Int.J.Curr.Microbiol.App.Sci (2024) *13*(04): 156-163

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 13 Number 4 (2024)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2024.1304.018

Study of Some Enzymatic Activities of Actinomycetes Isolated from Two Extreme Ecosystems in Southern Algeria

Harouna Maidoukia Abdoul Razack[1*], Garba Boubacar[1], Yahouza Zaneidou[1] and Boudemagha[2]

[1]Université Djibo Hamani de Tahoua, Faculté des Sciences d'Education, Département desDidactiques de Disciplines, Niger
[2]Université Constantine I, Faculté des Sciences de la Nature et de la Vie, Département de Microbiologie, Algeria

**Corresponding author*

Keywords

Actinomycetes, enzymatic activities, desert soil, chott Melghir

Article Info

Received:
28 February 2024
Accepted:
30 March 2024
Available Online:
10 April 2024

A B S T R A C T

Two samples from two extreme ecosystems (El Oued soil and Chott Melghir salt) were used in this study. Sixty-nine actinomycete colonies were collected from three actinomycete-selective media (ISP5, ISP2, AIA) supplemented with antibacterial and antifungal agents. These media were inoculated with these soil and salt samples. The strains were purified on the same media used for isolation. Twenty strains were selected for enzymatic activity testing. The enzymatic activities of these bacteria were tested using specific media for each activity test. The strains all showed activity on catalase, but the vast majority were not capable of reducing nitrate to nitrite, while most of the strains showed catalytic action on gelatin, starch, pectin, lecithinase, lipoproteinase, amylase and cellulose.

Introduction

Actinomycetes are Gram-positive filamentous bacteria that undergo morphological differentiation during their life cycle. In response to unfavourable conditions, such as a lack of nutrients and water. Actinomycetes sporulate. It is only when conditions become favourable again that the spores can germinate and form the vegetative mycelium again. They inhabit the soil and are important decomposers of organic matter, making the soil fertile and therefore improving harvests (Djaballah, 2010). In addition to soil, actinomycetes have been isolated from many aquatic environments, including marine ecosystems, freshwater and salt marshes (Al-Zarban *et al.,* 2002; Boughachiche *et al.,* 2005; Kitouni *et al.,* 2005). They are capable of metabolising several different compounds such as sugars (polysaccharides), alcohols, amino acids and aromatic compounds by producing extracellular enzymes. Their ability to degrade pesticides, herbicides and hydrocarbons has also been reported (Benimeli *et al.,* 2003). This metabolic diversity is due to their extremely large genome, which has around a hundred transcription factors that control the expression of the genes that enable them to meet their needs. Certain characteristics of actinomycetes make them important bio-control agents, mainly against phytopathological fungi in the soil. These characteristics include the production of different types of secondary metabolites

and biologically active substances, which are of high commercial value: namely enzymes that target the fungus wall directly and antibiotics. Actinomycetes are also known for their production of other secondary metabolites, such as antibacterials. Antiparasitic agents, anti-tumour agents and immunosuppressants (Angehrn *et al.,* 2004; Imada, 2005). The aim of this research is to study the antifungal and antibiotic properties of actinomycetes derived from the biodiversity of two extreme Algerian ecosystems that are very poorly, if not completely, unexploited. The samples studied in these investigations come from the sebkha of Chott Melghir and the Sol of El Oued, and all have different physico-chemical characteristics. It is interesting to note that all the samples examined represent extreme ecosystems, with very little vegetation and high salinity levels, especially in the case of the Chott Melghir sample.

Materials and Methods

Sampling and taking of samples

The samples studied in these investigations, as mentioned above, come from the sebkha of Chott Melghir and the Sol of El Oued. The Chott Melghir (Longitude: 607'30 to 6°30'02"E and Latitude: 34°00' to 3430'01"N) is located to the south-east of the town of Biskra. It is characteristic of the arid and hyper-arid Saharan regions and constitutes the lowest points of the Algerian Sahara (-35m altitude). The soil is saturated with salt due to heavy evaporation, and regularly becomes a salt desert. These soils are most often represented by hyper halophilic or gypso-psammophilic soils. The Wilaya of El Oued (Longitude 006°E53, Latitude033°N20') is located in south-eastern Algeria. A sandy region covering the whole of the Souf, as well as the eastern and southern parts of the Oued Righ. A rocky plateau that runs alongside the RN3 and extends southwards. A region of depression, a broad desert plateau sheltering a few eroded formations from the Miocene.

Taking samples

The salt samples from Chott Melghir were taken from the Sebkha under strict aseptic conditions. Two samples were taken (the first at the surface and the second at a depth of 22 cm). The samples were placed on a sheet of aluminium foil, then placed in a sterile bottle and transported to the laboratory for analysis. In the case of the El Oued soil, two samples were also taken using a large sterile spatula: the first five centimetres of the top layer of soil were removed. 100 to 150 grams of soil from the underlying layer (between 5 and 15 cm deep) were placed on a sheet of aluminium foil. Large debris (stones, roots, etc....) are removed, and around 100 g are placed in a sterile bottle and transported as quickly as possible to the laboratory for analysis (Pochon and Tardieux, 1962).

Physico-chemical characteristics of samples

The physico-chemical characteristics studied were carried out in the soil microbiological and physico-chemical analysis laboratory. They concern pH, electrical conductivity, salinity, organic matter content and total carbonate and nitrogen content.

Isolation of actinomycetes

Isolation of actinomycetes from the samples taken was carried out on three different culture media: AIA, ISP2, and ISP5. After preparation, 14% Nacl was added to part of each medium to promote the growth of halophilic bacteria. A concentration of 10 µg/ml of nalidixic acid was added to inhibit the growth of Gram-negative bacteria and 50 µg/ml of nystatin was used as an antifungal agent to eliminate invasive fungal flora (Larpent et Sanglier, 1989).

Seeding and incubation of samples

For each sample, 1 g of soil and salt is introduced into a tube containing 9 ml of sterile physiological water. A series of decimal dilutions was then carried out for the soil sample from $^{10-1}$ to $^{10-6}$ and for the salt sample from $^{10-1}$ to $^{10-4}$. Next, 0.1ml of each dilution was inoculated onto the surface of petri dishes containing one of the three culture media. The dishes were then incubated at 28°C for 21 days. The colonies of isolated actinomycetes were counted daily after the 14th day of incubation. A colony counting device was used.

Study of morphological characteristics and enzymatic activities

Once the actinomycetes had been isolated, only Gram-positive colonies with the characteristic filamentous appearance of actinomycetes were selected for biochemical testing. The biochemical characteristics tested were: caseinase; gelatinase; catalase; cellulase; lipases; esterase; lecithin and lipoproteins; nitrate reductase; and pectinase.

Results and Discussion

Physico-chemical characteristics of the samples

The samples studied come from the Chott Melghir sebkha and the El Oued soil, and all have different physico-chemical characteristics (Table 1). These physico-chemical factors may play an important role in the selection of microorganisms.

The samples are characterised by different pH values: the Salt sample, with a pH of 7.82, is an alkaline soil, while the pH of the second sample is 6.98, tending towards neutrality. These pH values are the result of the significant accumulation of limestone in these two samples. The aridity of the climate is also a factor that increases the pH. In many cases, trace element deficiencies are generally due either to a pH that is too low (acid soil) or, more frequently, too high (alkaline soil). The latter leads to the formation of insoluble hydroxides. According to Lee and Hwang (2002). The rate of organic matter (%) in a soil is considered to be: low at values between (4.0-7.0); moderate (7.1-9.0) and high (9.1-11.0). The very low level of organic matter in our two samples (0.55%) for the soil and (0.25%) for the salt allows us to conclude that the organic matter in these samples exists only in trace form. It is clear that soils located in arid regions are poor in organic matter.Electrical conductivity provides information on the overall content of dissolved salts; it only applies to salty soils and soils with a very high level of fertilisation (Aubert, 1978). Our samples are classified as very saline soils.

Isolation of Actinomycetes

Isolation of actinomycete strains from our samples of the Chott Melghir sebkha and El Oued soil, on the three selective media (AIA, ISP5 and ISP2) with the addition of antibacterial and antifungal agents and Nacl for halophiles, gave the results shown in Table 2.

From the three selective media used, 63 colonies were sampled. They were identified on the basis of their macroscopic and microscopic appearance (hard colonies embedded in the agar, filamentous appearance on Gram-positive staining). The results in Table 2 show that for the three media used, there was a significant difference in the number of Actinomycetes colonies isolated from the different samples studied. The number of colonies isolated was 47 for the soil sample, and 16 colonies for the salt sample. It is clear that a higher number of actinomycetes is obtained from the soil sample. This can be explained by the fact that, on the one hand, the soil sample is relatively richer in organic matter than the salt sample and, on the other hand, the degree of salinity of the salt sample is higher than that of the soil.

The number of actinomycetes is positively correlated with the level of organic matter, whatever the level of soil salinity (Lee and Hwang, 2002). The highest number of actinomycete colonies was obtained on culture media without Nacl. In fact, the ISP5 medium without Nacl proved to be more effective, allowing a good recovery of actinomycetes, i.e. 26 colonies compared with 9 for the ISP5 medium with Nacl. This was also true for the rest of the media.

In fact, 19 colonies were isolated from ISP2 medium without Nacl compared with 7 with Nacl in the same medium. Finally, 2 colonies were collected on AIA medium without Nacl compared with 0 colonies with Nacl.

Despite the richness of the three media ISP5, ISP2 and AIA in terms of carbon, nitrogen and selective elements for the growth of actinomycetes, ISP5 and ISP2 with or without Nacl enabled the isolation of a greater number of actinomycetes than AIA. Many techniques have been used for the selective isolation of actinomycetes, based essentially on heat treatment of samples and the addition to isolation media of inhibiting substances that stop the growth of invading germs (Ouhdouche *et al.,* 2001; Hilali *et al.,* 2002; Jung Yeop lee, 2002). Opinions on the use of antibiotics are, however, controversial. According to Porter *et al.,* (1960), most antibacterials used inhibit many actinomycetes. On the other hand, Dulaney *et al.,* (1955) recommend the use of a mixture of antibiotics and antifungals for the isolation of actinomycetes.

In the present study, the media were all supplemented with nystatin at a concentration of 50 µg/ml and nalidixic acid at a concentration of 10 µg/ml. The nystatin added to the medium inhibited the growth of the fungi. Williams and Davis (1965) tested this antifungal agent on fungi isolated from the soil, and found that it inhibited most of their growth at a concentration of 50 µg/ml. In addition, when tested on actinomycetes, this antifungal agent did not alter their growth, even when the concentration was increased to 100 µg/ml. The addition of nalidixic acid resulted in the elimination of Gram-negative bacteria, and is used in the work of Takizaw *et*

al., (1993) for the isolation of actinomycetes from marine environments. Suzuki *et al.,* (1999) found that actinomycetes can be resistant to nalidixic acid up to a concentration of 10ug/ml, beyond which the growth of certain genera can be reduced. In our study, a mixture of nystatin at 50 ug/ml and nalidixic acid at 10 ug/ml added to the selective media enabled the contaminants to be eliminated in large quantities and the actinomycetes to be selected. Although we took precautions, the combination of antibiotics and antifungals used allowed the growth of a few small colonies of fungi (reduced size and poorly developed mycelium), especially on the ISP2 medium. The most delicate problem concerns Gram-positive bacteria that we cannot eliminate and that hinder the growth of actinomycetes by invading the agar plates, as in the case of the presence of Bacillus patches.

Study of the macroscopic appearance and cultural characteristics of isolated actinomycetes

From the 63 isolated and purified actinomycetes strains, 20 strains were selected on the basis of their morphological characteristics, for the continuation of our work. The macroscopic characteristics of these actinomycete strains vary in shape, colour and texture (Table 3). They are generally round with irregular outlines, opaque and adhere to the surface of the agar, of different colours: brown, beige and white, this aspect is characteristic of the aerial mycelium of actinomycetes. Observation of the underside of the colony (back of the petri dish) helps to determine the colour of the substrate mycelium, which may be beige or dark brown.

Study of the microscopic appearance after Gram staining

According to the Gram stain, microscopic observation shows that most of the isolated and purified actinomycetes strains are filamentous bacteria with a positive Gram stain. Actinomycetes appear in the form of fine, branched, tangled filaments, some of which fragment or do not fragment into bacillary or ovoid elements. They are sometimes grouped together to form dense thallus, where the colouration is very similar. These results show that work on isolating actinomycetes is somewhat special. In fact, certain colonies should never be discarded on the pretext that their macroscopic appearance does not resemble actinomycetes. Microscopic observation after Gram staining is imperative.

Identification of certain enzymatic activities of actinomycete isolates

The results of the enzymatic activities of the 20 strains selected are shown in the table below

Table 4 shows that the majority of our strains produce lipid-degrading enzymes, in particular three lipolytic enzymes: esterase, lecithinase and lipoproteinase**.** Various lipases have been identified in actinomycetes. These are mainly Streptomyces fradiae and Streptomyces coelicolor (Hou *et al.,* 1988). According to these results, 12 of the 20 isolates showed good pectinolytic activity. The other strains do not have this ability. The production of pectinolytic enzymes has already been demonstrated by several genera of actinomycetes such as Micromonospora, Microbispora, Actinoplanes, Streptosporangium and Streptomyces (Demain and Solomon, 1985; Sanglier *et al.,* 1993). 15 strains show cellulolytic activity on liquid ISP9 medium containing cellulose as the sole source of carbon and energy. This is reflected in the digestion of Whatman No. 1 paper, indicating that the strains tested possess the enzyme responsible for cellulose hydrolysis (cellulase). These enzymes are generally produced by actinomycetes, particularly thermophilic species and streptomycetes (Sanglier, 1993). These enzymes have been used in many areas of biotechnology, including the food and textile industries (Ando *et al.,* 2002), the bioconversion of cellulosic waste, the paper industry, additives, animal feed, digestive aids and therapeutics. Of the 20 strains tested, only 9 showed nitrate reduction on the mannitol-mobility-nitrate medium.

This activity is reflected by a reaction coloured red after the addition of the two reagents, nitrate reductase 1 and nitrate reductase 2. This is explained by the presence of nitrite and the 9 strains reduced nitrate to nitrite using nitrate reductases. The presence of nitrate in the soil is the result of discharges from local authorities, industry and mainly agriculture following the spreading of massive doses of nitrogen fertiliser and slurry (livestock manure). Nitrates are now the major cause of pollution of large groundwater reservoirs and soil around the world. Table 4 shows 12 positive results out of the 20 strains tested. They grew moderately on casein medium with yeast extract and glucose, and the appearance of any light areas around the colonies indicates hydrolysis of the casein.

Table.1 Physico-chemical parameters of El Oued soil and Chott Melghir salt samples

Samples	pH	Electrical conductivity (uS /Cm)	Salinity (g/L)	CaCO3 Active	Organic Carbon (%)	Organic Matter (%)	Nitrogen (%)
The soilof El Oued	6.98	210000	90	11.70	0.31	0.55	**0.027**
Saltfrom Chott Melghir	**7.82**	**420000**	**180**	**12.60**	**0.14**	**0.25**	**0.012**

Table.2 Number of actinomycetes per soil and salt sample and per culture medium.

Samples	Number of colony		
	AIA	ISP5	**ISP2**
The soilof El Oued	2	26	**19**
Saltfrom Chott Melghir	0	9	**7**
Total	**2**	**35**	**26**

Table.3 Macroscopic appearance and cultural characteristics of some colonies isolated from sebkha and soil samples

Isolates	Shape	Size	Colour	Surface appearance
SO1	Irregular	Small	White	**Smooth**
SO2	Round	Very small	White	**Smooth**
SO3	Irrégulière	Very small	Dark brown	**Smooth**
SO4	Irrégulière	Very small	Dark brown	**Inlaid, shiny**
SO5	Round	Small	Dark brown	**Rough**
SO6	Serrated	Small	Beige	**Not inlaid**
SO7	Serrated	Large	brown	**Dry inlaid**
SO8	Serrated	Large	Dark brown	**Dry inlaid**
SO9	Serrated	Small	White	**Smooth, powdery**
SO10	Round	Large	Beige	**Creamy milky**
SO11	**Round**	**Large**	**Beige**	**Inlaid**

Table.4 Results of the enzymatic activities of the 20 selected strains

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Amylase	-	-	-	-	+	+	+	+	+	+	+	+	-	-	+	-	-	-	+	+
Pectinase	-	-	-	+	+	+	+	+	+	+	-	-	+	-	+	-	-	+	+	+
Gelatinase	+	+	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Caseinase	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-
Esterase	+	+	-	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	-
Cellulase	+	-	+	+	-	-	+	+	+	+	+	-	-	+	+	+	+	+	+	+
Lécithinase	-	-	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	-	+

Figure.1 Macroscopic appearance of some colonies isolated from the 2 samples

These results are in line with those presented by Gulve and Deshmukh (2011) for the study of the enzymatic activities of actinomycetes isolated from marine sediments, where they showed that the genera Streptomyces, Micromonospora, Nocardia and Saccharopolypora possess proteolytic activity. The work of Kurup *et al.,* (1975) showed the ability of a new strain of thermophilic actinomycetes, Thermoactinomyces candidus, to degrade casein.

For gelatin hydrolysis, 17 out of 20 strains were positive, resulting in the liquefaction of gelatin by an extracellular enzyme, gelatinase, after the tubes had been placed for approximately one hour at +4°C (figure). These results are similar to those found by de Djaballah (2010) who assigned the liquefaction of gelatin by halophilic and halotolerant strains isolated from the sebkha of Ain M'Lila which are close to the genera Actinopolyspora, Streptoaloteichus, Streptosporagium and Kitasatospora. The work of Iwasaki *et al.,* (1981) also showed that Streptomyces sannanensis liquefies gelatin at 4°C for 1 hour.

The main objective of our work was to study the metabolic biodiversity of actinomycetes from extreme ecosystems where the physicochemical conditions are unfavourable to microbial life (arid soil, extreme salinity, etc.). To do this, we had to isolate samples from the ecosystems we had explored (El Oued soil and Chott Melghir salt), and 63 actinomycete colonies were isolated from these two sites. Some of these isolates have macroscopic features characteristic of actinomycetes. Microscopically, these isolates appeared Gram-positive in the highly branched filamentous forms characteristic of actinomycetes. In this study, as the macroscopic observations commonly used were not sufficient, we focused on microscopic observation for all the isolates obtained. The use of several selective media for the isolation of actinomycetes was necessary in order to isolate as many of these bacteria as possible. The 63 colonies obtained in this study were isolated from ISP5 and ISP2 media. Only 2 colonies were isolated from AIA, so the variation in culture media is very significant. Of the 63 colonies isolated and purified, 20 were selected, including 3 strains from salt and 17 from soil.

The strains from the salt samples degraded almost all the compounds except starch and pectin, and only nitrate reductase was not degraded by the strains from the soil. Of the 20 strains tested, all showed positive catalase activity, 75% showed cellulolytic activity, 60% were pectinolytic, 75% were esterase positive, 60% had caseinase, gelatin was degraded by 85%, lipoproteinase was catabolised by 60%, and only 45% of the strains reduced nitrate to nitrite. Lecithinase was degraded by 75% and 55% were amylotic. These results are very encouraging and open up prospects in the field of medical and industrial research where these enzymes from extremophilic actinomycetes can make a major contribution. In view of these satisfactory results, we hope to continue our work in these arid regions, especially the Chott Melghir site, which we consider to be an extraordinary site since it is located at an altitude of -35 m.

Author Contribution

Harouna Maidoukia Abdoul Razack: Investigation, formal analysis, writing—original draft. Garba Boubacar: Validation, methodology, writing—

reviewing. Yahouza Zaneidou:—Formal analysis, writing—review and editing. Boudemagha: Investigation, writing—reviewing.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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How to cite this article:

Harouna Maidoukia Abdoul Razack, Garba Boubacar, Yahouza Zaneidou and Boudemagha. 2024. Study of Some Enzymatic Activities of Actinomycetes Isolated from Two Extreme Ecosystems in Southern Algeria. *Int.J.Curr.Microbiol.App.Sci.* 13(4): 156-163. **doi:** https://doi.org/10.20546/ijcmas.2024.1304.018