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Screening and isolation of halophilic bacteria producing extracellular hydrolytic enzymes from salterns of the protected ecosystem Khnifiss Lagoon (Morocco)

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ABSTRACT

Aims: The present study was aimed to determine the diversity of the halophilic and halotolerant bacteria with hydrolase activities from salterns of the protected ecosystem Khnifiss lagoon (Morocco).
Methodology and results: Bacteria were isolated from sediment samples collected at five hypersaline sites on three different growth media supplemented with various concentrations of NaCl. Thirty bacterial isolates were identified using the 16S rDNA sequencing method. Physiological characterization was examined on Columbia agar with different concentrations of NaCl and pH values and incubated at different temperatures. Enzymatic activities were tested in submerged cultures with appropriate substrates. A total of 246 halophilic bacterial isolates from the salines of the protected ecosystem of Khnifiss lagoon in the south of Morocco were able to produce different hydrolases (cellulases, amylases, DNases, lipases and proteases). Furthermore, the majority of the isolates were classified as moderately halophilic bacteria that grew optimally between 5% and 15% of NaCl, and they adapted to other extreme conditions than salt: some cases tolerated a pH range of 4.5 to 9.4, as well as a temperature range of 10 to 50 °C. The 16S rDNA sequencing and phylogenetic analysis results indicated that most of the bacteria isolated from the salt marshes studied belong to the genus *Bacillus*.
Conclusion, significance and impact of study: Phenotypic and phylogenetic characterization of halophilic and halotolerant bacteria isolated from the salines of the protected ecosystem of Khnifiss lagoon has shown great diversity. Screening of hydrolytic enzymes produced by these bacteria suggests that the salines of the Khnifiss lagoon are a rich source of technologically interesting bacteria.

Keywords: Halophilic bacteria, extracellular hydrolytic enzymes, screening, hypersaline environments, Khnifiss lagoon

INTRODUCTION

Hypersaline environments such as coastal lagoons, salterns and natural salt lakes are ecosystems where salinity is higher than seawater. These extreme conditions limit the survival of many organisms (Ma *et al.*, 2010; Poli *et al.*, 2017). To adapt to high salt concentrations, the microorganisms living in these environments have developed various biochemical strategies to keep cell structure and function (Ventosa *et al.*, 1998).

Moreover, bacteria isolated from these hypersaline environments have been classified into halophilic microorganisms and halotolerant microorganisms. Halophiles are microorganisms that need salt to grow, while halotolerant microorganisms can grow both in the presence and absence of salt. Depending on their halotolerance, halophiles are further divided into three groups: slight halophiles, able to grow optimally at 1-3% (w/v) NaCl; moderate halophiles at 3-15% (w/v) NaCl; and extreme halophiles at 15-30% (w/v) NaCl (Ventosa *et al.*, 1998; Ventosa, 2006; Maheshwari and Saraf, 2015). Furthermore, the halophilic and halotolerant bacteria investigated so far are of great interest for their ability to produce bioactive components with high biotechnological potential, especially extracellular hydrolytic enzymes. Halophilic enzymes such as nucleases, proteases, lipases and cellulases have potential opportunities in bioremediation, biosynthetic processes, food, textile and cosmetic industries (Dumorne *et al.*, 2017; Czech *et al.*, 2018).

In addition, there is increasing research on the biotechnological potential of halophilic bacteria enzymes

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because they are stable at low water activity, in the presence of salt and organic solvents at high temperatures and alkaline pH (Sánchez-Porro *et al.*, 2003; Gomes and Steiner, 2004; Lugani and Vemuluri, 2022). These characteristics enable them to catalyze reactions under difficult conditions characterizing many industrial processes (Moreno *et al.*, 2009). Despite all these advantages, until now, only a few halophilic enzymes have been utilized in biotechnological applications or industrial processes relative to the massive use of extremozymes from alkalophilic and thermophilic bacteria (Oren, 2010).

The Khnifiss lagoon is the most important wetland in the Moroccan desert. It was declared an internationally important wetland under the Ramsar Convention in 1980. It is located on the South Atlantic coast of Morocco, around 70 km east northeast of Tarfaya City. It extends upstream into a salt marsh called Sebkha Tazra. Moreover, the Khnifiss lagoon presents several unique characteristics of its geographical location. It is a biological reserve of world interest for desert flora and fauna and an important station for migratory birds (Bergier, 2009). The lagoon has potential attractions for eco-tourism and aquaculture, especially oyster and pectinid farming (Idrissi *et al.*, 2004). Therefore, several studies of animal and plant biodiversity have been carried out at Khnifiss lagoon; however, studies of microbial diversity are still limited. This lagoon is appropriate for such studies, especially for halophilic bacteria. Hence, the objective of this study was to determine the diversity of the halophilic and halotolerant bacteria with hydrolase activities.

MATERIALS AND METHODS

Isolation, purification and storage of isolates

Soil sample collection and physicochemical parameters measurement

During May and September 2017, samples of sediment were collected at five sites from salterns of the protected ecosystem Khnifiss lagoon located on the South Atlantic coast of Morocco (27°95'N 012°18'W) (Figure 1). Sediment samples were collected at 5 cm depth with a sterile stainless-steel spatula. Samples were then put in clean plastic containers, immediately transported into the laboratory in a cool box at 4 °C and cultured no later than 24 h after collection.

Temperature, salinity and dissolved oxygen were measured on-site using a multiparameter probe (Horiba, U-10, Kyoto, Japan), while pH was measured in the laboratory with a calibrated pH meter (PH50+; Labbox, France).

Isolation of halophilic bacteria strains

For the isolation of halophilic bacteria strains, 15 g of each sediment sample was blended with 15 mL of sterilized saline water with 5% NaCl and serially diluted

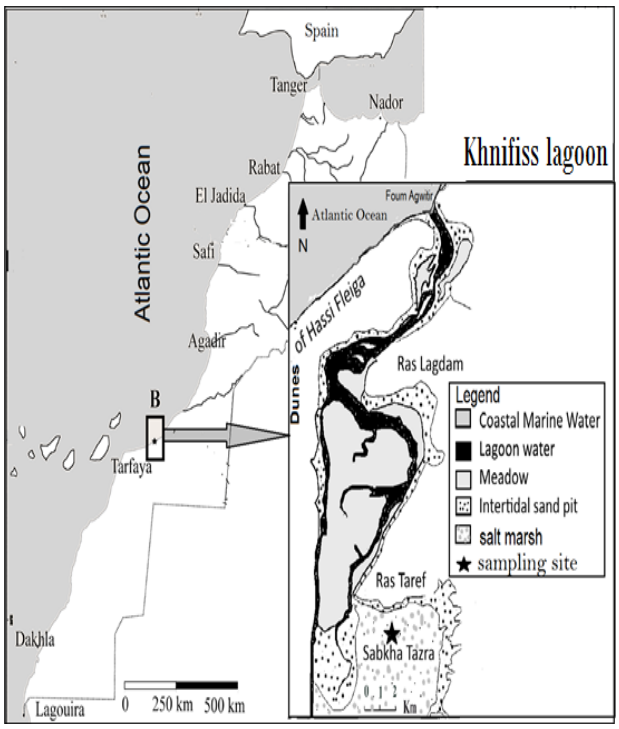

Figure 1: Location of sampling sites at Khnifiss lagoon.

from 10^{-1} to 10^{-4} in sterilized saline water (at 5% NaCl). Then, 0.1 mL of each dilution was spread on three different growth media, namely Columbia agar (Biokar Diagnostics, Beauvais, France), Tryptic soy agar (TSA; Difco, Detroit, USA) and Halophilic medium agar (HM agar) (Ventosa *et al.*, 1982), each with five different NaCl concentrations (2%, 5%, 10%, 15% and 20%) and incubated at 30 °C for 48 h under aerobic conditions. Bacterial counts were expressed as the number of colony-forming units per gram of soil, CFU/g. Based on the colony characteristics such as morphological features, pigmentation and size, distinct colonies were selected and successively subcultured on HM agar to ensure purity. The isolates were stored in 20% glycerol at -80 °C for further examination.

Influence of physicochemical conditions on the growth of isolated strains

To investigate the influence of physicochemical conditions on the growth of isolated strains, 246 strains were selected on the basis of their cell morphology and arrangement and the Gram stain reaction. Salt tolerance of isolates was examined on Columbia agar containing 5, 10, 15 and 20% of NaCl at 30 °C for up to 3 days. The effect of pH on bacterial growth was studied on Columbia agar with different pH (4.5, 7.5 and 9.2) adjusted by HCl-NaOH and incubated at 30 °C for up to 3 days. Growth at different temperatures was tested on Columbia agar after incubation at 10, 30 and 50 °C for 3 days. Growth was determined by visual observation.

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DNA extraction and 16S rRNA gene sequencing

Thirty strains were chosen for their potential to produce extracellular hydrolytic enzymes as well as their morphological, biochemical and physicochemical properties. Genomic DNA Mini Kit (Invitrogen, USA) was used to extract and purify the genomic DNA of these strains. PCR Supermix Kit (Invitrogen, USA) and universal primers FD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rP2 (5'-ACGGCTACCTTGTTACGACTT-3') were used to amplify the 16S rRNA gene (Weisburg *et al.*, 1991). The reaction mix was prepared in a final volume of 50 µL containing 1 µL of each primer, 3 µL of template DNA and 45 µL of PCR supermix.

The procedure of amplification was performed on MiniAmp thermocycler (Applied Biosystems, USA) under the following conditions: 94 °C for 2 min (initial denaturation), followed by 35 cycles of 94 °C for 15 sec (denaturation), 55 °C for 30 sec (annealing) and 72 °C for 1 min (extension); with final 3 min elongation cycle at 72 °C. PCR products were analyzed by electrophoresis on 1% agarose gels. Sanger sequencing was conducted at National Center for Scientific and Technical Research (CNRST, Morocco) using an ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems). Preliminary identifications were performed by submitting the FASTA sequences to the BLAST algorithm of the National Center for Biotechnology Information (NCBI) to search for homologous sequences in the GenBank database. The phylogenetic tree was constructed using the software MEGA 11 based on neighbor-joining (Kumar *et al.*, 2018). The 16S rRNA gene sequences of the isolated strains were deposited in the NCBI nucleotide database (accession numbers: OP647740-OP647769).

Determination of extracellular hydrolytic activities

Determination of extracellular hydrolytic activities was performed as follows:

Extracellular proteolytic activity

The proteolytic activity of halophilic strains was screened in skim milk agar containing 10% (w/v) skim milk, 2% (w/v) TSA agar and 5% (w/v) NaCl. The formation of a clear zone around the colonies after incubation at 30 °C for 3 days indicated hydrolysis of skim milk (Sánchez-Porro *et al.*, 2003).

Extracellular lipolytic activity

The lipase activity of the strains was detected using a TSA medium containing 1% Tween-80 and 5% of NaCl. The ability to create zones of hydrolysis around the growth after incubation at 30 °C for 2 days indicated the presence of lipolytic activity (Sierra, 1957).

Extracellular amylolytic activity

The presence of amylase activity on plates was determined following the method described by Cowan (1991), using TSA medium supplemented with 0.5% (w/v) soluble starch and 5% (w/v) NaCl. After incubation at 30 °C for 2 days, the plate was flooded with Lugol's iodine solution and a clear zone around colonies indicated starch hydrolysis.

Extracellular cellulolytic activity

To detect cellulase activity, the strains were cultured in TSA medium supplemented with 1% (w/v) soluble cellulose and 5% (w/v) NaCl. After incubation at 30 °C for 3 days, the plate was flooded with a 0, 1% (w/v) Congo red solution for 15 min, followed by rinsing with a 1 M NaCl solution for 10 min. The clear halos around the colonies showed cellulase activity (Sadfi-Zouaoui *et al.*, 2008).

Extracellular DNase activity

DNase activity of the isolate was determined using the DNase test agar medium, supplemented with 5% NaCl. After incubation at 30 °C for 7 days, the plate was flooded with 1 N HCl solution. The clear zone around the colonies indicated DNase activity (Jeffries *et al.*, 1957).

Statistics

The statistical analysis was performed using Microsoft Excel software version 2016 and XLSTAT software version 2020.1.1 (Addinsoft, New York, NY, USA).

RESULTS

Physicochemical parameters of the sampling sites

The results of the physicochemical parameters of the sampling sites (Table 1) showed that salinity ranged from 12.7% to 14.5% at the September sampling sites and from 14.9% to 15.2% at the May sampling sites. Temperatures ranged from 24.2 °C to 25.5 °C and from 25.7 °C to 26.1 °C at the September and May sampling sites, respectively. The measurement ranges are 6.25-7.12 for pH and 0.70-0.77 mg/L for dissolved oxygen.

Isolation of halophilic bacteria strains

The bacterial population of sediment samples

The bacterial colony count (Table 1) ranged from 5.1×10^4 to 1.2×10^6 CFU/g and from 1.5×10^4 to 1.6×10^5 CFU/g of sediment in the September sampling sites and May sampling sites, respectively. Furthermore, by increasing the salt content of the culture medium from 5 to 15%, the bacterial colony count was decreased. Compared to the medium containing 2% NaCl, the

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Table 1: Physicochemical parameters of the study sites and the bacterial population of sediment samples of the salterns of Khnifiss lagoon.

Sampling date	Sampling site	Salinity (%)	Temperature (°C)	pH	Dissolved oxygen (mg/L)	Bacterial count (CFU/g) NaCl (%) in HM agar medium			
						2	5	10	15
September 2017	Site 1	12.7	25.5	6.89	0.77	4.27×10^5	9×10^4	6.7×10^4	5.9×10^4
	Site 2	14.5	24.3	7.12	0.75	9.2×10^5	1.2×10^6	8.9×10^4	7.2×10^4
	Site 3	13.5	24.2	6.69	0.78	9.6×10^5	9.7×10^4	7.3×10^4	5.1×10^4
May 2017	Site 4	14.9	25.7	6.35	0.70	2.6×10^4	5.6×10^4	2.5×10^4	1.6×10^4
	Site 5	15.2	26.1	6.25	0.73	9.5×10^4	1.6×10^5	1.8×10^4	1.5×10^4

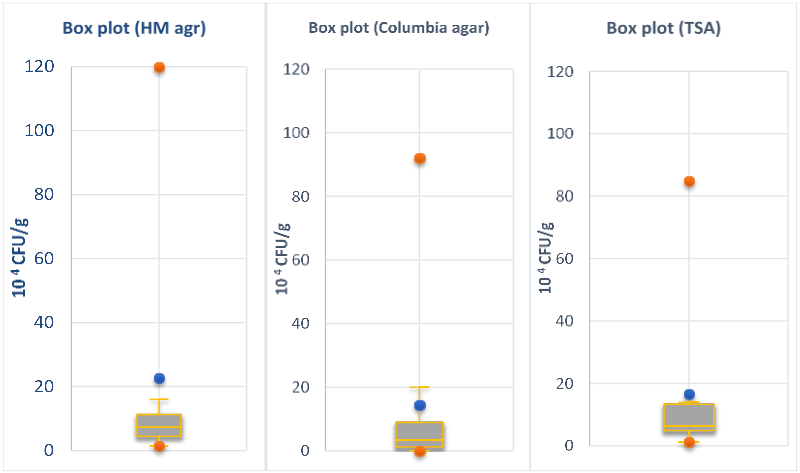

Figure 2: Box plot of bacterial load (CFU/g) by isolation medium with different concentrations of NaCl (2%, 5%, 10%, 15% and 20%).

bacterial colony count increased in the medium containing 5% NaCl at sites 2, 4 and 5, which are characterized by a salinity equal to or above 14.5%, while it decreased at sites 1 and 2, where salinity is less than or equal to 13.5%.

Comparison of the isolation medium for halophilic bacterial strains

According to the salt content (Figure 2), HM agar was characterized by CFU/g ranging from 1.5×10^4 to 120×10^4 with a mean of 22.5×10^4, a median of 7.25×10^4, an standard deviation equal to 35.96×10^4 and 50% of measured values comprised in the interval of 4.47×10^4 and 11.2×10^4; while Columbia agar was characterized by CFU/g ranging from 0.05×10^4 to 92×10^4 with a mean of 14.44×10^4, a median of 3.45×10^4 and a standard deviation equal to 26.36×10^4 and half of the measured values within a range of 1.25×10^4 and 8.92×10^4. Also, TSA was characterized by CFU/g from 1.2×10^4 to 85×10^4 with a mean of 16.47×10^4, a median of 6.3×10^4, an standard deviation equal to 24.2×10^4 and 50% of the measured values were in the range of 4.87×10^4 and 13.25×10^4.

Identification of bacterial strains

The identification of the strains according to their comparative partial 16S rDNA sequence analysis. Table 2 revealed the presence of 12 different species belonging to six genera, distributed as follows: *Bacillus spizizenii* (7 strains), *Bacillus vallismortis* (6), *Bacillus halotolerans* (3), *Cytobacillus oceanisediminis* (3), *Terribacillus saccharophilus* (3), *Bacillus subtilis* (2), *Bacillus inaquosorum* (1), *Bacillus licheniformis* (1), *Bacillus mojavensis* (1), *Oceanobacillus picturae* (1), *Priestia endophytica* (1) and *Virgibacillus salaries* (1). Similar results were also observed in the phylogenetic tree composed by the neighbor-joining method, as illustrated in Figure 3.

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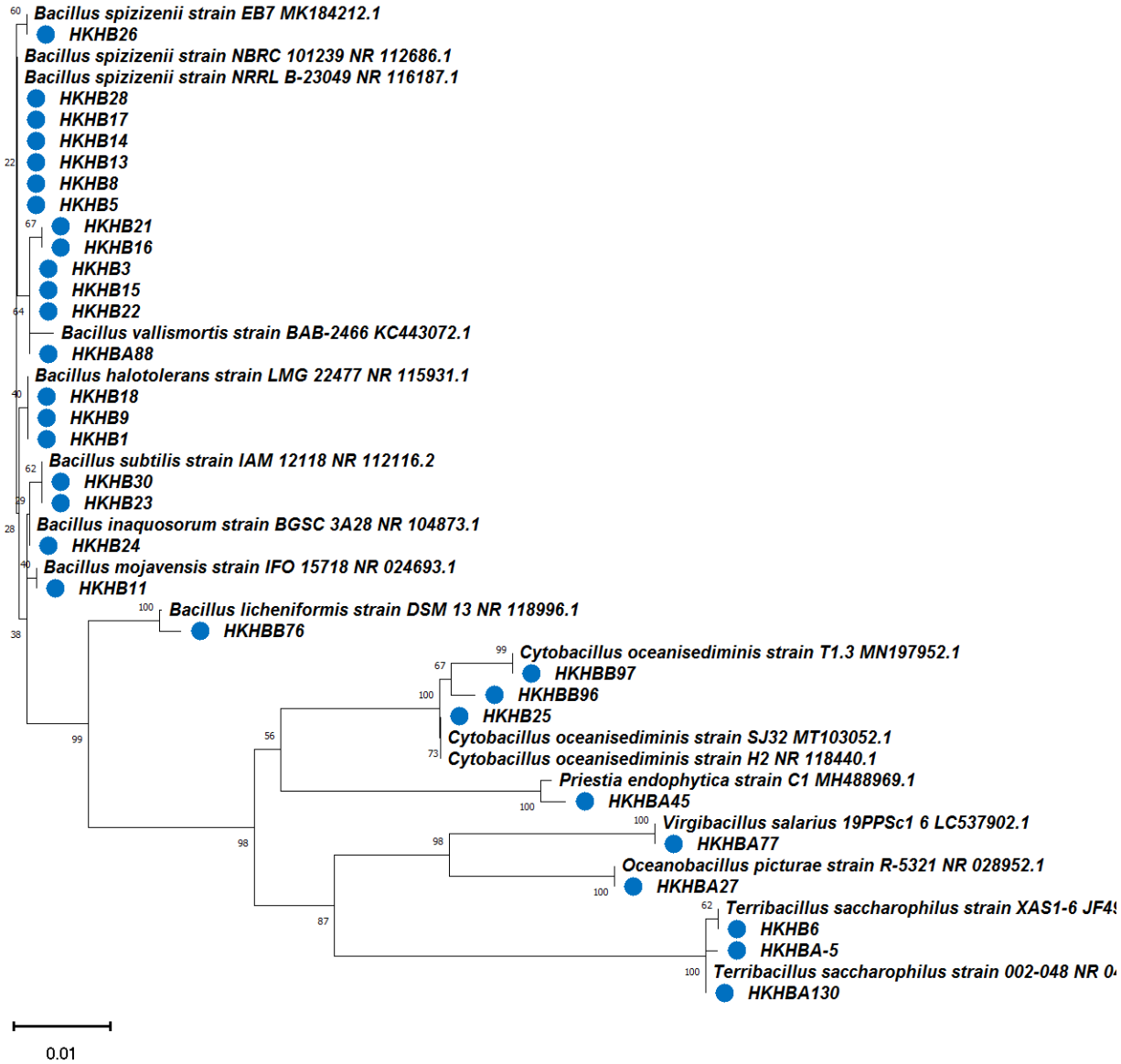

Figure 3: Unrooted neighbor-joining phylogenetic tree based on 16S rRNA gene sequences comparison of halophilic bacteria isolated from Khnifiss lagoon salterns. The blue circle indicates the 30 strains analyzed. Scale bar, 0.01 substitutions per nucleotide position.

Influence of physicochemical conditions on the growth of isolated strains

Influence of NaCl concentration

The percentage of bacterial growth in the presence of different NaCl concentrations is reported in Figure 4. All strains were able to grow on a medium containing 5% NaCl and 40% of these strains were able to grow on a medium containing 10% NaCl after 24 h of incubation. This percentage increases to 90% if incubation continues for 72 h. Thirty-two (32)% and 25% of strains were able to tolerate a concentration of 15% and 20% NaCl, respectively during 72 h of incubation. These results were not significantly changed when the incubation duration was reduced to 24 h.

Influence of temperature of incubation

The tolerance of the bacterial strains to incubation temperature is illustrated in Figure 5. All strains were able to grow at a temperature of 30 °C. While only 37% of the 264 strains were able to grow at a temperature of 10 °C within an incubation period of 48 h or more. Furthermore, 73% to 80% of the isolates could grow at a temperature of 50 °C, depending on the incubation period.

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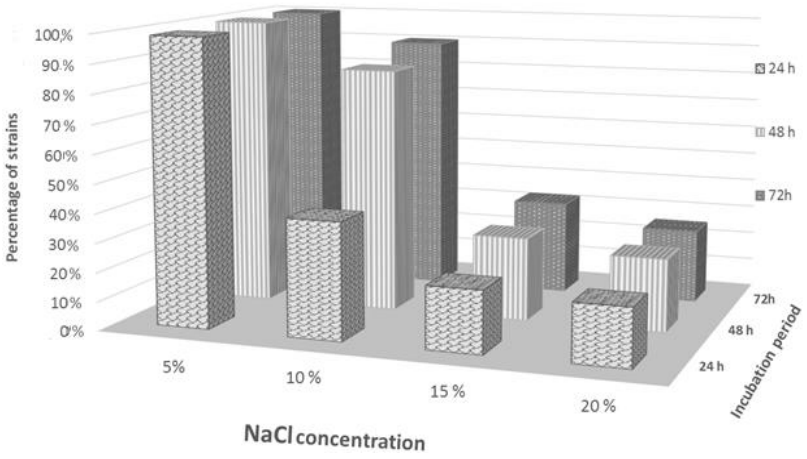

Figure 4: Tolerance of bacteria strains to different concentrations of NaCl at an incubation temperature of 30 °C during 24, 48 and 72 h.

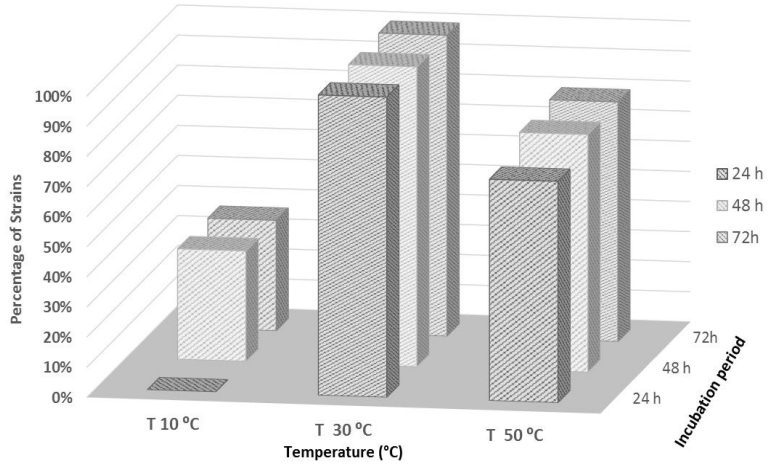

Figure 5: Tolerance of bacterial strains to incubation temperature during 24, 48 and 72 h.

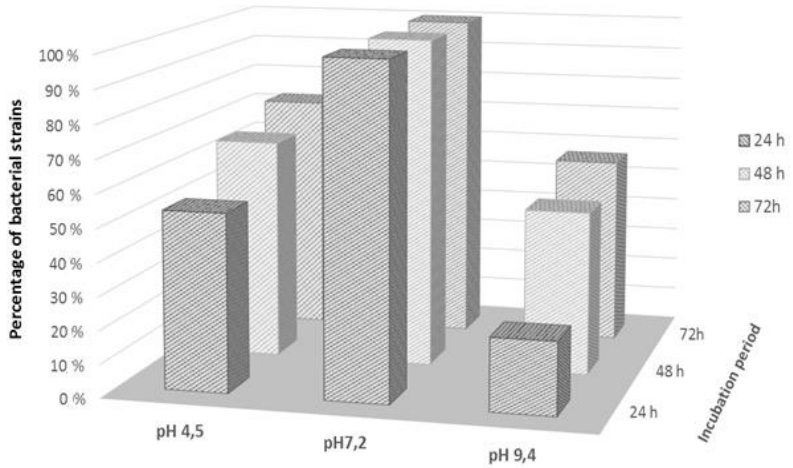

Figure 6: Tolerance of the bacterial strains to extreme pH at an incubation temperature of 30 °C for 24, 48 and 72 h.

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Table 2: Affiliation of the 16S rDNA sequences of isolated halophilic bacteria and their accession numbers.

Representative isolate	Tentative identification based on nearest neighbor	Similarity (%)	Sequence length (pb)	GenBank accession number
HKHB1	*Bacillus halotolerans*	100	1262	OP647740
HKHB18	*Bacillus halotolerans*	100	1461	OP647751
HKHB9	*Bacillus halotolerans*	100	1465	OP647745
HKHBB76	*Bacillus licheniformis*	99.66	1493	OP647766
HKHB11	*Bacillus mojavensis*	100	1395	OP647767
HKHB13	*Bacillus spizizenii*	100	1423	OP647746
HKHB14	*Bacillus spizizenii*	100	1420	OP647747
HKHB17	*Bacillus spizizenii*	100	1396	OP647750
HKHB26	*Bacillus spizizenii*	100	1436	OP647757
HKHB28	*Bacillus spizizenii*	100	1402	OP647758
HKHB5	*Bacillus spizizenii*	100	1403	OP647742
HKHB8	*Bacillus spizizenii*	100	1405	OP647744
HKHB23	*Bacillus subtilis*	100	1481	OP647754
HKHB30	*Bacillus subtilis*	100	1440	OP647759
HKHB24	*Bacillus inaquosorum*	100	1441	OP647755
HKHB15	*Bacillus vallismortis*	99.84	1466	OP647748
HKHB16	*Bacillus vallismortis*	100	1463	OP647749
HKHB21	*Bacillus vallismortis*	100	1480	OP647752
HKHB22	*Bacillus vallismortis*	100	1248	OP647753
HKHB3	*Bacillus vallismortis*	100	1449	OP647741
HKHBA88	*Bacillus vallismortis*	100	1320	OP647764
HKHBB96	*Cytobacillus oceanisediminis*	99.61	1244	OP647768
HKHBB97	*Cytobacillus oceanisediminis*	100	862	OP647769
HKHB25	*Cytobacillus oceanisediminis*	100	1426	OP647756
HKHBA27	*Oceanobacillus picturae*	99.79	1305	OP647761
HKHBA45	*Priestia endophytica*	99.77	1276	OP647762
HKHB6	*Terribacillus saccharophilus*	100	1395	OP647743
HKHBA130	*Terribacillus saccharophilus*	100	1477	OP647765
HKHBA-5	*Terribacillus saccharophilus*	99.88	1323	OP647760
HKHBA77	*Virgibacillus salaries*	100	1453	OP647763

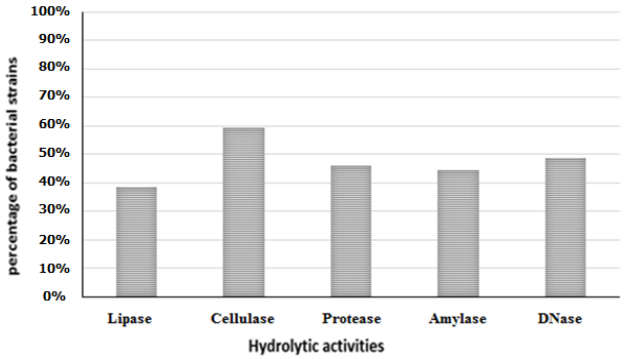

Figure 7: Hydrolytic activities of halophilic bacteria isolated from the salterns of Khnifiss lagoon.

Influence of pH

All isolates studied were tolerant of neutral pH, whereas depending on the incubation period of 24 to 72 h, only 22% to 56% were able to grow at alkaline pH and 28% to 46% tolerated acidic pH (Figure 6).

The results of the growth of the identified bacterial strains under different physicochemical conditions are shown in Table 3.

Extracellular hydrolytic activities

Extracellular hydrolytic activity percentage

In this study, five extracellular hydrolytic enzyme activities were screened for a total of 264 isolates. Ninety-three (93)% of strains were able to produce at least one of the hydrolytic enzymes, among which 39%, 59%, 46%, 44% and 49% of strains produced lipases, cellulases, proteases, amylases and DNases, respectively (Figure 7).

Combined hydrolytic enzyme activities

The results of combined hydrolytic enzyme activities show that 15 strains presented five hydrolytic activities, 29 strains were able to produce four hydrolytic activities, 60 strains had three hydrolytic activities, 60 strains showed two hydrolytic activities and 82 strains displayed one hydrolytic activity.

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Table 3: Growth of identified bacterial strains under different physicochemical conditions.

Species	Strains	NaCl (%)				pH			Temperature (°C)		
		5	10	15	20	4, 5	7, 2	9, 4	10	30	50
B. halotolerans	HKHB1	++	+	-	-	+++	++	+++	-	++	+++
	HKHB18	+++	++	+	-	+++	+++	-	-	+++	+++
	HKHB9	++	+	-	-	++	++	-	-	++	+++
B. licheniformis	HKHBB76	++	++	-	-	+	++	-	-	++	+++
B. mojavensis	HKHB11	+++	+	-	-	++	+++	-	-	+++	+++
B. spizizenii	HKHB13	++	+	-	-	+++	++	-	-	++	++
	HKHB14	+++	++	+	-	+++	+++	-	-	+++	+++
	HKHB17	++	+	-	-	+++	++	-	-	++	+++
	HKHB26	++	++	-	-	+++	++	-	-	++	++
	HKHB28	+++	+	-	-	+++	+++	+	-	+++	+++
	HKHB5	+++	+	-	-	+++	+++	+	-	+++	+++
	HKHB8	+++	++	-	-	+++	+++	-	-	+++	+++
B. subtilis	HKHB23	++	-	-	-	++	++	++	-	++	+++
	HKHB30	++	+	-	-	+	++	+	-	++	-
B. inaquosorum	HKHB24	+++	+	+	+	+	+++	-	+	+++	+++
B. vallismortis	HKHB15	++	+	-	-	++	++	-	-	++	++
	HKHB16	++	+	-	-	+++	++	-	-	++	++
	HKHB21	++	++	-	-	++	++	+	-	++	+++
	HKHB22	++	+	-	-	++	++	-	-	++	++
	HKHB3	+++	+	-	-	+++	+++	-	-	+++	++
	HKHBA88	+++	+	-	-	++	+++	-	-	+++	++
C. oceanisediminis	HKHBB96	++	-	-	-	-	++	+	+	++	-
	HKHBB97	+++	-	-	-	-	+++	+	+	+++	-
	HKHB25	+++	++	++	++	++	+++	-	-	+++	+++
O. picturae	HKHBA27	+	+	-	-	++	+	+	-	+	+++
P. endophytica	HKHBA45	-	+	-	-	-	-	-	-	-	+
T. saccharophilus	HKHB6	++	-	-	-	-	++	-	-	++	+
	HKHBA130	++	+	-	-	-	++	-	-	++	-
	HKHBA-5	+	+	-	-	-	+	+	-	+	+
V. salarius	HKHBA77	++	+	-	-	+++	++	++	-	++	++

Symbols: + growth; - no growth.

Extracellular enzyme profiles of the genetically identified bacteria

The results of the extracellular enzyme profiles of the genetically identified halophilic bacteria in this study are presented in Table 4.

In fact, *B. spizizenii* is the most representative species in this study with seven strains and has three strains producing five enzymes, three other strains producing all the enzymes studied except lipase and a single strain producing three enzymes: lipase, protease and DNase.

In addition, *B. vallismortis*, the second most abundant species with six strains, could produce all of the extracellular enzymes tested except for three strains unable to secrete cellulase.

Moreover, *B. halotolerans*, *C. oceanisediminis* and *T. saccharophilus* were represented by three strains, all able to produce at least one enzyme. Firstly, *B. halotolerans* strains produced all enzymes except for strains HKHB18 and HKHB9, which were unable to produce DNase and amylase, respectively. Secondly, two strains of *C. oceanisediminis* produced cellulase, amylase and DNase, while one strain was able to produce all enzymes except amylase. Lastly, two strains of *T. saccharophilus* were able to produce all enzymes except amylase, whereas one strain showed only lipase.

Furthermore, *B. subtilis* was presented by two strains, HKHB30, which produced all five enzymes studied, and HKHB23 which produced only lipase, cellulase and DNase.

Finally, the enzyme profiles of six species represented by a single strain were as follows: *B. mojavensis* was able to produce all the enzymes tested except amylase. On the other hand, only one enzyme, protease, lipase and amylase, respectively, has been produced by *B. licheniformis*, *O. picturae* and *B. inaquosorum*. Furthermore, *P. endophytica* was able to produce lipase and cellulase. Lastly, besides its ability to produce cellulase and amylase, *V. salaries* (Strain HKBA77) was the highest producer of lipase and DNase of all strains identified in this study.

DISCUSSION

Today, environmental sustainability is one of the main objectives of all manufacturing processes (Diaz-Tena *et al.*, 2013). So, new environmental laws for the protection of the environment are pushing industrial sectors to take

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Table 4: Hydrolytic activities of identified halophilic bacteria isolated from the salterns of Khnifiss lagoon.

Species	Strain number	Hydrolytic activity (Dh-Dc in mm)				
		Lipase	Cellulase	Protease	Amylase	Dnase
B. halotolerans	HKHB1	2	4	4	3	5
	HKHB18	4	10	4	6	0
	HKHB9	6	4	2	0	8
B. licheniformis	HKHBB76	0	0	2	0	0
B. mojavensis	HKHB11	8	8	4	0	10
B. spizizenii	HKHB13	4	10	2	5	1
	HKHB14	0	8	4	6	4
	HKHB17	0	8	2	4	1
	HKHB26	2	0	4	0	2
	HKHB28	0	4	8	3	1
	HKHB5	2	6	3	2	6
	HKHB8	2	2	8	10	4
B. subtilis	HKHB23	6	4	0	0	6
	HKHB30	2	8	3	4	2
B. inaquosorum	HKHB24	0	0	0	2	0
B. vallismortis	HKHB15	6	6	6	8	8
	HKHB16	2	6	10	10	8
	HKHB21	4	0	3	8	6
	HKHB22	6	0	6	6	6
	HKHB3	5	0	2	4	8
	HKHBA88	2	6	6	6	6
C. oceanisediminis	HKHBB96	0	4	0	4	10
	HKHBB97	0	6	0	4	10
	HKHB25	2	6	4	0	10
O. picturae	HKHBA27	0	0	0	0	6
P. endophytica	HKHBA45	1	6	0	0	0
T. saccharophilus	HKHB6	2	0	0	0	0
	HKHBA130	3	0	3	0	3
	HKHBA-5	5	8	1	0	1
V. salarius	HKHBA77	14	4	0	1	12

Note: After the incubation, the diameters of each halo (Dh) and each colony (Dc) were measured. (Dh-Dc) indicates the hydrolytic activity of each strain.

into account the importance of creating new technologies that are more eco-friendly (Díaz-Tena *et al.*, 2013). In this context, the current trend is to gradually replace chemical industrial molecules that pollute the environment with natural enzymes, particularly those extracted from extremophilic microorganisms (Adrio and Demain, 2014).

The enzymes of halophilic bacteria have recently gained interest due to their stable activity in high-salinity conditions. They are also characterized by the high use of acid residues, a low lysine frequency and an increased presence of amino acids that have slight hydrophobicity. These characteristics promote efficiency under the limiting water conditions that characterize saline environments (Britton *et al.*, 2006; DasSarma and DasSarma, 2015).

Morocco has a wide variety of hypersaline environments, including coastal lagoons (Nador, Oualidia, Moulay Bousselhem and Khnifiss lagoons). The importance of these environments as important genetic and biological resource treasures. In this regard, the Khnifiss lagoon was chosen in this work because it is an example of these unique ecosystems appropriate for the study of microbial diversity; moreover, no extensive study of this diversity has been carried out to date, particularly on halophilic bacteria with hydrolase activities.

Physicochemical analysis of the sampling sites and the isolation medium of the bacterial strains

Physicochemical analysis of the sampling sites

The results of the physicochemical parameters of the sampling sites show that the sediment salinity is relatively high, which is theoretically the condition for the presence of highly halophilic bacteria, but according to the results obtained, moderately halophilic bacteria are the most dominant of the strains isolated. Moreover, as sediment salinity increases, the bacterial population decreases, which is consistent with what was reported by Ventosa *et al.* (1998).

Temperatures measured at the sampling sites were moderate as a result of the proximity to the ocean, which explains the dominance of mesophilic strains in this study.

The dissolved oxygen in the sampling sites is relatively high, promoting aerobic bacteria proliferation. This can be explained by the fact that Khnifiss Bay is a

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protected ecosystem very far from the population and sources of pollution.

Bacterial population of sediment samples

The bacterial load varies according to the sampling site, the sampling date and the salt content of the isolation medium. The highest bacterial load (1.9×10^6 CFU/g of sediment) was observed at sampling site 2 on the HM agar isolation medium supplemented with 5% NaCl; this bacterial load is very close to those reported for hypersaline environments of similar salt concentrations in Morocco (Berrada *et al.*, 2012; Kaitouni *et al.*, 2020). The bacterial load decreased as the NaCl content of the isolation medium increased above 5%, while salinity was higher than 12.7% at the sampling sites. These results are contrary to what Forsyth and Kushner (1970) mentioned previously, that some bacterial cells are damaged when they are transferred from a lower to a higher salt concentration but not when they are transferred from a higher to a lower salt concentration. The explanation may be due to the different tolerance of the strains to in vivo and *in vitro* conditions, .i.e., several strains lost their ability to tolerate the original high salinity under the conditions of isolation, or they were originally collected as spores and proliferated when grown under low salinity conditions.

The isolation medium of the bacterial strains

Statistical analysis (Figure 2) of the isolation media with different concentrations of NaCl (2%, 5%, 10%, 15% and 20%) relative to bacterial load showed that primary isolation of halophiles was most successful with HM agar, followed by TSA and least successful with Columbia agar. This is because the HM agar medium is highly selective for moderate halophiles as a result of its content of low Mg^{2+} (Ventosa *et al.*, 1982). The high standard deviation of bacterial load (35.96×10^4 CFU/g) observed in the HM agar medium (in comparison with TSA and Columbia agar) may be explained by its suitability only for the isolation of halophilic bacteria at moderate salinity, which does not favor the selection of extremely halophilic bacteria (Ventosa *et al.*, 1982).

Identification of bacterial strains

Phylogenetic analysis based on 16S rDNA sequencing (Table 2) revealed that among the six genera obtained from saline sediments, the genus *Bacillus* is dominant, with 21 strains represented by seven species, which indicates that *Bacillus* is highly adapted to saline sediments. All other genera obtained are represented by at most three strains. Similarly, recent studies have proven that the genus *Bacillus* is very abundant in saline sediments (Ventosa *et al.*, 2008; Berrada *et al.*, 2012; Moreno *et al.*, 2012; Aanniz *et al.*, 2015; Kaitouni *et al.*, 2020). In fact, this could be explained by the ability of their spores to be transported and their considerable resistance and dormancy, which allow them to survive in harsh conditions for long periods (Ventosa *et al.*, 2008).

Furthermore, previous studies in Morocco, Algeria and Iran have reported the isolation of the same bacterial species identified in this study (Table 5): In Morocco, Aanniz *et al.* (2015) reported the dominance of *B. licheniformis* and also the presence of *B. subtilis* and *B. inaquosorum* in hot springs, salt marshes and desert soils. The study carried out in the same way by El berkaoui *et al.* (2022) in the salines of Sidi Moussa and Sidi El Abed allowed the identification of 15 strains belonging to eight species (*B. subtilis*, *B. spizizenii*, *V. salarius*, *B. mojavensis*, *B. licheniformis*, *B. halotolerans*, *C. oceanisediminis* and *B. aquimaris*). Similarly, Berrada *et al.* (2012) showed that *C. oceanisediminis* and *B. licheniformis* live in salt marshes in northern Morocco. In Algeria, Menasria *et al.* (2019) isolated 74 halophilic bacteria from the ecosystems of saline lakes "Sebkhas and Chotts," 18 of which are similar to the strains identified in the present study (*O. picturae* (5 strains), *P. endophytica* (4), *V. salarius* (3), *B. halotolerans* (2), *B. Spizizenii* (1), *C. oceanisediminis* (1), *B. inaquosorum* (1) and *B. vallismortis* (1). Similarly, a study conducted in the Debagh hot spring (Arab *et al.*, 2019) reported the presence of 16 *B. mojavensis*, 11 *B. licheniformis* and 3 *B. subtilis* among 41 bacterial strains identified. In Iran, Ghasemi *et al.* (2011) identified 13 moderately halophilic bacteria from Lake Maharla, 9 of which belong to the genus *Bacillus* and 5 of which are similar to the strains isolated in this study (*P. endophytica* (2), *B. subtilis* (2) and *B. vallismortis* (1)). Indeed, this similarity in the bacterial species isolated in these studies compared to our study is probably due to the similarity of some environmental conditions, such as the degree of salinity and temperature, which are controlled by the climate, especially since the climate of all these regions, except the Berrada *et al.* (2012) study, is semi-arid to desert.

This research was notable for the isolation of bacteria recently reclassified into new genera or species: *C. oceanisediminis* (Patel and Gupta, 2020) (formerly *Bacillus oceanisediminis*), *P. endophytica* (Gupta *et al.*, 2020) (formerly *Bacillus endophyticus*), *O. picturae* (Lee *et al.*, 2006) (formerly *Virgibacillus picturae*), *B. spizizenii* and *B. inaquosorum* (Dunlap *et al.*, 2020) (previously designed as subspecies of *B. subtilis*). Furthermore, several species have only recently been discovered: *C. oceanisediminis* was identified in 2010 (Zhang *et al.*, 2010), *V. salarius* in 2008 (Hua *et al.*, 2008), *T. saccharophilus* in 2007 (An *et al.*, 2007), *O. picturae* in 2003 (Heyrman *et al.*, 2003) and *P. endophytica* in 2002 (Reva *et al.*, 2002).

Influence of physicochemical conditions on the growth of isolated strains

Growth of the isolates under different physicochemical conditions showed the ability of strains to tolerate the salt range of 5-20%, with an optimum of less than 10% NaCl, indicating that they are moderately halophilic as classified

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Table 5: Previous studies that isolated the same species as the present study with hydrolase activity.

Isolation site	Number of identified strains	Species similar to the species isolated in this study (Number of strains)	Enzymatic activity	References
Natural and artificial hypersaline environments in the pre-Rif region (Morocco)	56	*B. subtilis* (6) *O. picturae* (1)	Amylase Protease Cellulase Pectinase Inulinase	Kaitouni *et al.* (2020)
Marsh and two salterns from Lower Loukkos (west of Morocco)	122	*C. oceanisediminis* (8) *B. licheniformis* (1)	Protease Cellulase Amylase DNase	Berrada *et al.* (2012)
Hot springs, salt marshes and desert soils (Morocco)	240	*B. licheniformis* (119) *B. spizizenii* (2) *B. inaquosurum* (6)	Amylase Protease Cellulase Lipase	Aanniz *et al.* (2015)
Salines of Sidi Moussa and Sidi El Abe (Morocco)	15	*V. salarius* (1) *C. oceanisediminis* (2) *B. subtilis* (4) *B. spizizenii* (1) *B. mojavensis* (1) *B. licheniformis* (3) *B. halotolerants* (2)	Protease Lipase Amylase DNase Cellulase	El berkaoui *et al.* (2022)
Ecosystems of saline lakes "Sebkhas and Chotts" (Algeria)	74	*O. picturae* (5) *P. endophytica* (4) *V. salarius* (3) *B. halotolerans* (2) *B. Spizizenii* (1) *C. oceanisediminis* (1) *B. inaquosorum* (1) *B. vallismortis* (1)	Amylase Cellulase Protease Nuclease Esterase Inulinase Gelatinase Pectinase Xylanase	Menasria *et al.* (2019)
Debagh hot spring (Algeria)	41	*B. mojavensis* (16) *B. subtilis* (3) *B. licheniformis* (11)	Not determined	Arab *et al.* (2019)
Maharla salt lake in Fars province (Iran)	13	*P.* endophytica (2) *B. subtilis* (2) *B.* vallismortis (1)	Lipase	Ghasemi *et al.* (2011)

conditions besides salt. Some cases have tolerated a wide pH range from 4.5 to 9.4 and a wide temperature range from 10 to 50 °C.

These characteristics give them great biotechnological potential in processes requiring difficult conditions.

Extracellular hydrolytic activities

The present study indicated that the most frequent hydrolytic activity detected was cellulase, followed by DNase, protease and amylase activities. Lipase was the least represented hydrolase.

In similar studies in Morocco, Berrada *et al.* (2012) investigated the diversity of halophilic bacteria, producing protease, lipase, amylase, DNase and cellulase in a marsh and two salterns a protected ecosystem of Lower Loukkos, concluding that lipase producers were also the least abundant isolates. Moreover, Boumhandi *et al.* (2018; 2020) studied the biodiversity of moderately halophilic bacteria producing extracellular hydrolytic enzymes in the south and central west of Morocco has also reported the most represented enzymatic activity in the south of Morocco was cellulase. Furthermore, these findings were consistent with those of Kaitouni *et al.* (2020), who studied the diversity of halophilic bacteria, producing cellulase, amylase, protease, pectinase and inulinase from both natural and artificial hypersaline regions in the pre-Rif area.

In contrast to the results of our studies, several researchers found that lipase was the most represented enzymatic activity among the halophilic bacteria studied, including Rohban *et al.* (2009), Baati *et al.* (2010), Rasooli *et al.* (2016) and Ruginescu *et al.* (2019). The first one studied the ability of halophilic bacteria to produce nine extracellular hydrolytic activities isolated from a hypersaline lake in Iran. The second one screened halophilic bacteria producing proteases, amylases and DNases in the salt mines of Sfax (Tunisia). The third one

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Table 6: The biotechnological potential of identified halophilic species.

Species	Bioactive components	Biotechnological potential	References
B. halotolerans	Enzymes	Bacterial biofertilizer	Jiménez-Gómez *et al.* (2020)
	L-asparaginase	Antioxidant and antitumor drug	El-Fakharany *et al.* (2020)
	Microbial biological control agents (MBCAs)	Bio-control of the management of plant-parasitic nematode	Xia *et al.* (2019)
	MBCAs	Bio-control of postharvest diseases of strawberries	Wang *et al.* (2021)
	Enzymes	Bioremediation of crude-oil-contaminated soil	Deng *et al.* (2020)
	Alkaline protease	Detergent formulations and in legume proteins hydrolysis and digestibility	Dorra *et al.* (2018)
B. licheniformis	Alkaline protease	Laundry detergents	Sellami-Kamoun *et al.* (2008)
	α-amylase	Pharmaceutical food sugar and paper industries	Ashraf *et al.* (2003)
	Lipase	Chemicals, detergents, food and pharmaceutical industries	Madan and Mishra (2014)
	DNase	Treatment and maintenance of tracheoesophageal speech valves	Shakir *et al.* (2012)
	Enzymes	Bioremediation of polycyclic aromatic hydrocarbons	Eskandari *et al.* (2017)
	Amylase and protease	Animal feed	Dumitru and Habeanu (2021)
	Antimicrobial metabolites	Pharmaceutical control of nosocomial and foodborne pathogens	Tsadila *et al.* (2021)
B. mojavensis	Alkaline protease	Detergent	Beg and Gupta (2003)
	Lipopeptide biosurfactants.	Food and pharmaceutical industries	Ayed *et al.* (2014)
	Biodemulsifier	Demulsification of oilfield emulsion	Hou *et al.* (2021)
	Alkaline serine-proteases	Laundry detergents	Haddar *et al.* (2009)
	Silver nanoparticles (AgNPs)	Pharmaceutical control of multidrug-resistant pathogens	Iqtedar *et al.* (2019)
	Enzymes	Bioremediation of polycyclic aromatic hydrocarbons	Eskandari *et al.* (2017)
	Lipopeptide antibiotics and cell-wall degrading enzymes (protease and cellulase)	Pharmaceutical control of *Candida albicans* (human fungal pathogens)	Youcef-Ali *et al.* (2014)
	α-amylase	Bread and baking industry	Hmidet *et al.* (2010)
	Cellulase	Anaerobic digestion of rice straw	Kumar *et al.* (2021)
B. spizizenii	α-amylase	Food fermentation	Soni *et al.* (2012)
	Siderophores, IAA, phosphate solubilization and hydrolytic enzymes (cellulase, glucanase and protease)	Improvement of growth, physiological and antioxidant parameters of tomato plants exposed to salt stress	Masmoudi *et al.* (2021)
	Biosurfactant	Recuperation of oil in a Limestone Petroleum Reservoir	Youssef *et al.* (2007)

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(Continued)

	Probiotic	Food industries	Lefevre *et al.* (2017)
	Compounds with antagonistic activity	Biocontrol of root rot diseases in alfalfa	Wen *et al.* (2015)
B. subtilis	Lipopeptide antibiotics and cell-wall degrading enzymes (protease and cellulase)	Pharmaceutical control of *Candida albicans* (human fungal pathogens)	Youcef-Ali *et al.* (2014)
	Protease and pectinase	The bio-scouring of cotton (textile)	Anab-Atulomah and Nwachukwu (2021)
	Halo-tolerant protease	Food, pharmaceutical, textile and detergent industries	Ali *et al.* (2016)
	Antimicrobial metabolites	Pharmaceutical control of nosocomial and foodborne pathogens	Tsadila *et al.* (2021)
	Extracellular amylase and cellulase	Tobacco fermentation	Dai *et al.* (2020)
B. inaquosorum	Extremely alkaline mannanase	The food and pharmaceutical industries	Regmi *et al.* (2017)
	Phospholipase A1	Degumming of oil	Gaganpreet *et al.* (2017)
	Pectinase	Improvement of pineapple leaf fiber quality	Vaithanomsat *et al.* (2021)
	Antimicrobial compounds	Treatment of hepatopancreatic necrosis disease (AHPND) in shrimp aquaculture caused by pathogen *Vibrio parahaemolyticus*	Avery *et al.* (2020)
B. vallismortis	Organic-solvent-thermostable alkalophilic cellulase	Generation of bioethanol	Gaur and Tiwari (2015)
	Thermosolvent stable xylanase	The detergent, food, pharmaceutical, leather and agricultural industries	Gaur *et al.* (2015)
	Laccase from the spores	Bioremediation of contaminated water by Malachite green residue (a dyestuff used in aquaculture)	Zhang *et al.* (2013)
	Biodemulsifier	Petrochemical industry	Hou *et al.* (2021)
	Thermotolerant amylase	Detergent	Suganthi *et al.* (2015)
	Extracellular polymeric substances (EPS)	Removal of heavy metal ions from wastewater	Ding *et al.* (2018)
C. oceanisediminis	Biosurfactant	Bioremediation of polycyclic aromatic hydrocarbon	Banerjee and Ghosh (2020)
	Thermo- and solvent-stable xylanase	Food industries	Boucherba *et al.* (2017)
	Halotolerant cellulase	Production of bioethanol using seawater	Indira and Jayabalan (2020)
O. picturae	Phosphatases and organic acids	Promotion of growth of mangrove vegetation in an arid environment deficient in phosphate	El-Tarabily and Youssef (2010)
P. endophytica	Polyhydroxyalkanoate	Bioplastic	Geethu *et al.* (2019)
	Azoreductase	Treatment of textile industry wastewaters (polluted by an azo dye)	Prasad and Rao (2011)

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(Continued)

	Protease inhibitor	Anticancer	Venkatachalam and Nadumane (2019)
	Silver nanoparticles (AgNPs)	Pharmaceutical control of *Candida albicans*, *Escherichia coli*, *Salmonella typhi* and *Staphylococcus aureus*	Gan *et al.* (2018)
	Compounds with antagonistic activity	Biocontrol of root rot diseases in alfalfa	Wen *et al.* (2015)
T. saccharophilus	Antimicrobial metabolites	Pharmaceutical control of nosocomial and foodborne pathogens	Tsadila *et al.* (2021)
	Lipases	Production of biofuel	Escobar-Nino *et al.* (2014)
	Rhizosphere associated bacteria	Promotion plant growth and reduce the incidence of plant stress	Salomon *et al.* (2016)
V. salarius	Exopolysaccharide (EPS)	Food, textile, cosmetics, bioenergy and petroleum industries	Gomaa and Yousef (2020)

examined the potential of halophilic strains to produce lipase, DNase, protease and amylase isolated from IncheBoroun Wetland. The last one showed the production of six hydrolases, including amylase, caseinase, gelatinase, lipase, pectinase, cellulase and inulinase by halophilic bacteria isolated in the Atacama Desert.

In addition, Sánchez-Porro *et al.* (2003) investigated the ability of halophilic strains isolated from solar salterns of Spain to produce five different extracellular hydrolases, including amylase, protease, lipase, DNase and pullulanase. As well, Dang *et al.* (2009) studied the diversity of culturable bacteria producing extracellular hydrolytic enzymes (amylases, proteases, lipases and DNases), isolated from deep-sea sediments of the Southern Okinawa Trough. Similarly, Moreno *et al.* (2009) determined the lipase, protease, amylase and nuclease production among extreme halophiles in hypersaline ecosystems in South Spain. However, in all these studies, the amylase producers were described as the most abundant isolates.

At the same time, Moreno *et al.* (2012) studied the potential of halophilic strains to produce various extracellular enzymes (protease, lipase, amylase, DNase, pullulanase and xylanase) in heavy-metal-contaminated soils from the Atacama Desert and they described DNase producers as the most abundant isolates.

In another context, the highest lipase, protease and DNase-producing strains in this study were *B. halotolerans* HKHB1, *B. vallismortis* HKHB16 and *V. salarius* HKHBA77, respectively. While, *B. halotolerans* HKHB18 and *B. spizenii* HKHB13 were the highest producers of cellulase. As well, *B. vallismortis* HKHB16 and *B. spizizenii* HKHB8 were the highest producers of amylase.

The biotechnological potential of identified halophilic species

This study showed the presence of higher activity of halophilic enzymes in all identified species isolated from the salterns of the Khnifiss lagoon, which could be a source of halophilic extremozymes. Several previous studies have highlighted the biotechnological potential of halophilic and halotolerant bacteria. Furthermore, all the species we have identified in this work have been reported to have biotechnological potential by the production of enzymes or other bioactive compounds that can be used in bioremediation, control of phytopathogenic diseases, and the food, detergent, textile and pharmaceutical industries (Table 6). These findings point to the need for further studies to evaluate the potential biotechnological of our strains and screen their potential to produce other bioactive compounds.

CONCLUSION

This study proved that the salines of the protected ecosystem of the Khnifiss lagoon (Morocco) presented a great diversity of halophilic and halotolerant

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bacteria with a dominance of the *Bacillus* genus, especially the species *B. spizizenii* and *B. vallismortis*. Moreover, isolated strains have shown their ability to adapt to extreme physicochemical conditions, as well as their capacity to produce extracellular hydrolysis enzymes (protease, lipase, amylase, DNase, cellulase). Therefore, investigations should be directed toward the optimization and characterization of the enzymes produced by isolated strains so explore their various industrial applications.

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