi elles 13 isolats ont montré une production considérable de biosurfactant (6 et 8.5cm de DDP ont été remarqués à 48 à 72h d'incubation).**Conclusion :** Ces microorganismes peuvent être utilisés comme une alternative biotechnologique pour diverses applications environnementales et industrielles. Cette étude sera suivie par la sélection des souches les plus performantes pour des études de production et d'optimisation de ces activités.
Mots-clés : Bactéries marines ; *Asparagopsis armata* ; activité antimicrobienne ; activité protéolytique ; hémolytique ; biosurfactants.

*Corresponding Author: BADIS Abdelmalek, E-mail: badisabdelmalek@yahoo.fr

INTRODUCTION

Competition among microorganisms for space and nutrients in the marine environment is a powerful selective force, which has led to the evolution [1, 2, 3]. The evolution prompts the marine microorganisms to generate multifarious enzyme systems to adapt against complicated marine environments. For this purpose, marine microbial biomolecules can offer novel applications with an extraordinary properties [1, 4, 5]. Seaweeds are one of the large and diverse ecosystems; it plays an essential role in the marine environment. It is mainly involved in global primary production and providing food and shelter for a variety of organisms [6]. Seaweed surface supplies protected and nutrient-rich conditions for the bacterial growth [7, 8]. Seaweed has a rich diversity of associated microorganisms compare with other multicellular organisms. These microorganisms may be beneficial or harmful to the seaweeds. Epiphytic bacterial communities have been reported as vital for the morphological development of seaweeds, and bacteria with antibacterial properties are thought to protect the seaweeds from pathogens and the other competition organisms [7]. Some bacterial species show host specificity and bactericidal activity against specific pathogens. This specificity engages complex biochemical interactions between seaweed and bacteria [7]. At present, the interactions between algae and bacteria are being widely investigated. However, the ecological significance of most such natural associations remains vague [9]. There is an important need to investigate the bacterial communities living on different coexisting algae using new technologies, but also to investigate the production, localization, and secretion of the biologically active metabolites involved in those possible ecological interactions [10]. Marine bacteria and fungi are of great interest as novel and rich sources of biologically active products [11]. Up till now, only a small number of microorganisms have been investigated for bioactive metabolites, yet a huge number of active substances with some of them featuring unique structural skeletons have been isolated [11]. The objective of this work was to evaluate the biotechnological properties of bacterial strains newly isolated from the red alga *Asparagopsis armata* collected from the Mediterranean coast of Algeria on the East of Tipaza (GPS coordinates: 2°39' 00 " East, 36°37' 12" North).

The screening was carried out based on qualitative measurements followed by a quantitative study of all the activities related to industrial and environmental interests, namely, the biosurfactant power (tensioactive character), proteolytic (protease), antimicrobial (antibiotic), and hemolytic activity. This study was completed by the selection of performing strains for the production and optimization of these activities in order to find new biotechnological applications.

MATERIALS AND METHODS

1. Chemicals

Unless specified otherwise, all chemicals and reagents were of the analytical grade or highest available purity and purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2. Sampling, Treatment, Isolation, and Cultivation of bacteria

Microbial strains (bacteria) were newly isolated on May 14, 2017, from the red alga *Asparagopsis armata*, collected from 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 Blida 1.

2.1. Sampling 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 [7, 9]. Within 3h 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 [7, 9, 12].

2.2. Isolation and Cultivation of bacterial strains

Several protocols for the isolation of microorganism derivatives from marine algae were used using VNSS as a selective medium to marine bacteria. This medium was exclusively used for the isolation and purification of bacterial strains of *Asparagopsis armata*. Before autoclaving (121°C for 20min), the pH of the medium was adjusted to 7.2 using a sodium hydroxide solution (2N) [3, 13, 14].

Three isolation methods were used in order to recover the maximum species associated with the algae:

-The first method consists of vigorously rubbing the surface of seaweed thallus with a sterile swab. And then, this swab was used to inoculate the Petri dishes containing the solid culture medium of VNSS and incubated at 30°C for 24 to 72h [12].

-The second method consists of cutting the algae into square pieces of about $1cm^2$ and placing them directly on the Petri dishes containing VNSS Agar plates and inoculated and incubated at 30°C for 24 to 72h [3, 15].

-The third method is to cut the thallus of seaweed into small pieces and placed them in test tubes containing filtered and sterilized seawater. They were then subjected to vigorous vortexing in order to recover the maximum number of bacterial strains. Decimal dilutions were prepared (10^{-1} to 10^{-5}), then 100µL of the algae suspension were spread using a rake on the VNSS solid culture medium and incubated at 30°C for 24 to 72h [3, 16, 17].

2.3. Purification and Conservation of bacterial strains

Colony Forming Units (CFUs) were discriminated and selected from a macromorphological study (form, size, color, opacity, surface, and contour appearance), as they appeared, to be inoculated individually on VNSS-agar in order to obtain pure bacterial cultures. All pure bacterial isolates were coded and then conserved for the short and long term.

-Short-term conservation: The different strains were subcultured on solid media and stored at +4°C.

-Long-term conservation: This is done in liquid specific media containing 30% of glycerol at -26°C in special cryotubes [18].

3. Phenotypic identification of the selected isolates

The identification of bacterial isolates has been accomplished through morphological and physiological studies based on macro-microscopic observations and biochemical tests [19, 20].

3.1. Morphological characterization of bacterial isolates

The morphological study of the bacteria is always an important step that allows a preliminary orientation for the identification. It is based on macroscopic aspect (shape, size, elevation, margin, and color of the colony were observed [21] and microscopic aspect (Examination in the fresh state [19] and Gram Staining [19, 21, 22].

3.2. Physiological characterization

The study of catalase, oxidase, mobility, and respiratory type was carried out according to standard protocols applied on poorly prepared bacterial cultures [19, 21].

4. Screening of antimicrobial activity

In the search for new bacterial strains producing diffusible antimicrobial metabolites [23], a screening program of twenty-five bacterial strains isolated from the red alga *Asparagopsis armata* was undertaken. Based on the protocols described in the literature, we tested the action of bacterial isolates with different biological targets against a panel of microorganisms identified with an ATCC (American Type Culture Collection) number, from the antibiotherapy and hygiene laboratory of the Pasteur Institute of Algiers approved by the WHO (World Health Organization), including nine bacteria, five Gram-positive, *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 25923), *Listeria monocytogenes* (ATCC 49594), *Micrococcus luteus* (ATCC 14110) and *Bacillus cereus* (ATCC 14975), and four Gram-negative, *Escherichia coli* (ATCC 25922), *Agrobacterium tumefaciens* (ATCC 23308), *Pseudomonas aeroginosa (*ATCC 25843) and *Salmonella enterica* (ATCC 14028), two yeasts, *Candida albicans* (ATCC 10231) and *Saccharomyces cerevisiae* (ATCC 9763) and one fungus *Aspergillus brasiliensis* (ATCC 16404). These isolates were tested for antimicrobial activity using the agar-diffusion method recommended by Patel and Brown [24]. The method consists of inoculating a bacterial layer of all strains on the screening media LB or VNSS and then incubating at 30°C for 24 to 48h. Plugs of 6mm in diameter were cut and put onto plates surface of the culture media (Mueller-Hinton for bacteria and yeasts, and Sabouraud for fungi) previously inoculated with the target germs [25, 26]. Cylinders of the medium not inoculated with the test bacteria were used as negative controls. The plates were then kept at +4°C for 4h to allow good diffusion of the antimicrobial substances and then incubated for 24h at 37°C for bacteria and yeasts, and 72h at 28°C for fungi and examined daily for the formation of inhibition zones around the agar plugs **[26, 27]**. Clear inhibition zone formed around plugs were considered indicative of antimicrobial activity.

The inhibitory activity was recorded by measuring the clear zone diameter in millimeters [12, 13, 16, 20, 26].

5. Screening of proteolytic activity

The evaluation of the proteolytic activity in a solid medium of bacterial strains newly isolated from the red alga *Asparagopsis armata* was carried out using a simple and practical method, widely used in screening programs for enzyme-producing microbial strains. It is based on the inoculation of bacterial strains onto skimmed milk agar plates containing casein as a protein substrate [19, 28, 29, 30]. It consists of 5g peptone; 3g yeast extract, and 15g agar in 750mL of distilled water [30]. The pH is adjusted to 7.2 with 1N NaOH or HCL. After sterilization and cooling to 60°C, 250mL of skim milk is added [30]. After homogenization of the medium, it was poured into Petri dishes, used to detect extracellular proteolytic activities. After incubation at 30°C, the inoculated Petri dishes are examined every 24h [19, 29, 30]. This qualitative test is visualized by the diffusion of secreted proteases into the agar while hydrolyzing the milk caseins, resulting in the appearance of a transparent halo around the colony [19, 29, and 30]. The diameter of the halo is proportional to the quantity of enzymes released by the bacteria. The ratio of halo diameter to colony diameter (dh/dc) allows a preliminary selection of isolated strains [19].

6. Screening of hemolytic activity and surface-active substances

Isolates obtained were screened for biosurfactant produced by using the Mineral Medium MM supplemented with carbon and energy sources (hydrophobic substrate inducing biosurfactant production). This medium is based on mineral salts, its composition (g/L): NH_4Cl (0.4), K_2HPO_4 (0.3), KH_2PO_4 (0.3), NaCl (10), $MgCl_2$ (0.33), $CaCl_2$ (0.05), yeast extract (0.1) and 1mL of a solution containing trace elements prepared in 1L distilled water (0.64g $CuSO_4.5H_2O$, 0.11g $FeSO_4.7H_2O$, 0.79g $MnCl_2.4H_2O$ and 0.15g $ZnSO_4.7H_2O$). The pH of the medium was adjusted to 7.2 and the medium was sterilized by autoclaving at 120°C for 20min [31, 32]. Four sources of carbon (1%, v: v) were used after their sterilization on a 0.22µm filter: olive oil, table oil, margine and crude oil. The experiments were performed into flasks of 250mL containing 50mL of medium: 25mL of MM and 1% (v: v) of the carbon source.

The medium was inoculated with 2% (v: v) of overnight bacteria culture and incubated at 30°C on a reciprocal shaker at 150 rpm [31, 32]. The culture broth was then tested for the production of extracellular biosurfactants by the determination of the oil displacement, and hemolytic activity [33]. The protocols applied are detailed as follows:

-The hemolytic activity was determined on solid GN or LB medium supplemented with human blood (5%) according to the protocol described by Arimi et *al*. [32, 34]. The fresh single colonies from the isolated cultures were taken and streaked on blood agar plates. These plates were incubated at 30°C for 24h [21, 32]. The plates were then observed and the presence of a clear zone around the colonies indicated the presence of biosurfactant-producing organisms [21, 32].

-For the detection of oil displacement activity (ODA) of biosurfactants produced by selected isolates, the method (Oil spreading technique) is based on the ability of the biosurfactants to change the contact angle at the oil-water interface [21, 35] was used. A portion of 100µL of crude oil was added to the surface of 10mL of distilled water in a Petri dish to form a thin oil layer. Then, 10µL of culture or culture supernatant was gently placed on the center of the oil layer [21, 31, 35]. The presence of biosurfactant would displace the oil and a clear zone would form. The diameter of the clearing zone on the oil surface would be visualized under visible light and measured after 30 seconds, which correlates to the surfactant activity, also known as an oil displacement activity [21].

RESULTS

1. Isolation of associated bacteria

In total, twenty-five strains of marine bacteria were obtained and they are included of 9 strains of the genus *Staphylococcus* and 16 strains of the genus *Bacillus*.

2. Phenotypic taxonomy of isolated bacteria

The approach that is proposed in this part provides an increasingly comprehensive taxonomic study of bacterial strains isolated from the red alga *Asparagopsis armata* using conventional methods that use classical identification characters, which allows a preliminary orientation for characterization, was thus carried out based on macroscopic observations via the determination of morphological characteristics of strains.

In general, the colonies obtained are round with Cream, Yellow, Orange, Red, and Brown colors. This preliminary macromorphological study was followed by microscopic and differentiation tests which allowed a complementary characterization of the different strains. The obtained results allowed us to classify the isolates into two different groups:

- The first group includes Gram-positive, Coccis, Immobile bacteria: 1R, 2R, 4R, 5R, 6R, 12R, 20R, 21R, 22R.
- The second group includes Gram-positive bacteria, Bacilli, and Mobile: 3R, 7R, 8R, 9R, 10R, 11R, 13R, 14R, 15R, 16R, 17R, 18R, 19R, 23R, 24R, 25R.

Regarding the biochemical and metabolic characteristics, all the isolated strains are catalase positive. Strains 3R, 7R, 8R, 9R, 10R, 11R, 13R, 14R, 15R, 16R, 17R, 18R, 19R, 23R, 24R, 25R are oxidase-positive while the other strains are oxidase-negative. The study of the respiratory type on meat-liver/Agar medium revealed that all strains are facultative aero-anaerobes. The analysis of the different morphological characters observed is presented in Table 1.

Table 1: Microscopic characteristics and biochemical tests.

morphological characters	*Bacterial strains*												
	1R	2R	3R	4R	5R	6R	7R	8R	9R	10R	11R	12R	13R
Gram	+	+	+	+	+	+	+	+	+	+	+	+	+
Forme	cocci	cocci	bacilli	cocci	cocci	cocci	bacilli	bacilli	bacilli	bacilli	bacilli	cocci	bacilli
Mobility	-	-	+	-	-	-	+	+	+	+	+	-	+
Mannitol	+	+	+	+	-	+	+	+	+	-	-	+	-
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	-	+	-	-	-	+	+	+	+	+	-	+
Respiratory type	FAA	FAA	FAA	FAA	FAA	FAA	FAA	FAA	FAA	FAA	FAA	FAA	FAA
morphological characters	*Bacterial strains*												
	14R	15R	16R	17R	18R	19R	20R	21R	22R	23R	24R	25R	
Gram	+	+	+	+	+	+	+	+	+	+	+	+	
Forme	bacilli	bacilli	bacilli	bacilli	bacilli	bacilli	cocci	cocci	cocci	bacilli	bacilli	bacilli	
Mobility	+	+	+	+	+	+	-	-	-	+	+	+	
Mannitol	+	-	+	+	+	-	+	+	+	-	+	+	
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	
Oxidase	+	+	+	+	+	+	-	-	-	+	+	+	
Respiratory type	FAA	FAA	FAA	FAA	FAA	FAA	FAA	FAA	FAA	FAA	FAA	FAA	

+: Represents a positive result; **- :** Represents a negative result; **FAA:** Represents a facultative aero-anaerobic respiratory type.

3. Screening of antimicrobial activity

Twenty-five bacterial isolates from the red algae *Asparagopsis armata* were tested by the agar diffusion method for their ability to inhibit the growth of targets microorganisms. Screening has resulted in a total of 13 isolates from 25 bacterial strains screened for their potential to produce antimicrobial molecules were found to be active against the target germs, while the other bacteria (1R, 4R, 5R, 6R, 10R, 11R, 12R, 13R, 15R, 20R, 22R, 24R) showed an absence of activity. Strains 3R, 7R, 8R, 9R, 17R, and 25R showed a relatively broad spectrum of action, which extends as well to Gram-positive, Gram-negative bacteria and yeasts as to fungi. Mainly, against *Listeria monocytogenes* (20mm), *Bacillus subtilis* (15mm), *Staphylococcus aureus* (10mm), *Micrococcus luteus* (9mm), (Gram-positive bacteria), *Agrobacterium tumefaciens* (18mm), *Pseudomonas aeroginosa* (14mm), *Escherichia coli* (13mm), (Gram-negative bacteria), and against two yeasts *Saccharomyces cerevisiae* (21mm) and *Candida albicans* (20mm), while, the two bacteria *Salmonella enteric* and *Bacillus cereus* were the most resistant. On the other hand, the strains 2R, 14R, 16R, 18R, 19R, 21R, and 23R have been shown to inhibit the growth of one target germs, the filamentous fungi *Aspergillus brasiliensis* (15mm). The results obtained allowed us to classify the isolates into two groups, the antibacterial activity group includes (3R, 7R, 8R, 9R, 17R, and 25R) and the antifungal activity group includes (2R, 14R, 16R, 18R, 19R, 21R, and 23R).

4. Screening of proteolytic activity

The isolation led to the selection of 25 new strains. The strains are named 1R to 25R. During the subsequent screening experiments, various kinds of microbial colonies formed large and clear halos on skimmed milk agar medium were observed. Based on the ratio of halo diameter (mm) to colony diameter (mm) (dh/dc) (Table 2),

we showed that among the 25 strains tested: 11 (1R, 4R, 5R, 6R, 10R, 12R, 13R, 15R, 19R, 20R, 21R) strains do not show protease activity, and 14 strains producing proteases with the largest halos, namely 2R, 3R, 7R, 8R, 9R, 11R, 14R, 16R, 17R, 18R, 22R, 23R, 24R, and 25R, were found to be more interesting with very appreciable results in comparison with the results of several studies reporting the enzyme-producing potential of bacterial strains. Among them, bacterial isolates 3, 7, 9, 14, 16, 17, 18, 23 and, 25 showed the highest protease activity with a dh/dc ratio varied between 1.55 and 1.59. The results obtained are grouped in table 2.

Table 2: proteolytic activity on skimmed milk agar medium of 25 newly isolated bacterial strains

Strain	dh/dc (mm)	Strain	dh/dc (mm)	Strain	dh/dc (mm)
1R	-	10R	-	19R	-
2R	1.18	11R	1.02	20R	-
3R	1.57	12R	-	21R	-
4R	-	13R	-	22R	1.15
5R	-	14R	1.56	23R	1.56
6R	-	15R	-	24R	1.08
7R	1.55	16R	1.59	25R	1.56
8R	1.30	17R	1.57		
9R	1.56	18R	1.58		

5. Screening of hemolytic activity and surface-active substances

The various applications of biosurfactants require an easy, fast, and reliable method for the selection of biosurfactant-producing bacteria. In this part of the study, we were interested in the selection of the performing biosurfactant-producing strains and the selection of the carbon source by the determination of the oil displacement and hemolytic activities. A total of 25 isolates were tested for their hemolytic activity on blood agar plates, a clear zone was observed around the colony confirming the positive test for hemolytic activity, namely: 2R, 3R, 4R, 7R, 8R, 9R, 10R, 13R, 14R, 16R, 17R, 18R, 19R, 21R, 22R, 23R,and 25R. Hemolysis-positive strains were tested for their production of surface-active substances by oil displacement activity (ODA) produced by selected isolates. Table oil and olive oil are the two best carbon sources for biosurfactant production, which is demonstrated by a maximum crude oil displacement diameter for most strains. The results obtained (Fig.1) show that the strains 2R, 3R, 4R, 7R, 8R, 9R, 14R, 16R, 17R, 18R, 19R, 21R, and 25R have a very interesting biosurfactant production potential. The highest halo zone value was noticed at 48 to 72 hours of incubation in every strain with a variant diameter between 6 and 8.5cm. The strains 2R, 4R, 8R, 9R, 17R, and 21R have a very interesting biosurfactant producing potential with table oil as a carbon source and the bacteria 3R, 7R, 14R, 16R, 17R, 18R, 19R, and 25R with olive oil as a carbon source.

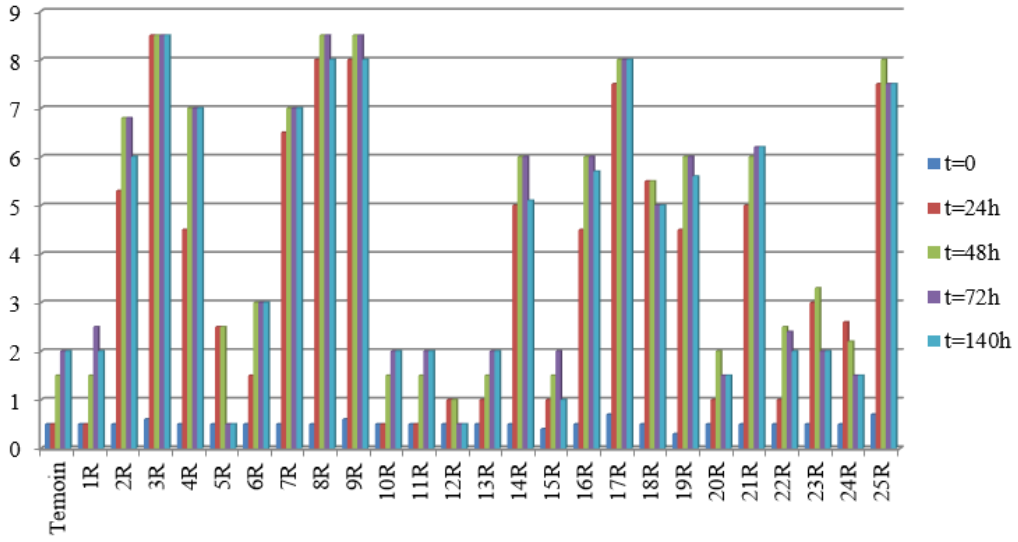

Figure 1: Oil displacement diameters of different bacterial strains according to the time.

DISCUSSION

To our knowledge, our study is the first report about taxonomic composition and biological activity of culturable bacteria associated with the marine alga *Asparagopsis armata* collected from the Mediterranean coast of Algeria on the East of Tipaza. It was found, that alga specimen are colonized by a community of diverse culturable microorganisms. A total of twenty-five strains were isolated and investigated phenotypically. The predominance of isolates belonging to the two groups; the first group includes 9 strains of the genus *Staphylococcus*, and the second group includes 16 strains of the genus *Bacillus*. Bacteria of *Bacillus* and *Staphylococcus* species are widely distributed in marine environments, especially, in Seaweeds. Having applied a culture-based method we could survey a part of culturable bacterial groups, not microbial community taken as a whole. At the same time, the culture-dependent method could be useful to better explore the physiological peculiarities of isolates since the present study was aimed to screen bacteria for their production of bioactive compounds (an antimicrobial, proteolytic, hemolytic, and surface activity). The number of strains synthesizing antimicrobial metabolites and biosurfactant production was moderate 13 strains, whereas strains with hemolytic and proteolytic activities were more frequently observed 17 and 14 strains respectively. Ten strains showed surface, hemolytic, proteolytic, and antimicrobial activities, which resulted evidently from the production of the low-molecular-weight substances. Some works have been mentioned on the isolation and screening of marine associated bacteria for antimicrobial, proteolytic, hemolytic, and surface activities. A study conducted by Lyudmila A. Romanenko under the theme "Isolation, phylogenetic analysis and screening of marine mollusc-associated bacteria for antimicrobial, hemolytic and surface activities" with the objective to characterize the culturable heterotrophic bacteria recovered from *A. broughtoni* and evaluate the potential of isolates to produce metabolites with antimicrobial, hemolytic or surface activities. The screening of these microorganisms announces the prospect of discovering new natural products which can be developed as a biotechnological resource [13].
It is conceivable, that microorganisms-associated may supply their host bivalve with bioactive metabolites providing vital functions or chemical protection from colonization by opportunistic microorganisms. The study of marine bacteria associated with the red alga is of importance for our understanding of their ecological role in the interaction with animals and between themselves, and also for their biotechnological application as producers of bioactive compounds. Our investigation is carried out following the request of the producing sectors to invest in the field of biotechnology using our local strains, especially those isolated from marine environments. To do this, our current study is part of the objectives of our laboratory on biomolecules derived from the microbial origin which we want to make a collection of marine strains associated with algae.

CONCLUSION

The first objective of our work focused on the isolation and phenotypic identification of marine bacteria newly isolated from the red alga *Asparagopsis armata* collected from the Mediterranean coast of Algeria on the East of Tipaza. The total associated bacteria with the red algae were twenty-five strains. The identification of these isolates was based on morphological characteristics (macroscopic and microscopic study) and the different biochemical tests (Catalase, Oxidase, Mannitol-mobility, and the respiratory type). The results obtained allowed us to classify the isolates in two different micromorphological genres, 9 strains of the genus *Staphylococcus* and 16 strains of the genus *Bacillus*. Regarding the biochemical and metabolic characteristics, all the isolated strains are catalase positive, 16 strains are oxidase-positive while the other strains are oxidase-negative and all strains are facultative aero-anaerobes. Thus, the second objective of this study deals with the evaluation of their ability to exert an antimicrobial, proteolytic, hemolytic, and surface activity. Screening has resulted in a total, 13 isolates from 25 bacterial strains screened for their potential to produce antimicrobial molecules were found to be active against the target germs, while the other bacteria showed an absence of activity: 6 strains have antibacterial activity while 7 strains have antifungal activity. Fourteen strains producing proteases with the largest halos, Seventeen strains have hemolytic-positive activity while the 13 isolates showed a considerable biosurfactant production.

REFERENCES

[1] Das, S.; Lyla, P. S.; and Khan, S.A. (2006). Marine microbial diversity and ecology: importance and future perspectives. *Current Science.*, 1325-1335.

[2] Zhang, C.; and Kimn, S.K. (2012). Application of Marine Microbial Enzymes in the Food and Pharmaceutical Industries. *Advances in Food and Nutrition Research.*, 65, 423-435., doi: 10.1016/B978-0-12-416003-3.00028-7.

[3] Menezes, C.B.A.; Bonugli-Santos, R.C.; Miqueletto, P.B.; Passarini, M.R.Z.; Silva, C.H.D.; Justo, M.R.; Leal, R.R.; Fantinatti-Garboggini, F.; Oliveira, V.M.; Berlinck, R.G.S.; and Sette, L.D. (2010). Microbial diversity associated with algae, ascidians and sponges from the north coast of Sao Paulo state, Brazil. *Microbiological Research.*, 165, 466-482.

[4] Zhang, C.; and Kim, S.K. (2010). Research and Application of Marine Microbial Enzymes: Status and Prospects. *Marine Drugs.*, 8, 1920-1934, doi: 10.3390/md8061920.

[5] Putri, D.A.; Radjasa, O.K.; and Pringgenies, D. (2015). Effectiveness of marine fungal symbiont isolated from soft coral *Sinularia* sp. from Panjang Island as antifungal. *Procedia Environmental Sciences.*, 23, 351-357.

[6] Shannon, E.; and Abu-Ghannam, N. (2016). Antibacterial Derivatives of Marine Algae: An Overview of Pharmacological Mechanisms and Applications. *Marine Drugs.*, 14(4), 81; doi: 10.3390/md14040081.

[7] Susilowati, R.; Sabdono, A.; and Widowati, I. (2015). Isolation and characterization of bacteria associated with brown algae *Sargassum spp.* from Panjang Island and their antibacterial activities. *Procedia Environmental Sciences.*, 23, 240-246.

[8] El Gamal, A. A. (2010). Biological importance of marine algae. *Saudi Pharmaceutical Journal.*, 18, 1-25.

[9] Beleneva, I.A.; and Zhukova, N.V. (2006). Bacterial communities of some brown and red algae from Peter the Great Bay, the Sea of Japan. *Microbiology*., 75(3), 348-357.

[10] Goecke, F.; Labes, A.; Wiese, J.; and Imhoff, J.F. (2010). Chemical interactions between marine macroalgae and bacteria. *Marine Ecology Progress Series*., 409, 267-300, doi: 10.3354/meps08607.

[11] Debbab, A.; Aly, A.H.; Lin, W.H.; and Proksch, P. (2010). Bioactive compounds from marine bacteria and fungi. *Microbial biotechnology*., 3(5), 544-563, doi:10.1111/j.1751-7915.2010.00179.x.

[12] Kanagasabhapathy, M.; Sasaki, H.; Haldar, S.; Yamasaki, S.; and Nagata, S. (2006). Antibacterial activities of marine epibiotic bacteria isolated from brown algae of Japan. *Annals of microbiology*., 56(2), 167-173.

[13] Romanenko, L.A.; Uchino, M.; Kalinovskaya, N.I.; and Mikhailov, V.V. (2008). Isolation, phylogenetic analysis and screening of marine mollusc-associated bacteria for antimicrobial, hemolytic and surface activities. *Microbiological research*., 163(6), 633-644, doi:10.1016/j.micres.2006.10.001.

[14] Wang, Z.; Xiao, T.; Pang, S.; Liu, M.; Yue, H. (2009). Isolation and identification of bacteria associated with the surfaces of several algal species. *Chinese Journal of Oceanology and Limnology*., 27(3), 487-492, doi: 10.1007/s00343-009-9165-4.

[15] Singh, R.P.; Mantri, V.A.; Reddy, C.R.K.; Jha, B. (2011). Isolation of seaweed-associated bacteria and their morphogenesis-inducing capability in axenic cultures of the green alga *Ulva fasciata*. *Aquatic Biology*., 12(1), 13-21, doi: 10.3354/ab00312.

[16] Boottanun, P.; Potisap, C.; Hurdle, J.G.; and Sermswan, R.W. (2017). Secondary metabolites from *Bacillus amyloliquefaciens* isolated from soil can kill *Burkholderia pseudomallei*. *AMB Express*., 7:16, doi: 10.1186/s13568-016-0302-0.

[17] Cao, H.; He, S.; Wei, R.; Diong, M.; and Lu, L. (2011). *Bacillus amyloliquefaciens* G1: A Potential Antagonistic Bacterium against Eel-Pathogenic Aeromonas hydrophila. *Evidence-Based Complementary and Alternative Medicine*., Article ID 824104, 7 pages, doi:10.1155/2011/824104.

[18] Tyc, O.; Zweers, H.; de Boer, W.; and Garbeva, P. (2015). Volatiles in Inter-Specific Bacterial Interactions. *Frontiers in microbiology*., 6:1412, doi: 10.3389/fmicb.2015.01412.

[19] Benkiar A., (2016). Isolement et criblage de souches bactériennes productrices de protéase alcaline en vue d'une application industrielle. Thèse de Doctorat, Universite Saad Dahleb De Blida, Faculté de Technologie Département de Génie des Procédés, Alger.

[20] Frikha Dammak, D.; Zarai, Z.; Najah, S.; Abdennabi, R.; Belbahri, L.; Rateb, M.E.; Mejdoub, H.; and Maalej, S. (2017). Antagonistic Properties of Some Halophilic Thermoactinomycetes Isolated from Superficial Sediment of a Solar Saltern and Production of Cyclic Antimicrobial Peptides by the Novel Isolate *Paludifilum halophilum*. *BioMed Research International.*, Article ID 1205258, 13 pages, doi.org/10.1155/2017/1205258.

[21] Chakrabarti S., (2012). Bacterial Biosurfactant: Characterization, Antimicrobial and Metal Remediation Properties. National Institute Of Technology, Department Of Life Science.

[22] Almohsen J.S.H., (2014). Isolation and Characterisation of Halotolerant Bacteria and Algae and their Potential for Biofuel Production. Thesis submitted in part fulfillment of the requirement for the degree of Doctor of Philosophy, Department of Molecular Biology and Biotechnology, The University of Sheffield, UK.

[23] Penesyan, A.; Kjelleberg, S.; and Egan, S. (2010). Development of novel drugs from marine surface associated microorganisms. *Marine Drugs*., 8, 438-459, doi: 10.3390/md8030438.

[24] Boudjelal, F.; Zitouni, A.; Mathieu, F.; Lebrihi, A.; Sabaou, N. (2011). Taxonomic study and partial characterization of antimicrobial compounds from a moderately halophilic strain of the genus *Actinoalloteichus*. *Brazilian Journal of Microbiology*., 42(3), 835-845.

[25] Phongsopitanun, W.; Suwanborirux, K.; and Tanasupawat, S. (2014). Identification and antimicrobial activity of *Streptomyces* strains from Thai mangrove sediment. *The Thai Journal of Pharmaceutical Sciences.*, 38 (1), 1-56.

[26] Hamiche, S.; Badis, A.; Jouadi, B.; Bouzidi, N.; Daghbouche, Y.; Utczás, M.; Mondello, L.; and El Hattab, M. (2019). Identification of antimicrobial volatile compounds produced by the marine bacterium *Bacillus amyloliquefaciens* strain S13 newly isolated from brown alga *Zonaria tournefortii*. *Journal of Essential Oil Research.*, 31(3), 203-210, doi: 10.1080/10412905.2018.1564380.

[27] Sihem B.M.; Rafik, E.; Florence, M.; Mohamed, C.; and Ahmed L. (2011). Identification and partial characterization of antifungal and antibacterial activities of two *Bacillus sp.* strains isolated from salt soil in Tunisia. *African Journal of Microbiology Research*., 5(13), 1599-1608.

[28] Schinner, F.; Margesin, R.; and Pumpel, T. (1992). Extracellular protease-producing psychrotrophic bacteria from high alpine habitats. *Arctic and Alpine Research*., 24(1), 88-92, doi: 10.2307/1551325.

[29] Jo, G.H.; Jung, W.J.; Kuk, J.H.; Oh, K.T.; Kim, Y.J.; Park, R.D. (2008). Screening of protease producing *Serratia marcescens* FS-3 and its application to deproteinization of crab shell waste for chitin extraction. *Carbohydrate Polymers*., 74(3), 504-508.

[30] Hadjidj, R.; Badis, A.; Mechri, S.; Eddouaouda, K.; Khelouia, L.; Annane, R.; El Hattab, M.; Jaouadi, B. (2018). Purification, biochemical, and molecular characterization of novel protease from *Bacillus licheniformis* strain K7A. *International Journal of Biological Macromolecules*., 114, 1033-1048.

[31] Eddouaouda, K.; Mnif, S.; Badis, A.; Younes, S. B.; Cherif, S.; Ferhat, S.; Mhiri, M.; Chamkha, M.; Sayadi, S. (2011). Characterization of a novel biosurfactant produced by *Staphylococcus* sp. strain 1E with potential application on hydrocarbon bioremediation. *Journal of Basic Microbiology*., 52(4), 408-418.

[32] Ferradji, F.Z.; Mnif, S.; Badis, A.; Rebbani, S.; Fodil, D.; Eddouaouda, K.; and Sayadi, S. (2014). Naphthalene and crude oil degradation by biosurfactant producing *Streptomyces* spp. isolated from Mitidja plain soil (North of Algeria). *International Biodeterioration & Biodegradation*., 86, 300-308, doi:10.1016/j.ibiod.2013.10.003.

[33] Elazzazy, A.M.; Abdelmoneim, T.S.; and Almaghrabi, O.A. (2015). Isolation and characterization of biosurfactant production under extreme environmental conditions by alkali-halo-thermophilic bacteria from Saudi Arabia. *Saudi Journal of Biological Sciences.*, 22(4), 466-475, doi:10.1016/j.sjbs.2014.11.018.

[34] Arimi, S.M.; Park, R.W.; Fricker, C.R.J. (1990). Study of haemolytic activity of some *Campylobacter* spp. on blood agar plates. *Journal of applied bacteriology*., 69(3), 384-389.

[35] Morikawa, M.; Daido, H.; Takao, T.; Murata, S.; Shimonishi, Y.; and Imanaka, T. (1993). A new lipopeptide biosurfactant produced by *Arthrobacter*sp. strain MIS38. *Journal of bacteriology*, 175(20), 6459-6466.