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RESEARCH ARTICLE

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Isolation, Identification and Enzymatic Activity of Halotolerant and Halophilic Fungi from the Great Sebkha of Oran in Northwestern of Algeria

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ABSTRACT

The Great Sebkha of Oran is a closed depression located in northwestern of Algeria. Despite the ranking of this sebkha among the wetlands of global importance by Ramsar Convention in 2002, no studies on the fungal community in this area have been carried out. In our study, samples were collected from two different regions. The first region is characterized by halophilic vegetation and cereal crops and the second by a total absence of vegetation. The isolated strains were identified morphologically then by molecular analysis. The biotechnological interest of the strains was evaluated by testing their ability to grow at different concentration of NaCl and to produce extracellular enzymes (i.e., lipase, amylase, protease, and cellulase) on solid medium. The results showed that the soil of sebkha is alkaline, with the exception of the soil of cereal crops that is neutral, and extremely saline. In this work, the species *Gymnoascus halophilus, Trichoderma gamsii,* the two phytopathogenic fungi, *Fusarium brachygibbosum* and *Penicillium allii,* and the teleomorphic form of *P. longicatenatum* observed for the first time in this species, were isolated for the first time in Algeria. The halotolerance test revealed that the majority of the isolated are halotolerant. *Wallemia sp.* and two strains of *G. halophilus* are the only obligate halophilic strains. All strains are capable to secrete at least one of the four tested enzymes. The most interesting species presenting the highest enzymatic index were *Aspergillus sp.* strain A4, *Chaetomium sp.* strain H1, *P. vinaceum*, *G. halophilus*, *Wallemia sp.* and *Ustilago cynodontis.*

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1. Introduction

Sebkha is an Arabic word referring to a closed depression temporarily occupied by a salt lake. It is characterized by an abundance of soluble salts concentrated on the surface that prevents any vegetation [1]. Halophilic vegetation appears in less salty soils that surround the sebkha. In Algeria, several sebkha or salt lakes stretch from the Algerian north coast to the Sahara. The Great Sebkha of Oran is the largest sebkha in northwestern of Algeria with an area of 1890 km^2. It is temporarily occupied by a salt lake of 300 km^2 whose salt concentration is estimated at more than 100 $g.L^{-1}$ of dissolved salts [2].

A specific fauna and flora characterize saline ecosystems. Beside this population, several studies have shown that other organisms such as eubacteria, archaea, algae, and fungi can grow under salt stress and populate saline ecosystems [3,4]. Various research have been carried out on the fungal diversity of the saline environments in the world in particular the solar salterns [5–8], Dead Sea [9–13], arid desert [14,15], and some sebkha [16,17]. In general, fungal communities in hypersaline environments are dominated by *Aspergillus*, *Penicillium* and some of their related teleomorphic genera (formerly *Eurotium*, *Emericella*, and *Eupenicillium*). Other genera such as *Alternaria*, *Cladosporium*, *Fusarium*, *Chaetomium*, *Wallemia,* and *Hortaea* were also reported [18–20]. Some new species were also described from hypersaline environments including three species of the genus *Wallemia* [21], twelve species of the genus *Cladosporium* [22,23], two species of the genus *Emericella* [24] and three species of the genus *Gymnoascus* [25].

The main reasons for studying extremophiles including halophilic microorganisms are to understand their mechanisms involved in stress adaptation and for the biotechnological application of their metabolites capable of activity under extreme conditions. Low water activity and high salt concentration of hypersaline environments make these habitats an important source of halophilic microorganisms that

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can provide enzymes of industrial interest [26]. Several researches on halophilic hydrolases such as amylases, cellulases, lipases and proteases have been reported from halophilic bacteria and fungi [27,28] including few investigations on enzymes from obligate halophilic fungi [29]. In addition, the increasing need for bioremediation of hypersaline environments and for biocontrol agents that can be used in agriculture irrigated by saline water stimulate the search for these halophilic organisms [30,31].

Although the Great Sebkha of Oran is considered an extreme environment due to its high salt content, no studies on its fungal community have been published. In this present work, we report the first study on fungal diversity of the Great Sebkha of Oran by isolating halotolerant and halophilic fungi from the saline soil in two zones of the Great Sebkha. No isolation was done at the lake level because of the difficulty of access to the center of the sebkha. Fungal isolates were identified by morphological and microscopical observations and by the use of molecular techniques. In order to select strains of biotechnological interest, the salt tolerance of isolates and their ability to produce hydrolytic enzymes were evaluated.

2. Materials and methods

2.1. Sampling site and isolation

Samples were collected from the soil of the Great Sebkha of Oran located in northwestern of Algeria at 12 km from the Mediterranean Sea (Figure 1). Sampling was carried out in an area of 5 km^2 between Boutlelis and Al Amria in 9 sites divided in two zones: zone1 (Figure 1: sites B, C, D, R, G, S) is characterized by halophilic vegetation and cereal crops and zone2 (Figure 1: sites A, E, H) is characterized by a total absence of vegetation.

Soil samples were collected after removing the surface layer of the soil at a depth of 5 to 15 cm, placed in a sterile bottle and transported to the laboratory where fungi were isolated.

Fungal isolations were made by using the dilution plate method on potato dextrose agar medium (PDA) prepared with different concentrations of NaCl (5%, 10% and 15%). Plates were then incubated at 25 °C for a month.

After one month, small agar plugs containing fungal mycelium, identified as different species by macroscopic and microscopic observations, were transferred to different fresh PDA plate (containing the same salt concentration than the isolation plate) then incubated in the dark at 25 °C for 3–4 weeks to assess the purity of each obtained isolate.

2.2. Physicochemical analysis of soil

Soil suspensions were prepared for physicochemical analysis by mixing 10 g of the soil in 50 ml of distilled water. Electrical conductivity, salinity, and pH of soil suspensions were determined using a conductivity meter and a pH meter.

2.3. Morphological identification of fungal isolates

The morphological identification of the isolates was based on a macroscopic observation of the cultural characteristics and a microscopic study of the morphological characters of the mycelium and of sexual and asexual reproductive organs. The identification keys of Pitt [32], Barnett and Hunter [33], Samson et al. [34] and Samson and Frisvad [35] were used to classify isolates in different genera.

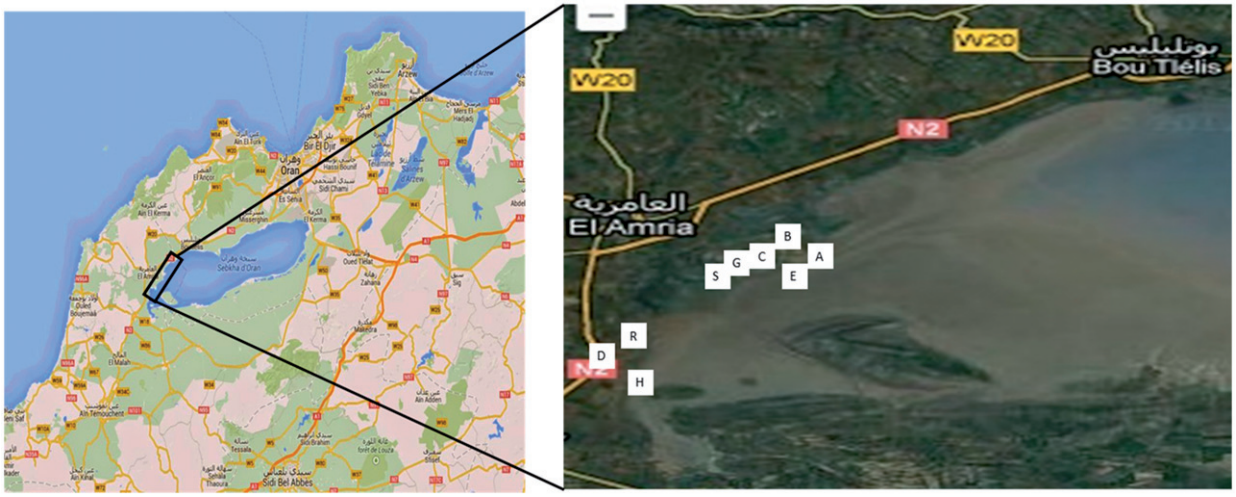

Figure 1. Map of the Great Sebkha of Oran. Letters indicate sites of sampling. (Image ©2018 DigitalGlobe, CNES/Airbus, DigitalGlobe, Données cartographiques ©2018 Google.)

2.4. DNA extraction and molecular identification of isolates

30-days-old fungal mycelium was scraped from the surface of a PDA plate using a sterile scalpel and transferred into a sterile 2 mL tube. Genomic DNA was then extracted using the FastDNA® SPIN kit (MP Biomedicals, Santa Ana, CA) following the manufacturer's instructions with an initial homogenization step using the Retsch MM400 instrument (Retsch GmbH, Haan, Germany) at 30 Hz for 30 sec, for two times. The DNA was re-suspended in 100 µL of sterile nuclease-free water, quantified and checked in quality using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). After extraction, all DNA extracts were stored at −20 °C.

In order to establish the species designation, different DNA regions were amplified according to the fungal genera. The 1α translation elongation factor gene (TEF-1α) was amplified for strains belonging to the genus *Fusarium* and *Curvularia* using primers EF1F/EF1R [36], *β*-tubulin for strains of *Aspergillus, Penicillium,* and their teleomorphic forms using primers Bt2a/Bt2b [37], actine for the genus *Cladosporium* using primers ACT-512F/ACT-783R [38]. The ITS1-5.8S-ITS2 region of rDNA was amplified for the rest of genera using primers ITS4 and ITS5 [39]. Amplification reactions were performed in 25 µL volume using 0.025 U/µL of GoTaq Flexi DNA polymerase (Promega, Madison, WI) and 1 × GoTaq Flexi buffer (Promega), 25–50 ng of template DNA, 0.08 µM of each primer, 2 mM of $MgCl_2$ and 0.2 mM of 10 mM dNTP mix (Promega). Amplification products were analyzed by electrophoresis in 1 × TAE buffer (40 mM Tris-acetate, 1 mM EDTA) with 1% (w/v) agarose gel (LE, analytical grade agarose; Promega) prepared using 1 × TAE buffer and detected by UV fluorescence after GelRed™ (Biotium Inc., Fremont, CA) staining, according to manufacturer's instructions. The BenchTop 100-bp DNA ladder (Promega) was used as molecular size marker. PCR products were sent to Eurofins MWG (Ebersberg, Germany) for purification and sequencing in forward and reverse, using the same primers used for PCR. The sequence obtained from each isolate was further analyzed by Basic Local Alignment Search Tool (BLAST) at the National Center of Biotechnology Information (NCBI) website.

DNA extraction, amplification and sequencing were realized during two stays at Laboratory of Biodiversity and Microbial Ecology (LUBEM) Brest, UBO, France.

2.5. Halotolerance test

The halotolerance test was studied on PDA medium prepared with different concentrations of NaCl (from 0% to 20% with an interval of 2.5). Strains were inoculated at the center of the Petri dishes containing 15 ml of culture medium and incubated at 25 °C for 10 days. The measurement of the radial growth of each thallus was carried out every 48 h.

2.6. Extracellular enzymes production

Enzymes production was evaluated on solid medium. In order to visualize the enzymatic activity a specific substrate of each enzyme was added to the culture medium as a carbon source. After inoculation and incubation of cultures for 2–5 days depending on the growth rate of the strains, the appearance of a clear halo or precipitation around the thallus indicates enzyme production.

Amylase activity was evaluated on nutrient agar medium supplemented with 2 $g.L^{-1}$ of soluble starch. After incubation, the cultures were flooded with a solution of iodine. The appearance of a clear zone around the thallus reveals the presence of amylase [40].

Cellulase activity was tested on medium supplemented with 1% cellulose (7.0 $g.L^{-1}$ KH_2PO_4, 2.0 $g.L^{-1}$ K_2HPO_4, 0.1 $g.L^{-1}$ $MgSO_4 7\,H_2O$, 1.0 $g.L^{-1}$ $(NH_4)_2SO_4$, 0.6 $g.L^{-1}$ yeast extract, 10 $g.L^{-1}$ microcrystalline cellulose and 15 $g.L^{-1}$ agar) [41]. At the end of the incubation period, the cultures were incubated at 50 °C for 16 h to accelerate the action of the enzyme [42]. The cultures were then flooded with 5 ml of iodine and rinsed with distilled water to visualize the hydrolysis zone [43].

Protease activity was detected on milk agar medium containing 30% skim milk and 2% agar. After incubation, the degradation of casein was reflected by a clear zone around the thallus [44].

Lipase activity was determined on culture medium containing tween 80 as a lipid substrate (10 $g.L^{-1}$ peptone, 5 $g.L^{-1}$ NaCl, 0.1 $g.L^{-1}$ $CaCl_2 2H_2O$, 17 $g.L^{-1}$ agar and 10 $mL.L^{-1}$ Tween 80). Tween 80 was sterilized separately then added to the sterile medium. After incubation, the cultures were put at 4 °C for 12 h to better visualize the appearance of an opaque precipitation around the thallus [40].

For each enzyme, the activity was evaluated by an enzymatic index (EI) where EI = R/r (R being the diameter of the halo and r the diameter of the thallus). Strains with an EI equal to or greater than 2 are considered as good producers of the studied enzyme [40].

3. Results

3.1. Physicochemical properties of soil samples

The results of the physicochemical analyses presented in Table 1 show that the soil of the zone1 (dominated by halophilic vegetation) and the soil of

Table 1. Physicochemical analyses of soil samples.

Site sampling		Strain code	Date of sampling	pH	Electrical conductivity ms.cm^{-1}	Salinity g.L^{-1}
Zone1	Halophilic plants	B, C, D, R	December 2012	8.0	57.5	37
	Cereal crops	G, S	January 2015	7.2	6	3.8
Zone2	Total absence of vegetation	A, E	December 2012	7.7	43	28
		H	July 2015	8.2	71.5	46

the zone2 (characterized by a total absence of vegetation) are alkaline and have a high salinity rate. Nevertheless, it should be noted that in the sites G &S (zone1) characterized by the presence of cereal crops, the soil is neutral and less saline. According to the scale of Durand [45], all these soils are classified as extremely saline (EC $>$ 4 ms.cm^{-1}).

3.2. Strains identification

A total of 136 isolates were isolated from both zones. One hundred and twenty three isolates were identified to the genera level by macroscopic and microscopic observation of cultures while 13 isolates were not identified (sterile mycelia). Fifty isolates representing the different morphologically identified genera as well as the 13 unidentified isolates were selected for molecular identification.

For the majority of the 50 assayed strains, the obtained sequences showed high similarity (≥97%) with fungal species sequences deposited in GenBank database (Table 2); 33 strains have been affiliated to 29 species belonging to 14 genera of Ascomycota division and one species of Basidiomycota division, the other 17 strains were identified only at the genus level. Among those 17 unidentified strains, 6 belonging to the genera *Penicillium* (R32, R33), *Trichoderma* (G15), *Alternaria* (G5), *Chaetomium* (H1) and *Wallemia* (H15) showing high similarity (≥97%) with sequences of more than one species. The sequenced gene used to identify these 6 strains does not allow distinction between species. Therefore, other genes need to be studied to have a more precise identification of those species. Four others strains belonging to the genera *Chrysosporium* (H18), *Tritirachium* (B2), *Lecanicillium* (R29) and *Pleospora* (H43) showing a high similarity (100%) with unidentified species of these genera. The last 7 unidentified strains belonging to the genera *Aspergillus* (A4, E2, E7), *Arachnomyces* (H10), *Fusarium* (R1) and *Chaetomium* (H38, H42) but presented only low similarity with sequences of fungal species available in GenBank database. These strains could represent new species but more molecular and phylogenetic studies will be necessary to confirm their correct taxonomic position.

3.2.1. Strains isolated from zone1

A total of 83 isolates belonging to 17 genera and 26 identified species and 8 unidentified species were isolated from the soil of sebkha where halophilic plants and cereal crops dominate (Table 3). The most dominant genus was *Fusarium* (32.5%) represented by 5 species: *F. oxysporum*, *F. equiseti*, *F. brachygibbosum*, *F. acuminatum* and one unidentified species belonging to dimerum clade (*Fusarium sp* strain R1). The species *F. equiseti* was the most frequently isolated representing 18% of all isolates and 55.5% of *Fusarium* isolates. The genera *Penicillium* and *Aspergillus* were isolated with a frequency of 26.50% and 13.25% respectively. Their teleomorphic form were represented by at least four species: *A. amstelodami*, *P. egyptiacum,* which were the dominant species, *P. longicatenatum* and two unidentified species *Penicillium sp.* strain R32 showing high similarity with *P. egyptiacum* or *P. sinaicum*, and *Penicillium sp.* strain R33 showing high similarity with *P. egyptiacum* or *P. molle* that were isolated with low frequency.

Two strains of *Trichoderma*, *T. gamsii* and *Trichoderma sp.,* were isolated from the soil of cereal crops. These two species and the rest of the strains belonging to the orders of Hypocreales, Pleosporales, Microascales, and Capnodiales were the least frequently isolated in this area.

3.2.2. Strains isolated from zone2

A total of 53 isolates belonging to 14 genera and 13 identified species and 8 unidentified species were isolated from the soil of sebkha characterized by a total absence of vegetation (Table 3). Unlike zone1, where the genus *Fusarium* was the most frequently isolated, this genus and all the genera of the order of Hypocreales isolated from zone1 were not isolated from zone2 with the exception of the species *Sarocladium strictum* which has been isolated from both zones. The most frequently isolated species were *A. amstelodami*, *P. egyptiacum*, *Alternaria sp.* and *Gymnacella denkaliensis*. The species isolated with lower frequency belonged to the genera *Penicillium*: *P. flavigenum*, *P. griseofulvum* and *P. alii*, *Aspergillus*: *A. subramanianii*, *A. calidoustus,* and three strains of an unidentified species *Aspergillus sp.* strain A4, E2, and E7 having 95% to 96% similarity with the species *A. micronesiensis*. Unidentified species of *Chaetomiaceae* represented by three strains H1, H38 and H42 were also isolated, the strain H1 has a high similarity with the two species *C. murorum* and *C. piluliferum,* and, in

Table 2. List of fungal isolates obtained from the sebkha and their closest match with the NCBI GenBank database.

Strains code	Locus	*Closest match in GenBank*	Max ident / Query coverage	Accession number
A1	β tubulin	*Aspergillus subramanianii*	99/98	MK361155
A2	β tubulin	*Aspergillus subramanianii*	99/97	MK361156
S11	β tubulin	*Aspergillus terreus*	100/100	MK361157
S15	β tubulin	*Aspergillus calidoustus*	99/100	MK361158
S20	β tubulin	*Aspergillus europaeus*	99/100	MK361159
A4	**β tubulin**	***Aspergillus micronesiensis***	**95/97**	**MK361160**
E2	**β tubulin**	***Aspergillus micronesiensis***	**96/98**	**MK361161**
E7	**β tubulin**	***Aspergillus micronesiensis***	**95/98**	**MK361162**
H12	β tubulin	*Aspergillus amstelodami*	97/100	MK361163
S16	β tubulin	*Penicillium flavigenum*	99/100	MK361164
S17	β tubulin	*Penicillium griseofulvum*	100/99	MK361165
S18	β tubulin	*Penicillium canescens*	98/98	MK361166
S19	β tubulin	*Penicillium mariae-crucis*	100/93	MK361167
H22	β tubulin	*Penicillium allii*	100/100	MK361168
R7	β tubulin	*Penicillium vinaceum*	99/100	MK361169
E9	β tubulin	*Penicillium egyptiacum*	99/100	MK361170
S12	β tubulin	*Penicillium longicatenatum*	99/91	MK361171
R32	**β tubulin**	***Penicillium sinaicum***	**99/95**	**MK361172**
		Penicillium egyptiacum		
R33	**β tubulin**	***Penicillium egyptiacum***	**98/100**	**MK361173**
		Penicillium molle	**97/100**	
H2	ITS	*Gymnacelladankaliensis*	100/98	MK361132
H18	**ITS**	***Chrysosporium sp.***	**100/99**	**MK361133**
H10	**ITS**	***Arachnomycesperuvianus***	**93/99**	**MK361134**
H19	ITS	*Gymnoascus halophilus*	100/100	MK361135
H20	ITS	*Gymnoascus halophilus*	100/100	MK361136
B8	TEF1	*Fusarium oxysporum*	99/100	MK361174
D3	TEF1	*Fusarium equiseti*	100/97	MK361175
R38	TEF1	*Fusarium brachygibbosum*	99/99	MK361176
S8	TEF1	*Fusarium acuminatum*	99/100	MK361177
S7	TEF1	*Fusarium brachygibbosum*	97/99	MK361178
R1	**TEF1**	***Fusarium sp***	**95/92**	**MK361179**
		Fusarium cf. dimerum	**90/61**	
R8	ITS	*Sarocladiumstrictum*	100/100	MK361137
S2	ITS	*Trichoderma gamsii*	100/100	MK361138
G15	**ITS**	***Trichoderma koningii***	**99/100**	**MK361139**
		Trichoderma koningiopsis		
		Trichoderma hispanicum		
		Trichoderma sp.		
B2	**ITS**	***Tritirachium sp***	**100/100**	**MK361140**
C3	ITS	*Gibellulopsisnigrescens*	100/100	MK361141
R13	ITS	*Beauveriabassiana*	100/100	MK361142
R29	**ITS**	***Lecanicillium sp.***	**100/100**	**MK361143**
B1	ITS	*Purpureocilliumlilacinum*	100/100	MK361144
R5	ITS	*Myrothecium verrucaria*	99/100	MK361145
S3	ITS	*Clonostachysrosea*	100/100	MK361146
H1	**ITS**	***Chaetomium piluliferum***	**98/100**	**MK361147**
		Chaetomium murorum	**98/100**	
H38	**ITS**	***Chaetomium retardatum***	**93/100**	**MK361148**
H42	**ITS**	***Chaetomium retardatum***	**93/100**	**MK361149**
B5	ITS	*Microascusmanginii*	99/100	MK361150
G5	**ITS**	***Alternaria sp.***	**100/100**	**MK361151**
		Alternaria alternate		
		Alternaria tenuissema		
		Alternaria chartarum		
H43	**ITS**	***Pleospora sp.***	**100/100**	**MK361152**
R20	TEF1	*Curvulariaspicifera*	100/97	MK361180
R36	ACT	*Cladosporium ramotenellum*	100/100	MK361181
H14	ITS	*Ustilagocynodontis*	99/100	MK361153
H15	**ITS**	***Wallemia sp.F53 (related to Wallemia sebi)***	**98/100**	**MK361154**
		Wallemia Canadensis		
		Wallemia mellicola		

Strains not identified to the species level are mentioned in bold.

GenBank database, the closest homologue species to strains H38 and H42 was *C. retardatum* with a similarity of 93%. The rest of the isolates belonged to 7 species or genus of Ascomycota division: *Gymnoascus halophilus*, *Chrysosporium sp*, *Arachnomyces sp.*, *S. strictum*, *Pleospora sp.*, *Curvularia spicifera* and *Cladosporium ramotenellum,* and two species of Basidiomycota division: *Ustilago cynodontis* and an unidentified species *Wallemia sp.* belonging to *W. sebi* complex.

3.3. Halotolerance test

The salt tolerance test represented in Table 3 showed that all strains could grow on PDA medium without NaCl with the exception of *Wallemia*

Table 3. Number of isolates in each site and their salt tolerance.

		Number of isolates		Salt tolerance (NaCl %)	
Strains identity	Strains code	Zone 1	Zone 2	growth interval	optimal growth
Eurotiales					
Aspergillus subramanianii	A1	–	1	0–17.5	2.5
Aspergillus subramanianii	A2	–	2	0–15.0	2.5
Aspergillus terreus	S11	1	–	0–12.5	2.5
Aspergillus calidoustus	S15	3	2	0–12.5	2.5
Aspergillus europaeus	S20	1	–	0–12.5	5
Aspergillus sp	A4	–	1	0–17.5	2.5
Aspergillus sp	E2	–	1	0–15.0	2.5
Aspergillus sp	E7	–	1	0–15.0	[2.5–7.5]
Aspergillus amstelodami	H12	6	5	0–15.0	7.5
Penicillium flavigenum	S16	3	2	0–12.5	2.5
Penicillium griseofulvum	S17	4	3	0–12.5	2.5
Penicilliumcanescens	S18	2	–	0–12.5	2.5
Penicillium mariae-crucis	S19	2	–	0–12.5	2.5
Penicillium allii	H22	–	1	0–12.5	5
Penicillium vinaceum	R7	2	–	0–17.5	5
Penicillium egyptiacum	E9	5	6	0–15.0	2.5
Penicillium longicatenatum	S12	2	–	0–15.0	5
Penicillium sp	R32	1	–	0–15.0	5
Penicillium sp	R33	1	–	0–12.5	5
Onygenales					
Gymnacelladankaliensis	H2	–	6	0–12.5	2.5
Chrysosporium sp.	H18	–	1	0–7.50	2.5
Arachnomyces sp.	H10	–	1	0–12.5	2.5
Gymnoascus halophilus	H19	–	1	2.5–17.5	10
Gymnoascus halophilus	H20	–	1	2.5–17.5	10
Hypocreales					
Fusarium oxysporum	B8	6	–	0–12.5	2.5
Fusarium equiseti	D3	15	–	0–12.5	2.5
Fusarium brachygibbosum	R38	2	–	0–12.5	2.5
Fusarium acuminatum	S8	1	–	0–10.0	2.5
Fusarium brachygibbosum	S7	2	–	0–12.5	2.5
Fusarium sp	R1	1	–	0–12.5	2.5
Sarocladiumstrictum	R8	2	2	0–7.50	5
Trichoderma gamsii	S2	1	–	0–5.0	0
Trichoderma sp.	G15	1	–	0–5.0	0
Tritirachium sp	B2	1	–	0–12.5	[2.5–5.0]
Gibellulopsisnigrescens	C3	2	–	0–7.50	0
Beauveriabassiana	R13	1	–	0–7.50	0
Lecanicillium sp.	R29	1	–	0–12.5	2.5
Purpureocilliumlilacinum	B1	2	–	0–7.50	2.5
Myrothecium verrucaria	R5	1	–	0–7.50	0
Clonostachysrosea	S3	1	–	0–7.50	0
Sordariales					
Chaetomium sp	H1	–	1	0–7.50	2.5
Chaetomium sp	H38	–	1	0–10.0	[2.5–5.0]
Chaetomium sp	H42	–	1	0–12.5	5
Microascales					
Microascusmanginii	B5	1	–	0–15.0	2.5
Pleosporales					
Alternaria sp.	G5	3	5	0–12.5	0
Pleospora sp.	H43	1	1	0–12.5	2.5
Curvulariaspicifera	R20	1	1	0–12.5	0
Capnodiales					
Cladosporium ramotenellum	R36	4	3	0–12.5	5
Basidiomycètes					
Ustilagocynodontis	H14	–	2	0–5.0	0
Wallemia sp.	H15	–	1	2.5–20	[2.5–7.0]
Total number of isolates	136	83	53		
Total number of genera	24	17	14		
Total number of identified species	30	26	13		
Total number of unidentified species	14	8	8		

*sp.*H15 and the two strains of *G. halophilus* H19 and H20 that are obligatorily halophilic. 74% of the strains could grow at 12.5% NaCl and 5 strains (*A. subramanianii* strain A1, *Aspergillus sp.* strain A4, *P. vinaceum* and the two strains of *G. halophilus*) at 17.5%. The only strain that could grow at 20% was *Wallemia sp.* The optimum growth of most strains is 2.5% or 5% NaCl. The concentration of 10% is optimal for the growth of *G. halophilus*.

3.4. Extracellular enzymes production

The 50 isolates previously selected for molecular analysis were assayed for extracellular enzyme activity. The secretion of the extracellular enzymes

Table 4. Enzymes activities of fungal isolates.

		Enzymatic index (EI)			
Strains	Strain code	lipase	amylase	protéase	cellulase
Aspergillus subramanianii	A1	–	–	0.21	1
Aspergillus subramanianii	A2	0.5	0.57	0.2	1.6
Aspergillus terreus	S11	0.2	–	0.2	1.8
Aspergillus calidoustus	S15	0.33	0.21	–	3
Aspergillus europaeus	S20	–	0.3	1	2
Aspergillus sp	A4	0.5	2.0	2.4	4.66
Aspergillus sp	E2	–	0.5	1	3
Aspergillus sp	E7	–	–	0.3	1.2
Aspergillusamstelodami	H12	0.8	–	–	2.5
Penicillium flavigenum	S16	0.2	0.28	0.3	0.8
Penicillium griseofulvum	S17	1.4	0.8	–	1.42
Penicilliumcanescens	S18	1	0.15	1	2
Penicillium mariae-crucis	S19	–	0.5	0.08	0.87
Penicillium allii	H22	0.28	0.6	0.23	2
Penicillium vinaceum	R7	5	1.8	0.8	4.33
Penicillium egyptiacum	E9	0.5	0.5	1.42	2
Penicillium longicatenatum	S12	0.3	1.14	0.75	1
Penicillium sp	R32	0.25	0.55	1.28	1.6
Penicillium sp	R33	0.5	0.4	1.16	1.16
Gymnacelladankaliensis	H2	0.25	0.5	0.2	–
Chrysosporium sp.	H18	3	–	1	3
Arachnomyces sp.	H10	–	–	0.8	3.33
Gymnoascus halophilus	H19	–	2	4	5
Gymnoascus halophilus	H20	–	1.6	4.33	6
Fusarium oxysporum	B8	–	–	–	2.66
Fusarium equiseti	D3	–	–	–	3.33
Fusarium brachygibbosum	R38	–	–	–	3.33
Fusarium acuminatum	S8	–	0.33	–	1.5
Fusarium brachygibbosum	S7	–	–	–	3.66
Fusarium sp	R1	0.33	0.3	0.1	3.33
Sarocladiumstrictum	R8	0.8	0.5	0.83	3
Trichoderma gamsii	S2	–	–	–	0.75
Trichoderma sp.	G15	0.5	–	–	–
Tritirachium sp	B2	–	–	1	2.5
Gibellulopsisnigrescens	C3	0.83	0.37	–	1.4
Beauveriabassiana	R13	–	0.2	0.36	2.5
Lecanicillium sp.	R29	–	–	0.41	1.66
Purpureocilliumlilacinum	B1	–	–	0.2	1.6
Myrothecium verrucaria	R5	–	–	0.2	0.71
Clonostachysrosea	S3	–	0.3	0.06	0.8
Chaetomium sp	H1	–	–	–	4
Chaetomium sp	H38	–	–	2	1.66
Chaetomium sp	H42	–	–	0.5	3.5
Microascusmanginii	B5	–	–	0.3	1.5
Alternaria sp.	G5	–	–	–	1
Pleospora sp.	H43	–	–	–	1.8
Curvulariaspicifera	R20	–	–	–	0.46
Cladosporium ramotenellum	R36	1.2	–	1.4	1.66
Ustilagocynodontis	H14	5	–	4	4.66
Wallemia sp	H15	5	–	–	–

(lipase, amylase, protease, and cellulase) by the strains was detected on solid medium containing the specific substrate for each enzyme. The enzymatic index of strains is represented in Table 4.

All strains secrete at least one enzyme. The production of cellulase was observed in all strains except *Gymnascella denkaliensis*. The strains that have the highest cellulase activity were the two strains of *G. halophilus* H19 and H20 with an EI of 5 and 6 respectively, *P. vinaceum, Aspergillus sp.* strain A4, *Chaetomium sp.* strain H1 and *Ustilago cynodontis* with an EI between 4 and 4.66. A lower cellulase production was observed in *Fusarium* species, *Chrysosporium sp.*, *Arachnomyces sp.*, *S. strictum*, *A. calidoustus*, *Aspergillus sp.* strain E2 and *Chaetomium sp.* strain H42 with an EI around 3.

About 46.15% of the strains produce lipase enzyme. The highest lipolytic activity was observed in *P. vinaceum, U. cynodontis* and *Wallemia sp.* with an EI of 5.

67.30% of the strains secrete the enzyme protease and 48.07% the enzyme amylase. The two strains of *G. halophilus* and *U. cynodontis* have the highest proteolytic activity with an IE of 4. All amylase-producing strains showed a low activity, the higher production was by *G. halophilus* strain H19 with an EI of 2.

The production of lipase, protease, and amylase enzymes was not detected in 10 strains. However, a negative result does not confirm the inability of a strain to produce the enzyme. This may mean that the medium is inadequate for the detection of the enzyme or that the enzyme has not been released

from the mycelium or the enzyme has been secreted into the medium but the mycelium predominated and covered the visualization area of the enzymatic activity.

4. Discussion

The sebkha of Oran is the greatest sebkha in western Algeria with a superficies of 1890 km^2. Our study on fungal diversity was conducted at only 2 locations because of the difficulty of accessing the sebkha. Zone1 (Figure 1: sites B, C, D, R, G, S) is characterized by halophilic vegetation and cereal crops and zone2 (Figure 1: sites A, E, H) is characterized by a total absence of vegetation. Sampling was done in an area of 5 km^2 representing only 0.26% of the total area of the sebkha. According to our results, 44 species (30 identified species and 14 unidentified species) belonging to 24 genera were isolated from both zones. To the best of our knowledge, this study is the first report on the mycoflora of the Great Sebkha of Oran.

The most dominant genera found in the sebkha were *Penicillium*, *Aspergillus*, their teleomorphic forms and *Fusarium*. Several species of the genera *Aspergillus* and *Penicillium* have already been reported as the most frequently isolated species in hypersaline environments [16,17,19,46,47]. *P. egyptiacum* (formerly *Eupenicillium egyptiacum*) found among the dominant species in zone2 was also isolated from the soil of the western shore of the Dead Sea [10,12]. *P. longicatenatum*, a second teleomorphic form of the genus *Penicillium* isolated in our study, has not been previously isolated from saline soils. This species was described by Visagie et al. [48]; but these authors only described the asexual form, so this is the first observation of the sexual reproduction for this species. A third teleomorphic form was represented by an unidentified species *Penicillium sp.* Strains R32 and R33. In the blast analysis, these strains appear quite close to *P. egyptiacum* but other studies will be necessary to identify them in a definite way. The species *P. allii* previously found in hypersaline environments [49,50] was also isolated from garlic as a phytopathogenic fungus [51–53]. Although Algeria is a garlic producing country, *P. allii* has never been isolated or reported as a pathogen of garlic in Algeria. Moreover, this is the first isolation of this species in Algeria.

The genus *Fusarium* isolated only from zone1 was represented by the species *F. oxysporum, F. equiseti, F. brachygibbosum, F. acuminatum* and the unidentified species *Fusarium sp.* strain R1 belonging to dimerum clade. The two species *F.oxysporum* and *F. equiseti* were previously found in several hypersaline soils [9,14,47,54]. In the studies of Mandeel [18] on the biodiversity of the genus *Fusarium* in the saline soil of Bahrain and the one of Macià-Vicente et al. [55] on fungal root endophytes from Mediterranean environments, both species *F. oxysporum* and *F. solani* were found to be the most dominant and the frequency of isolation of *F. equiseti* was lower. These results do not agree with our result where the species *F. equiseti* was found to be the most dominant and where none *F. solani* has been isolated. The species *F. acuminatum* and *Fusarium* spp of dimerum clade were rarely isolated from hypersaline soil [54,56]. *F. brachygibbosum* has been isolated from saline soils such as salt marshes [57] and native desert flora of Oman [58]. This species has also been reported as a pathogen of palm trees in Oman [59] and olive trees in Tunisia [60]. In Algeria, this species has never been reported and this is the first strain of *F. brachygibbosum* isolated in Algeria. As Algeria is the 7th world producer of olive this species should be watched carefully.

Two strains of *Trichoderma*, *T. gamsii* and *Trichoderma sp.* were isolated from cereal crops soil where salinity is lower and the pH is neutral. The two strains are halotolerant growing at a salinity of 5% NaCl. The species *T. gamsii*, generally isolated as endophytic fungi having a high ability to be used as a biological control agent and as plant growth promoting [61–63], was also isolated from marine sediments [64]. No studies have shown that this species has been isolated from terrestrial saline soils or its use as a biocontrol agent under salt stress. Several studies have been conducted on the search for halotolerant strains of *Trichoderma* spp. that could be used as biological control agents under salt stress because of the use of saline water for irrigation in agriculture in arid zone [31,65,66]. This is the first isolation of *T. gamsii* in Algeria. This opens the way for their possible use as biological control agents in agriculture irrigated by saline water.

Three obligate halophilic strains (H15, H19 and H20) were isolated from the zone2. The two strains, H19 and H20, were identified as *G. halophilus*, the new obligate halophilic species isolated for the first time from the sediments of the Chaka salt lake in China by Zhou et al. [25]. The strain isolated in China is more tolerant to the salt concentration with a maximum growth at 22.5% NaCl while our two strains have a maximum growth at a NaCl concentration of 17.5%. This is the second isolation of this species in the world.

The other halophilic strain (i.e., H15) belongs to the genus *Wallemia*. This basidiomycetous genus is a xerophilic food borne fungi repeatedly isolated from hypersaline environments. Until some years the genus *Wallemia* included three species *W. sebi*, *W. ichthyophaga* and *W. muriae* [21] but its classification was recently revised. According to Jančič et al.

[67], the species *W. sebi* presents a complex of four species: *W. sebi sensu stricto*, *W. mellicola, W. canadensis* and *W. tropicalis*. Moreover in 2016, Jančič et al. [68] added a new species to the genus: *W. hederae*, a phylogenetic sister of *W. ichthyophaga*, and recently, another new species was described: *W. peruviensis* that is closely related to *W. hederae* [69].

The two species *W. ichthyophaga* and *W. muriae* are obligate halophilic while the *W. sebi* species complex, *W. hederae* and *W. peruviensis* are halotolerants species. Despite the fact that our strain H15 is an obligate halophilic, it belongs to *W. sebi* species complex. Additional studies will be needed to determine the exact species of this strain.

U. cynodontis, another basidiomycetous fungus, was also isolated from zone2. This inflorescence smut fungus has never been reported from hypersaline environments, its presence in our isolation will require further studies.

The halotolerance test revealed that the majority of the strains isolated in our study are halotolerant as they can grow in the absence of NaCl and tolerate a salt concentration of 12.5% with a maximum growth at 17.5% for only four species, and one species, *Wallemia sp.*, can grow at 20% NaCl. Fungi usually isolated from hypersaline environments have different levels of salinity tolerance, ranging from the low halotolerant to the extremely halotolerant growing at 25% NaCl or more [7]. Despite the large number of sebkha in Algeria, no study has been published on the mycoflora of these ecosystems or their rate of halotolerance. *Wallemia sp*. and the two strains of *G. halophilus* are the only obligate halophilic strains isolated during our study. These three strains were isolated from site H of zone2 characterized by the absence of any vegetation. Relative to the other two sites in zone2 (A and E), site H is the furthest from zone1 (where there is a halophilic vegetation) and this is also the one where the higher salinity was measured.

A few obligate halophilic fungi have been previously reported from extreme saline environments as *Gymnascella marismortui* [11], two species of *Wallemia* (*W. muriae* and *W. ichthyophaga*) [21], four species of *Aspergillus* (*A. gracilis*, *A. penicillioides*, *A. restrictus*, *A. unguis*) [13,70,71] and the yeast *Sterigmatomyces halophilus* [70].

Halophile and halotolerant fungi have adopted several adaptation mechanisms to be able to live in hypersaline environments. To adapt, the fungal cell must first be able to detect the evolution of salt concentration in the environment; the main mechanism involved in this detection is the high-osmolarity glycerol (HOG) pathway. This pathway is also involved in the response to salinity and osmoadaptation of fungal cells [72]. Under salt stress, most fungi accumulate solutes such as polyols and free amino acids to increase their internal osmolality and allow water to enter their cells. Other strategies are used by fungi such as changes in ion transport or plasma membrane fluidity that play an important role in adaptation to high salt concentration [72,73]. Moreover, one of the results obtained in the molecular studies of salt tolerance of the obligate halophile *W. ichthyophaga* was the observation of a significant increase in salt-responsive genes coding for hydrophobins. Compared to other fungi, the main difference in the amino-acid compositions of hydrophobins from *W. ichthyophaga* is the high number of acidic amino acids [74]. This property, which is considered to be a characteristic of proteins exposed to high salinity, could multiply the fields of biotechnological application of hydrophobins especially under high salt concentration. To summarize, all salinity adaptation strategies adopted by fungi are of great importance and it will be worthy to study in more detail the adaptation mechanisms of our halophilic strains.

The biotechnological interest of the 50 strains was evaluated by testing their ability to produce extracellular enzymes (i.e., lipase, amylase, protease, and cellulase) on solid medium. In general, halophilic fungi are an important source of polyextremophilic metabolites. Their thermotolerant and halophilic properties allow them to be stable and applicable in wide range of pH and temperature of industrial process [75,76].

In our study, the most interesting species presenting the highest enzymatic index were *Aspergillus sp.* strain A4, *Chaetomium sp.* strain H1, *P. vinaceum*, *G. halophilus* and the two basidiomycetous *Wallemia sp.* and *U. cynodontis.*

The two unidentified species *Aspergillus sp.* strain A4 and *Chaetomium sp.* strain H1 are good producers of cellulase. Further studies will be required to confirm the identification of these species and their biotechnological interest. *Wallemia sp.* has a high lipase activity with an EI of 5 and no cellulolytic, amylolytic or proteolytic activity was detected. This result was also obtained by Jančič et al. [68] who studied the enzymatic profile of the four species of *Wallemia* (*W. sebi, W. ichthyophaga, W. muriae*and *W. hederae*). They found that *Wallemia* spp. secrete several enzymes including lipase and esterase but no cellulolytic, amylolytic, and proteolytic activities were observed. *P. vinaceum* secretes the four tested enzymes but have only a high lipase and cellulase activity with an EI of 5 and 4.33 respectively. *P. vinaceum* is studied the most as marine derived fungi and has rarely been isolated from the soil. Several studies indicated that this species is an important source of bioactive molecules [77,78]. The two strains of *G. halophilus* H19 and H20

secrete amylase (EI near of 2), protease (EI of 4), and cellulase (EI of 5 and 6). This species was isolated for the first time in 2016 [25] and no study was realized on the enzymatic profile of this species until now. Our study is the first enzymatic characterization of *G. halophilus* in which both strains H19 and H20 were found to have a high amylolytic, cellulolytic, and proteolytic activity. This species could prove to be an interesting source of enzymes. The yeast *U. cynodontis* secretes three enzymes cellulase, protease and lipase with an EI of 4.66, 4 and 5 respectively. Several studies conducted on yeasts from Ustilaginaceae family have shown that these yeasts are a promising source of molecules of industrial interest including enzymes [79–81], organic acids [82] and biosurfactants [83].

In conclusion, the results of our study are consistent with the studies already carried out on hypersaline environments and confirm once again that the fungal flora of these extreme environments is of remarkable diversity. A total of 24 genera and 30 identified species were isolated including four species isolated for the first time in Algeria: *F. brachygibbosum, P. allii T. gamsii, G. halophilus,* and a first isolation of the teleomorphic form of *P. longicatenatum.* We also have isolated 17 strains that have not been identified at the species level either by morphological study or in our molecular analysis. Further studies will be necessary to clarify their identification to the species level and to know if whether they are new species or not.

Our study also showed that six species *Aspergillus sp.*strain A4, *Chaetomium sp.* strain H1, *P. vinaceum*, *G. halophilus* and the two basidiomycetous *Wallemia sp.* and *U. cynodontis* have significant enzymatic activity requiring further studies to determine their biotechnological potential.

It must be pointed out that all these fungi were obtained from a region located at the margin of the sebkha representing only 0.26% of the total area of the Great Sebkha of Oran, which does not reflect the true fungal diversity preserved at the level of the central lake that has remained unscathed from any human activity and which should be very interesting to discover in our further studies.

Disclosure statement

The authors declare that they have no conflict of interest.

This article does not contain any studies with human participants or animals performed by any of the authors.

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