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Exploring Actinomycetes Diversity in Extreme Ecosystems of Algeria: Isolation, Morphological Characterization, and Enzymatic Capacities

Exploring Actinomycetes Diversity in Extreme Ecosystems of Algeria: Isolation, Morphological Characterization, and Enzymatic Capacities

Aguis Hayat^{1,2}, Benreguiég Mokhtar^{1,2}, Halla Noureddine^{1,2}

¹Department of Biology, Faculty of Natural and life Sciences, University of Saida, Dr Moulay Tahar, 20000 Saida, Algeria.

²Laboratory of Biotoxicology, Pharmacognosy and Biological Valorisation of Plants, Department of Biology, Faculty of Natural and life Sciences, University of Saida, Dr Moulay Tahar, 20000 Saida, Algeria.

* Correspondence: hayat.aguis@univ-saida.dz / aguisdpu@gmail.com

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Abstract

This study aimed to isolate actinomycetes strains from a wide space and rhizosphere zone in arid and semi-arid environments and to determine their enzymatic potential. Using a combination of different pre-treatments and selective media, 94 distinct strains were collected and identified based on their morphological characteristics. Predominantly, these strains were recovered from the rhizosphere of *Fraxinus excelsior* 40%, followed by *Casuarina equisetifolia* 29%, and then 16% from Ain Sefra soil, 10% from Naama soil, and 5% from Bechar soil. A qualitative screening test was conducted to assess amylase, protease, and lipase activity. Out of the isolated strains, 77 and 75 displayed significant effectiveness in producing lipase and amylase, whereas only 43 strains showed the capacity to produce protease. Interestingly, a group of 27 strains exhibited positive results for co-production of all examined enzymes. Based on these findings, there is considerable potential for further investigation to exploit these enzymes or isolates strains in various industrial sectors.

Keywords: Microbial enzymes, Actinomycetes, Industrial applications, Enzymatic potential, Co-production.

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Introduction

Due to their impressive features, microbial enzymes have experienced a significant increase in industrial demand over the years. They are highly productive and manifest superior biochemical diversity, activity and stability compared to those derived from plants, animals or chemical catalysts. Notably, microbial enzyme catalysis has the ability to accelerate processes, reduce

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energy requirements, provide cost-effective alternatives, and exhibit eco-friendly properties. Furthermore, microbial strains and microbial natural products (NPs) can be enhanced through traditional or modern techniques for example, CRISPR-Cas9 genome editing, have shown significant promise for industrial enzyme production. Currently, there are approximately 200 different types of microbial enzymes available in the global enzyme market, used in various industrial processes such as leather, detergent, food, pharmaceutical, agriculture, etc. Almost 75% of microbial enzymes belong to the hydrolase class, which is responsible for decomposing diverse natural substances. Hydrolases, including proteases, lipases, and amylases, are widely produced by different microorganisms [1, 2, 3]. Actinobacteria or actinomycetes are one of the most well-known sources of valuable enzymes exploited in various bio-industrial processes [3, 4] since they are ubiquitous and possess a noteworthy capacity for synthesizing numerous hydrolase enzymes that play an essential role in decomposing complex organic matter found in soil or residue and mineralization of substances [5]. They are classified as Gram-positive bacteria with a high content of cytosine and guanine (60–70%) [6] and are placed in the phylum of actinobacteria, known for being one of the most common and diverse bacterial phyla in terms of morphological, physiological, biochemical, and metabolic characteristics [7]. Large number of this phylum are characterized by developing two types of mycelium: aerial and substrate, with the ability to form spores and produce pigments [8]. These morphological characteristics play an important role in the identification and taxonomy of species [9]. Actinomycetes species exist in both terrestrial and aquatic environments, whereas, their primary habitat was soil, representing about 20-60% of the microbial communities [10]. In ecological terms, they significantly contribute directly and indirectly to soil quality and plant growth and health through for instance, biogeochemical cycles, fertility enhancement, plant growth promotion operations, nitrogen fixation, and bio-control activities [5, 11]. However, environmental factors significantly affect the diversity, metabolic efficiency, biological activities, and mechanisms of adaptation of actinomycetes species [12], In this context, it has been described that arid and semi-arid soils show promising potential for actinomycetes biodiversity, which could lead to the discovery of potent enzymes [13]. In Algeria, the semi-arid and arid regions represent large areas of the country [14]. As a result, multitudes of species with biotechnological advantages have been isolated mainly from soils [13, 15, 16, 17]. In this study, the main objective is to isolate highly efficient actinomycetes for enzyme production, particularly protease, lipase, and amylase through primary screening. Moreover, to highlight the diversity of the actinomycetes community present in arid and semi-arid soils in three regions (Naama, Bechar, and Saida) in Algeria.

1. Material and methods

1.1. Soils Sampling and Pre-treatment

The samples were collected from three regions of Algeria in January 2020. They were obtained from both a wide space and the rhizospheric zone of *Fraxinus excelsior* and *Casuarina equisetifolia*, at a depth of 10 cm below the surface. These samples were carefully placed in sterile glass containers and transported to the laboratory for further analysis [18]. The air-dried soils

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taken from Ain Sefra (AS), Naama (NS), and Bechar (BEC) were dry-heated at 55°C for 10 minutes [18, 19]. The rhizospheric air-dried soil samples taken from *Fraxinus excelsior* (FRP) and from *Casuarina equisetifolia* (CEq) in Saida were pre-treated with 1% CaCO₃ [20].

1.2. Actinomycetes strains

Strain isolation was accomplished using the conventional serial dilution method [6, 21, 22]. In this process, from every single pretreated sample, 1g of soil was suspended in 9 ml of sterile saline solution (0.9% NaCl). Subsequently, serial decimal dilutions were prepared up to (10⁻⁴), and then 100 µl from each dilution was evenly spread across Starch casein agar (SCA) [23], Glucose Yeast Extract Malt Extract (GYM) agar, and Bennett agar plates [20]. These plates were incubated at 30°C for 14 to 21 days. Afterward, the colonies were picked and purified according to the phenotypic characteristics. The isolates were then maintained on isolation agar at 4°C until use, and the selected strains were stored in glycerol (30%, v/v) at -80°C.

1.3. Morphological characterization

The isolates underwent a phenotypic examination following the descriptions of Bergey's Manual of Systematic Bacteriology (Goodfellow et al., 2012) [24] and the International Streptomyces Project. This involved the macroscopic and microscopic aspects of the colony, encompassing surface texture, elevation, margin, aerial and substrate mycelium color, as well as pigment production [25].

1.4. Screening of enzymatic potential

The enzymatic examination process includes two distinct steps: a qualitative screening (primary) for testing various enzymatic activities (lipolytic, proteolytic, amylolytic), followed by a quantitative screening (secondary) specifically applied to the selected isolates showing higher enzymatic activities identified in the initial examination.

Qualitative screening (primary): All the strains were evaluated on specific media using the spot inoculation method, as detailed in Tab1. Enzyme activity was identified by the presence of a halo around the colonies, signifying a specific characteristic. The results were recorded by measuring the zone diameters in millimeters [26].

Table 1 : Qualitative enzymatic screening of actinomycetes strains

Enzyme	Medium	Incubation	indication of enzyme activity	References
Lipase	tween 80 agar (10% w/v)	5days /30°C	Presence of a white halo formed by crystals of the calcium salt.	[27]
Protease	basal medium with skim milk (1% w/v)	7days /30°C	Appearance of a colorless zone circling the colony	[28]

Amylase basal media 5 days /30°C Foramtion of a clearyellow halo around the colonyafteradding iodine solution (1%) [29]

2. Results

2.1. Actinomycetes strain

Totally, 94 actinomycetes strains were found in the soil samples (Fig 1), of which 40 % of actinomycetes strains were isolated from the rhizospheric zone of Fraxinus excelsior tree, and approximately 29% of strains were acquired from Casuarina equisetifolia tree rhizosphere zone. Whereas, the lowest total number of isolates was obtained in the soil of Ain sefra, Naama and Bechar with 16%, 10% and 5% respectively.

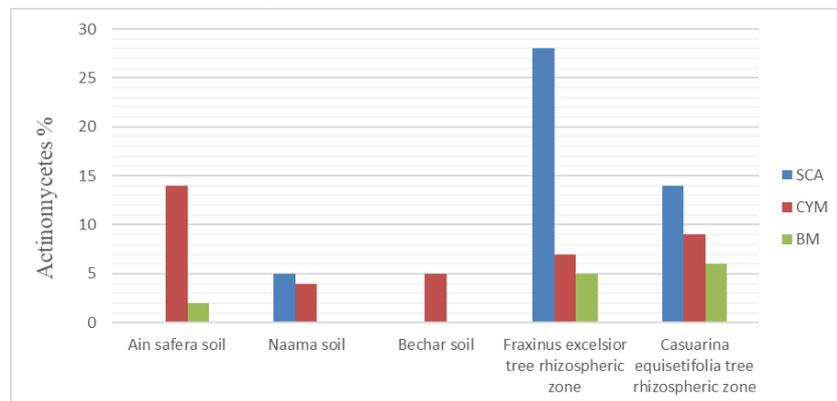


Fig 1. Percentage of actinomycetes strains isolated from soil samples using three types of selective media.

2.2. Morphological characterization

The diversity of isolated actinomycetes was studied morphologically, as summarized in the Tab2. Various colonies were observed on the selective media (Fig 2), where the aerial and substrate mycelium exhibited distinct colors, recorded in a simple manner. The predominant color of the aerial mycelium was white, while the substrate mycelium appeared as light yellow. All colonies displayed a dry consistency and a dull appearance, with only 26 strains demonstrating the ability to produce pigments.

Table 2: Morphological characterization of isolated actinomycetes on different media.

Characteristics	Growth pattern	Number of isolates
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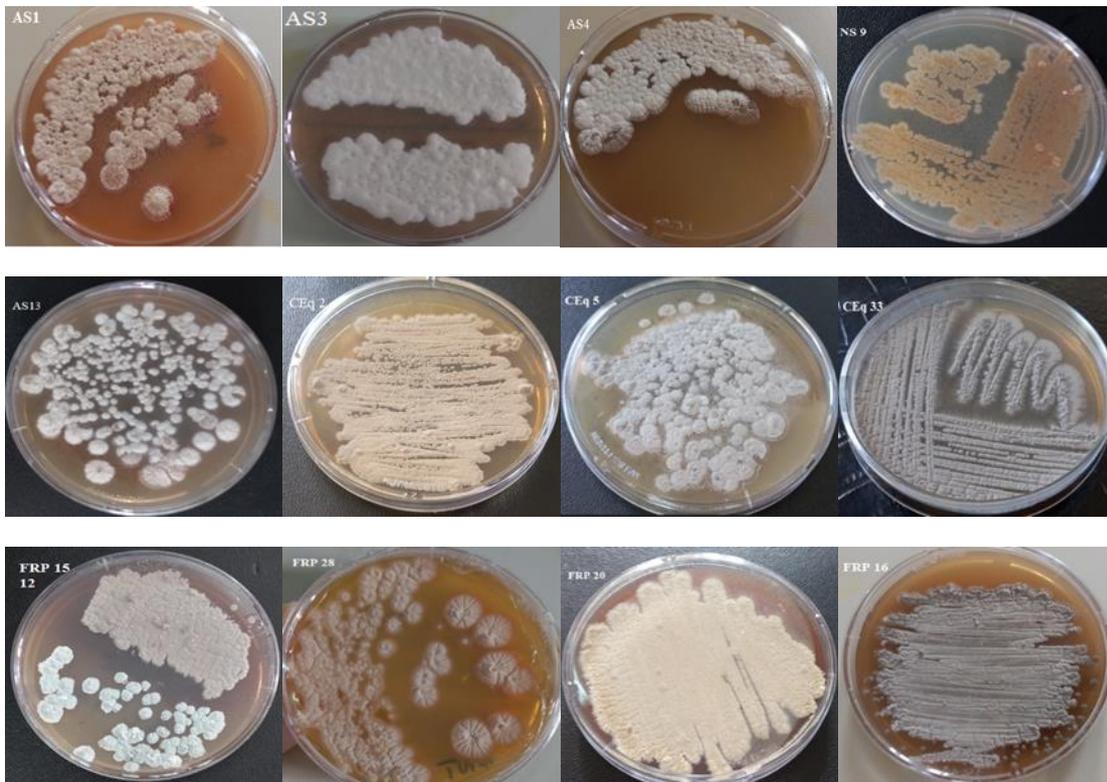
Growth	Good	70
	Moderate	23
	weak	1

Texture Surface	Granular	27
	Rough	30
	Wrinkled	18
	Spongy	4
	Cottony	9
	Powdery	6

Aerialmyceliumcolor	Pinkish white	4
	White	20
	light brown	3
	purple-grey	4
	grey	15
	white-green	3
	grey-blue	4
	pale-green	3
	white-grey	6
	yellow-orange	6
	yellow-brown	2
	beige	5
	brown	3
	grey-green	3
	red-brown	2
	pale yellow	4
	creme	4
	pink	3

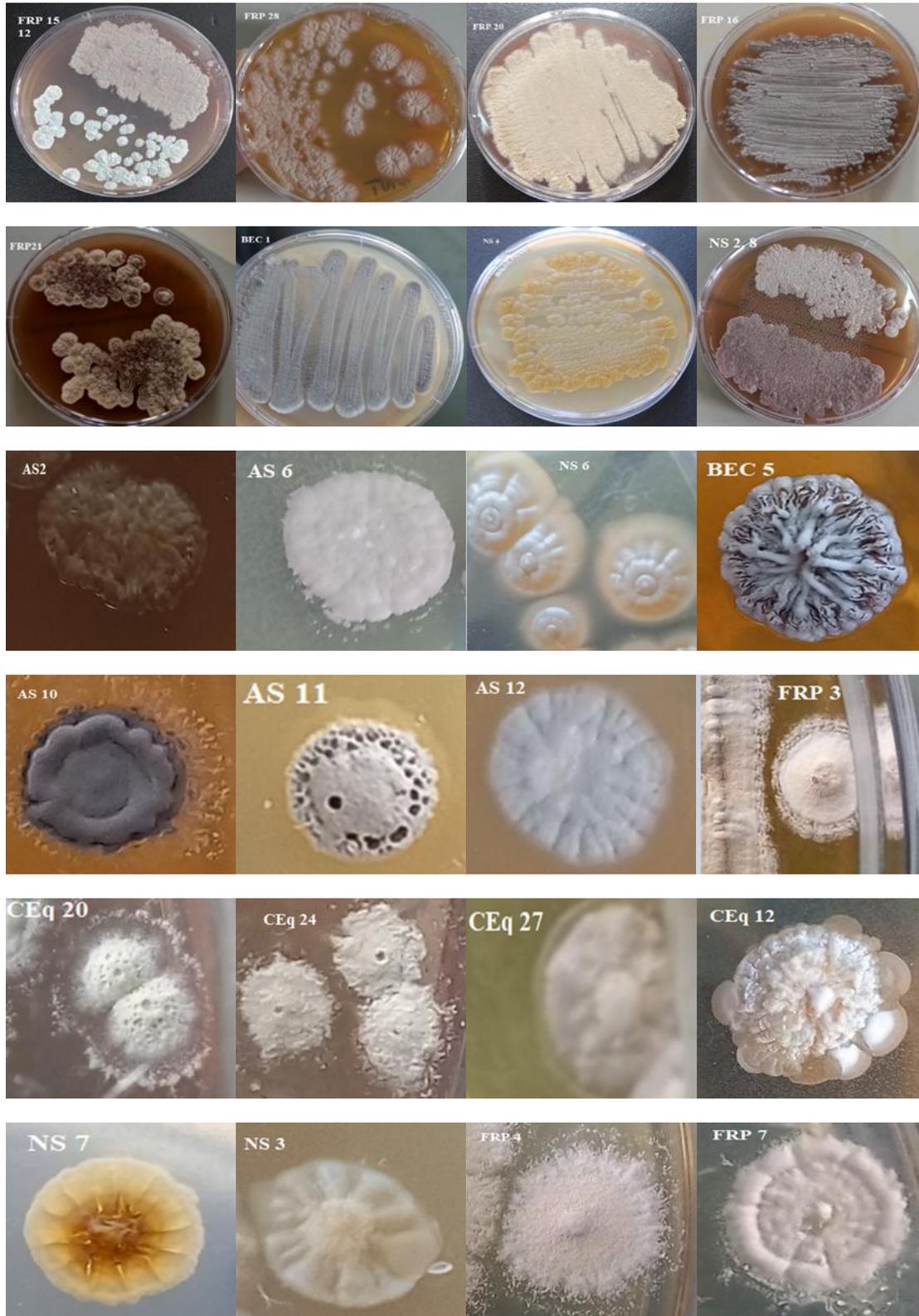
Substratemyceliumcolor	Orange-red	2
	Light yellow	23
	Yellow-brown	14
	Red-brown	2
	Yellow-orange	19
	Beige	5
	Dark-brown	9
	Orange	5
	Grey-brown	2
	Light brown	4
	Incolor	9
Margin	Entire	47
	Irregular	33
	Filamentous	10
	undulate	04
Elevation	Raised	38
	Falt	12
	Umbonate	18
	Convex	22
	Falt- Raised	4

Pigment	Light brown	3
	brownish orange	3
	Reddish Brown	4
	Light yellow	4
	Light brown	1
	Deep orange yellow	3
	Light Orange	2
	Strong Brown	2
	yellowishpink	1
	Pink	2
	Non detected	68



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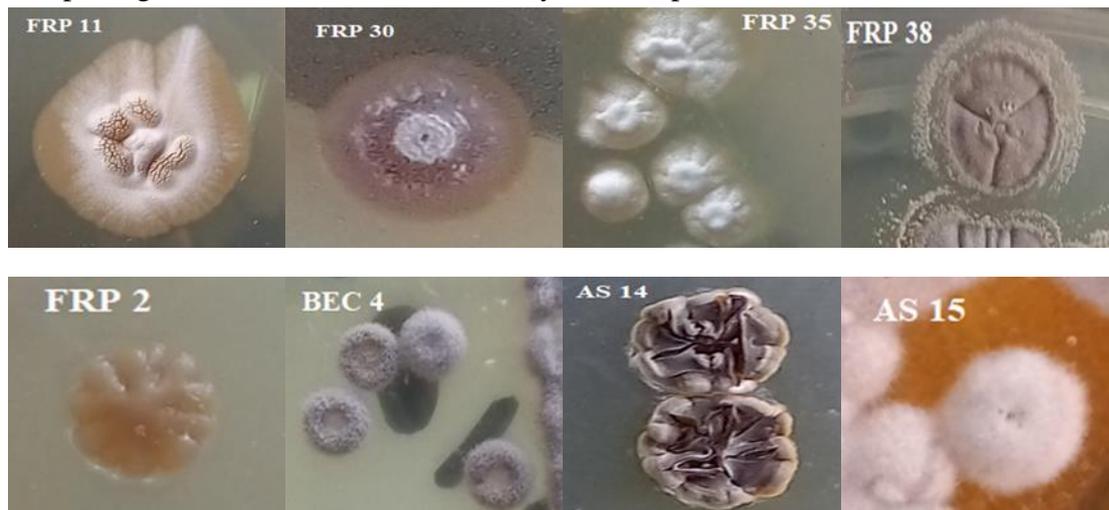


Fig 2 : Isolated actinomycetes strains

2.3. Screening of enzymatic potential

Out of the 94 strains examined for their enzymatic efficiency in producing amylase, protease, and lipases, it was found that 75 isolates showed activity only for amylase, 77 for lipase, and 43 for protease. Interestingly, 27 strains exhibited co-production of all enzymes (Fig. 3).

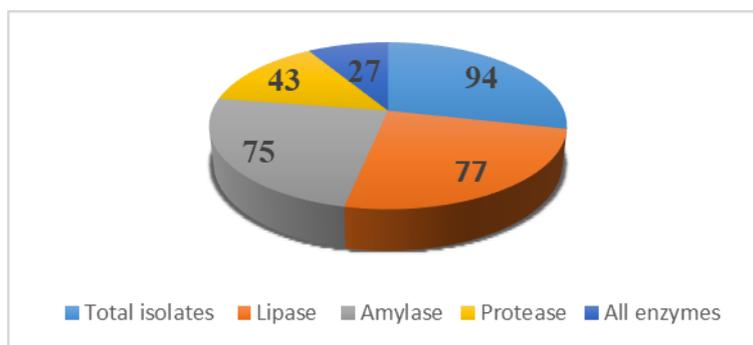


Fig 3 : The number of isolates producing the studied enzymes

The range of activity zones for each enzyme varied: amylase showed a range of 5 mm to 33 mm, lipase between 21 mm to 40 mm, and protease from 9 mm to 31 mm. Isolate FRP22 recorded the largest amylase activity zone at 33 mm in diameter. For lipase, both Isolate FRP38 and AS13 achieved the maximum zone diameter of 40 mm. Strain BEC5 showed the largest diameter of 31 mm for protease activity Tab 3.

Table 3: Enzyme activities of actinomycetes isolates

Strain	Enzyme activity (mm)		
	Amylase	Protease	Lipase

AS1	30	weak	29
AS2	26	12	27
AS3	/	weak	31
AS4	29	weak	28
AS5	28	weak	30
AS6	25	27	24
AS7	18	20	30
AS8	weak	22	31
AS9	11	19	32
AS10	30	28	22
AS11	weak	weak	26
AS12	16	weak	33
AS13	20	16	40
AS14	16	19	13
AS15	18	Non detected	weak
NS1	24	21	31
NS2	30	weak	28
NS3	30	09	21
NS4	16	30	30
NS5	weak	10	Non detected
NS6	Non detected	weak	32
NS7	25	20	29
NS8	30	weak	30
NS9	21	weak	33
BEC1	30	Non detected	25

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BEC2	21	26	23
BEC3	28	16	31
BEC4	32	22	30
BEC5	18	31	20
CEq1	24	20	33
CEq2	22	Non detected	26
CEq3	16	Non detected	22
CEq4	23	Non detected	27
CEq5	21	Non detected	30
CEq6	22	Non detected	19
CEq7	Non detected	weak	22
CEq8	16	Non detected	Non detected
CEq9	weak	21	Non detected
CEq10	22	Non detected	Non detected
CEq11	20	Non detected	15
CEq12	19	Non detected	Non detected
CEq13	17	10	25
CEq14	22	Non detected	13
CEq15	23	12	28
CEq16	weak	15	21
CEq17	30	weak	20
CEq18	20	weak	16
CEq19	18	25	25
CEq20	Non detected	Non detected	30
CEq21	weak	24	Non detected

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CEq22	24	28	Non detected
CEq23	20	Non detected	27
CEq24	weak	Non detected	18
CEq25	27	Non detected	22
CEq26	10	weak	Non detected
CEq27	21	Non detected	23
ERP1	Non detected	20	24
ERP2	Non detected	weak	13
ERP3	28	21	25
ERP4	23	16	32
ERP5	weak	20	19
ERP6	32	weak	26
ERP7	18	Non detected	33
ERP8	20	Non detected	29
ERP9	22	weak	18
ERP10	20	Non detected	25
ERP11	33	Non detected	26
ERP12	20	10	Non detected
ERP13	Non detected	Non detected	25
ERP14	27	Non detected	22
ERP15	Non detected	Non detected	19
ERP16	16	Non detected	18
ERP17	23	21	19
ERP18	22	weak	19
ERP19	22	28	18

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ERP20	33	Non detected	weak
ERP21	05	29	Non detected
ERP22	33	weak	23
ERP23	30	26	Non detected
ERP24	17	Non detected	25
ERP25	19	16	22
ERP26	18	21	33
ERP27	22	25	20
ERP28	21	15	weak
ERP29	Non detected	Non detected	weak
ERP30	23	25	16
ERP31	23	20	26
ERP32	21	Non detected	28
ERP33	20	Non detected	23
ERP34	weak	weak	34
ERP35	weak	weak	27
ERP36	26	18	weak
ERP37	28	20	Non detected
ERP 38	22	28	40

Discussion

The current study mainly describes the diversity and enzymatic activity of actinomycetes isolated from arid and semi-arid soil in four regions (Bechar, Ain Sefra, Naama, and Saida) in Algeria. 94 different actinomycetes strains were successfully obtained from a wide space and rhizosphere soil of two tree species, *Fraxinus excelsior* and *Casuarina equisetifolia*. Actually, according to several research findings in the literature, actinomycetes could be extensively found in all environments but mainly soil, where they are considered as soil dominant microorganisms. Usually, they are highly abundant in the upper layer with a density range of (106–109 cells/g of soil), and this

density is often inversely correlated with depth [9, 30]. The most prevalent genus in the soil is *Streptomyces* (70%). Additionally, other genera such as *Nocardia*, *Microbispora*, *Micromonospora*, *Actinomyces*, *Actinoplanes*, and *Streptosporangium* can also be found [5]. Evidence suggests that novel actinobacteria primarily originate from terrestrial soil, and secondly from plants [8]. Different morphology appearances was observed between the obtained strains table 2. Various researchers indicated that these morphological features are vital for the identification of actinomycetes species. The observation of both aerial and substrate mycelia was crucial in distinctly differentiating actinomycetes from other soil bacteria [9, 31]. Findings in the preceding studies reported the isolation of different actinomycetes [20, 32, 33], which are almost similar to the results of this study. The isolation process revealed a significant disparity in the number of actinomycetes strains between the samples (Fig 1). Probably, this could be illustrated by the influence of geographical location and ecological factors of the regions, such as climatic conditions [12, 34]. Arid and semi arid environments are known by the low moisture levels and high temperature [35]. Consequently, these conditions considerably promote the growth and germination of actinomycetes spores [9, 36, 37]. Moreover, competition with fast growing microorganisms is limited rather than damp lands [37]. Actinomycetes, including endemic species, are more able to adapt to unfavorable environments compared to other microorganisms [37, 38]. They exhibit diverse adaptations, including the ability to form spores highly resistant to desiccation [11] and produce potent antimicrobial compounds as part of their natural defense mechanisms [5, 36, 39]. Unlike other microorganisms, actinomycetes contribute to increasing soil humidity retention [34]. Furthermore, these environments are characterized by a lower of dissolved organic matter (DOM), Recognized as the most mobile part of organic matter, DOM plays a vital role as a source of carbon (C), nitrogen (N), phosphorus (P), and energy for soil microbial populations [40, 41]. Microorganisms are highly dependent on DOM. However, certain are unable to utilize available organic matter, either due to their specific substrate preferences or when it becomes concentrated [41]. Consequently, this provides an advantage to actinomycetes species due to their filamentous growth structure, enabling efficient nutrient access and their capacity to utilize a wide range of nutrients, including complex organic matter [42]. Beside, in winter, which is the sampling period, the count of actinomycetes decreases to around 13% and reaches nearly 20% in the spring. In contrast, during autumn, their number exceeds 30% [43]. The diversity as well as the number of the actinomycetes strains in soil could be explained by various important factors such as vegetation, pH and temperature, soil type and texture, organic carbon source and mineral, salinity, aeration, interactions with other organisms, and geogenic factors [9, 12, 30]. On the other hand, previous studies have also reported that the rhizosphere zone shown to be an important source for most actinomycetes species compared to other soil habitats [5, 30, 44, 45]. The rhizosphere provides a nutrient-rich environment enriched with organic compounds from roots exudates, plant residues and accumulation of essential substances (e.g., amino acids, fatty acids, organic acids, sugars, and vitamins) for actinomycetes growth [45]. Actinomycetes species produce different types of enzymes, such as

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protease, α -amylase, lipase, cellulase, and chitinase, etc., to efficiently break down plant debris [11, 45]. Various actinobacteria genera, including *Streptomyces*, *Nocardia*, and *Micromonospora* are ubiquitous and have the ability to use substrates originating from the decomposition of plant biomass [12]. Kingchan Malisorn et al. [46] isolated 17 actinomycetes belonging to *Streptomyces*, *Micromonospora*, and *Kitasatospora* from the rhizosphere soil of plants. Furthermore, investigations have reported that certain actinomycetes genera like *Frankia* and some species of *Streptomyces*, *Micromonospora*, named diazotrophic actinomycetes, can establish symbiotic relationships with actinorhizal plants and contribute to the biological nitrogen fixation (BNF) process by forming nitrogen-fixing nodules in the plant roots [45]. Moreover, previous research reported that *Frankia* have been isolated from *Casuarina* species [47]. The association of actinomycetes with plants is fostered by their filamentous structure, which establishes robust connections with soil particles in the rhizosphere [48]. The findings from the present study agree with several other research, clearly indicating that actinobacteria thrive in the rhizosphere and proliferate in soils rich with organic matter under alkaline conditions [9, 12, 30]. In this study, also the number of isolated actinomycetes found in the rhizosphere soil of two tree species, *Fraxinus excelsior* and *Casuarina equisetifolia*, was similar to a previously reported result, where a total of 65 actinomycetes strains were isolated from the rhizospheric soil of plants in Rajasthan, India [49]. In another study, 11 different actinomycetes strains were isolated from the rhizosphere of olive trees [50]. Furthermore, the results of this study agree with earlier findings, indicating that in the rhizosphere, the abundance and diversity of actinomycetes are considerably influenced by the plant species through the root exudates and type of soil [30, 38, 51]. The culture media and pre-treatment methods were also found to play a significant role in the isolation process of actinomycetes, as indicated by several studies [18, 52]. Therefore, the use of the pre-treatment methods focus on selecting actinobacteria genera by stimulating their growth or/and inhibiting or removing most of the undesired bacteria and fungi [18]. Treating the sample with Calcium Carbonate induces desiccation, leading to a substantial decrease in vegetative forms. Subsequently, This facilitates the isolation and cultivation of actinomycetes [53, 54]. In this study, the findings of the rhizosphere soils are nearly similar to the results obtained by Sailaja Rani et al. (2022) [55], where isolated 50 distinct actinomycetes from the rhizosphere soils of groundnut pretreated with calcium carbonate. Dry heating is also a selective and effective method for eliminating non-sporulating bacteria and inhibiting the growth of many spore-forming bacteria and fungi, while also promoting the growth of certain actinomycetes [18]. Many actinobacteria produce heat-resistant spores, some of which may be flagellated. These characteristics grant them two crucial ecological advantages: persistence and efficient dispersal. Actinomycetes are among the first microorganisms to recover rapidly following extended dry periods or heat and show swift responses when the soil is rewetted [34]. Bidhayak Chakraborty et al. [27] indicated that actinomycetes spores can resist desiccation well and exhibit slightly higher tolerance to dry or moist heat opposed to other microbes. The findings from samples (Ain sefra, Naama and Bechar) treated with dry heating are comparable to

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a previous study where only 6 actinomycetes were isolated using heat treatment and serial dilutions [19]. According to previous studies, various media have been suggested for the cultivation and selection of actinomycetes species [56]. SCA (Starch Casein Agar), GYM (Glucose Yeast Extract Malt Extract Agar), and Bennett Agar are commonly employed for the isolation of actinomycetes in different studies due to their nutrient-rich compositions and selective properties [54]. These media are provided with starch and glucose as the carbon sources, casein, nitrate, yeast extract, and malt extract as the nitrogen sources, and peptone as source of amino acids and other mineral [37]. Numerous studies recommend using media containing a high proportion of carbon to nitrogen and complex sources of these elements, such as starch, casein, and yeast extract, which is appropriate for isolating actinomycetes, particularly the *Streptomyces* genus [37, 54, 56]. This might explain why SCA achieves a high count of actinomycetes in this study, a similarity observed in findings reported by Anupama Sapkota et al. (2020) [57]. Apart from that, most of the isolates show a high aptitude for producing lipase and amylase, with a moderate ability to produce protease (Fig 3). The natural capacity of actinobacteria to produce extracellular hydrolytic enzymes comes from the natural selection process of microorganisms, by which they adapt and survive in competitive environments [18]. In prior studies, several researchers have demonstrated that actinomycetes found in soil manifest a notably high capacity for the production of various enzymes responsible for the degradation/ decomposition of organic matter [58]. Abdullah Abdulkareem Hassan et al. (2022) [59] also reported that actinomycetes secrete extracellular enzymes to break down organic biopolymers and reconstruct it from the residues of dead plants and animals. Additionally, it has been indicated that actinomycetes, despite their slower growth compared to bacteria and fungi, possess the ability to readily degrade highly resist and difficult-to-decompose organic matter. As a consequence of their degradation mechanisms, they secrete dark brown pigments that modify the soil humus color [5]. Hydrolytic enzymes produced by actinomycetes are vital contributors to soil fertility as they break down complex polysaccharides and proteins into simpler compounds [48]. Furthermore, they enhance soil quality by participating in nutrient recycling and protective mechanisms [11]. The current study provides findings that are comparable to several studies that reported the production of enzymes by actinomycetes. For example, Gargi Sarkar and Suthindhiran K (2020) [60] extracted alkaline protease from *Streptomyces* sp. GS-1 for use as a dehairing agent. In addition, Anwesha Gohain et al. (2020) [36] indicated that amylase synthesized by *Streptomyces erumpens* is used in the bakery. In another study, it was found novel extracellular alkaline and thermostable amylase synthesized by *Actinomyces keratinilytica* sp. Cpt29 holds potential for use in the detergent industry. While, Naif Abdullah Al-Dhabi et al. (2019) [61] isolated *Streptomyces* sp. Al-Dhabi-49 with the co-production of lipase and protease.

Conclusion

In this study, the primary focus was to isolate a diverse range actinomycetes strains from a wide space and rhizosphere soil of *Fraxinus excelsior* and *Casuarina equisetifolia* trees in arid and semi-

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arid environments, as well as to evaluate their enzymatic capabilities. A total of 94 distinct strains were isolated, mostly obtained from the rhizosphere soil than the wide space. Among these, a significant number of strains displayed high efficacy in producing amylase and lipase, while nearly half demonstrated the ability to produce protease. Intriguingly, only 27 strains exhibited positive results for all the enzymes studied. These findings reveal that the isolated actinomycetes represent a promising resource for producing valuable enzymes for various bio-industrial applications. It is also proposed that further optimization of culture conditions and characterization of these enzymes be carried out.

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