Isolation and characterization of bacteria producing hydrolase enzymes from two moroccan hypersaline environments

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Abstract. Halophilic bacteria are a group of microorganisms living in saline environments and in some cases need the salinity to survive. Furthermore, these bacteria species have the potential for interesting and promising applications. In fact, this is due not only to their ability to adapt to extreme physico-chemical conditions but also because many of them they produce interesting substances for the industry such as extracellular hydrolytic enzymes. The present study aims to isolate and characterize halophilic bacteria producing hydrolase enzymes from two saline sites in Sidi Moussa-Oualidia complex (Morocco) and to screen their potential to produce hydrolase enzymes. In this study, 15 halophilic bacteria were identified and analyzed for their ability to produce extracellular enzymes. The phylogenetic analysis based on 16S rDNA sequencing indicated that the 15 isolated strains belong to the genera *Bacillus*, *Oceanobacillus* and *Virgibacillus*. The study of enzymatic activity revealed that all isolates studied were capable of producing at least one extracellular hydrolytic enzyme of which 9 produced amylase, 6 cellulase, 13 DNase, 11 lipase, and 8 protease. In addition to their ability to produce extracellular hydrolytic enzymes, our isolates have demonstrated their potential adaptation to extreme Physico-chemical conditions. These properties could allow them to be used for many industrial applications.

1 Introduction

Halophilic and halotolerant bacteria are a group of microorganisms living in saline environments and in some cases need the salinity to survive. To maintain cell structure and function in high salt concentration, these bacteria have developed various biochemical strategies [1]. Indeed most halophilic and halotolerant bacteria studied to date are attracting interest for their capacity to produce compounds with high potential in industrial processes,

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such as extracellular hydrolytic enzymes[2, 1], including protease, amylase, lipase, cellulase, and DNase. These enzymes have very diverse potential uses in different biotechnological and industrial applications such as food, biomedical, pharmaceutical, detergents, cosmetic and textile industries[3-5]. Moreover, research on the biotechnological potential of halophilic bacterial enzymes is increasing because of their stable activity at a high salt concentration as well as at high temperature and alkaline pH. These properties would allow halophilic enzymes to be used in industrial processes requiring harsh conditions[6-8]. However, to date, very few halophilic enzymes have been used in biotechnological applications or industrial processes compared to enzymes from alkalophilic and thermophilic bacteria[9]. The Sidi Moussa-Oualidia complex is an important Moroccan wetland including several hypersaline environments: Sidi El Abed salines, Sidi M'barek salines, Sidi Moussa lagoon and salines, and Oualidia lagoon and salines. It is located on the Atlantic coast of Morocco, in the province of El Jadida. Accordingly, it was designated as a wetland of international importance under the Ramsar Convention in 2005. However, until now, no environmental study has focused on the phylogenetic diversity of halophilic bacteria in the Sidi Moussa-Oualidia complex. Therefore, the present study aimed to isolate and characterize the halophilic bacteria producing hydrolase enzymes from the Sidi El Abed and Sidi Moussa salines.

2 Materials and methods

2.1 Study area

Sediment samples were collected from two sites in the Sidi El Abed (33°02'N, 08°42'W) and Sidi Moussa (33°01'N, 08°43'W) salines located on the Atlantic coast of Morocco (Figure 1).

Figure 1: Geographical location of the Sidi El Abed and Sidi Moussa salines with the sampling sites (Google Earth image).

2.2 Isolation of bacterial strains

15 g of each sediment sample was collected and homogenized in 15 mL of sterilized saltwater with 5% NaCl and serially diluted up to 10^{-4} dilution. For the isolation of the halophilic bacteria, 0.1 ml of each serially diluted sample was plated on one of five different salt concentrations (2%, 5%, 10%, 15% and 20%) in the Columbia medium containing the following composition (g per L): polypeptones (17); pancreatic heart peptone (3); corn starch (1); sodium chloride (5); yeast extract (3); agar (13.5); pH 7.3 +/-

0.2 (Biokar Diagnostics, Beauvais, France) and incubated at 30°C for 48 h. 192 Distinct colonies were selected and subcultured several times on Columbia agar to obtain high purity. The isolates were stored in 20% of glycerol at -80°C for further analysis.

2.3 16S rDNA sequencing and phylogenetic analysis

Fifteen strains were randomly selected according to their phenotypic, biochemical and physicochemical characteristics and to their capacity to synthesize extracellular hydrolytic enzymes. The genomic DNA of these strains was extracted and purification by Genomic DNA Mini Kit (Invitrogen, USA) following the procedure suggested by the manufacturer. The 16S rRNA was amplified using PCR Supermix Kit (Invitrogen, USA) and the universal primers FD1 (AGAGTTTGATCCTGGCTCAG) and rP2(ACGGCTACCTTGTTACGACTT) synthesized by Bio Basic Inc[10]. The amplification procedure was performed according to the manufacturer's recommendations on the MiniAmp thermal cycler (Applied Biosystems, USA). Sanger sequencing was performed at National Center for Scientific and Technical Research (CNRST, Morocco). Preliminary identifications were realized based on sequence assembly and by query in the NCBI database. Phylogenetic analysis was performed using the software MEGA 7 [11]. The partial sequences of the 16S rDNA gene have been deposited in the NCBI database under the following accession numbers MZ317300-MZ317314.

2.4 Morphological and physiological characterization

The morphological characteristics of the isolates were determined by standard methods [12]. The salt tolerance of the bacterial strains was tested on Columbia agar with 5%, 10%, 15% and 20% NaCl and incubated at 30°C. The pH effect on isolates was examined by growing them on Columbia agar with a three pH value (4.5, 7.5 and 9.2) and incubating them at 30°C. Growth at different temperatures was evaluated on Columbia agar after incubation at 10, 30 and 50°C. the growth was measured by visual observation after incubation for 24 to 72 hours.

2.5 Determination of extracellular hydrolytic activities

Screening of strains for extracellular hydrolytic activities was performed as follows: Extracellular cellulase activity was revealed as described by Sadfi-Zouaoui et al [13]. DNase activity was tested according to Jeffries et al [14]. Amylolytic, lipolytic and proteolytic activities were assayed as described by Cowan [15], Sierra [16] and Sánchez-Porro et al [8] respectively. The halo diameter of hydrolytic activity (Hd) and colony diameter (Cd) of each strain were measured and the difference between Hd and Cd was calculated and considered as an estimate of hydrolytic activity (Figure 2).

Figure 2:Method used to measure hydrolytic activity.

3 Results and discussion

3.1 Identification of bacterial strains.

Comparative analysis of the 16S rRNA partial sequences of the strains isolated from the sediments of Sidi El Abed and Sidi Moussa salterns revealed the following distribution: *Bacillus subtilis* has four strains, *Bacillus licheniformis* has three strains, *Cytobacillus oceanisediminis*(The new name for *Bacillus oceanisediminis*[17])and *Bacillus halotolerans* each have two strains, and *Bacillus mojavensis*, *Bacillus aquimaris*,*Virgibacillus salarius* and *Bacillus spizizenii*(Previously considered a subspecies of *Bacillus subtilis* but was promoted to species status byDunlap et al. [18]) each have one strain (Table 1).The phylogenetic tree constructed byUPGMAMethod, presented in Figure 3, showed similar results.

This result reveals that *Bacillus* is the most dominant genus, with 12 strains representing 6 species; the other two genera, *Cytobacillus* and *Virgibacillus*, were represented by two and one species, respectively. Therefore,*Bacillus* is very adapted to saline sediments, as proven by this study.

Table 1: Affiliation of the 16S rRNA gene sequences and accession numbers of isolated halophilic bacteria.

Representative isolate	Tentative identification based on nearest neighbor	16S similarity (%)	GenBank accession number
HBFO11	<i>Virgibacillus salarius</i>	99.88%	MZ317305
HBSW27	<i>Cytobacillus oceanisediminis</i>	100%	MZ317311
HBFW7	<i>Cytobacillus oceanisediminis</i>	100%	MZ317310
HBFW2	<i>Bacillus subtilis</i>	100%	MZ317309
HBSO12	<i>Bacillus subtilis</i>	100%	MZ317302
HBFW4	<i>Bacillus subtilis</i>	100%	MZ317313
HBFW17	<i>Bacillus subtilis</i>	100%	MZ317308
HBSO7	<i>Bacillus spizizenii</i>	100%	MZ317301
HBSW4	<i>Bacillus mojavensis</i>	100%	MZ317307
HBSO2	<i>Bacillus licheniformis</i>	100%	MZ317300
HBSO20	<i>Bacillus licheniformis</i>	99.69%	MZ317304
HBFW20	<i>Bacillus licheniformis</i>	100%	MZ317314
HBSO19	<i>Bacillus halotolerans</i>	100%	MZ317303
HBSW29	<i>Bacillus halotolerans</i>	100%	MZ317312
HBFO12	<i>Bacillus aquimaris</i>	100%	MZ317306

Figure 3:Tree of phylogenetic based on16S rRNA gene sequences analysis from the halophilic bacteria isolated from Sidi El Abed and Sidi Moussa salterns. The red circle indicates the 15 strains analyzed. Bar, 0.01 substitutions per nucleotide position.

3.2 Effect of physico-chemical conditions on bacterial strain growth

The growth of bacterial strains under different physico-chemical conditions is reported in Table 2. All strains were able to grow optimally on medium with 5% NaCl,thirteen of them were able to grow on medium with 10% NaCl,only two strains belonging to *B. subtilis* were able to tolerate a concentration of 15% and no strain tolerate 20% NaCl. These results indicated that these strains are moderately halophilic as classified byKushner and Kamekura[19].

The results of the Influence of temperature of incubation showed that all strains were able to grow at 30°C and 50°C except three strains, that could not tolerate 50°C, and only two strains: *C.oceanisediminis* HBSW27 and *B.aquimaris* HBFO12 were able to grow at 10°C. Hence, the strains identified in this study are designated as mesophilic and, in some cases, thermotolerant. Moreover, all isolates studied were tolerant of neutral pH, with 8 strains designated as acid-tolerant and 8 strains also considered alkaline-tolerant [20]. The pH tolerance of *V.saliarius* HBFO11 and *B.subtilis* HBSO7 was quite wide (4.5 to 9.4).

The physico-chemical characterization of the isolated bacteria proved the great biotechnological potential of our strains, due to their ability to tolerate other extreme conditions than salt.

Table 2: Growth of halophilic bacteria identified at different physico-chemical conditions.

Species	Strains	[NaCl]				pH			Temperature		
		5%	10%	15%	20%	4.5	7.2	9.4	10 °C	30 °C	50 °C
<i>V.salarius</i>	HBFO11	++	+	-	-	+++	++	++	-	+++	++
<i>C. oceanisediminis</i>	HBSW27	++	+	-	-	-	++	-	-	++	+
	HBFW7	+++	-	-	-	-	+++	+	+	+++	-
<i>B.subtilis</i>	HBFW4	+++	++	-	-	++	++	-	-	++	++
	HBFW17	+++	++	-	-	+++	+++	-	-	+++	+++
	HBSO12	+++	++	-	-	++	+++	+	-	+++	-
	HBFW2	+++	++	+	-	+++	+++	-	-	+++	+++
<i>B.spizizeni</i>	HBSO7	++	++	+	-	+++	++	++	-	++	++
<i>B.mojavensis</i>	HBSW4	+++	+	-	-	-	+++	++	-	+++	+
<i>B.licheniformis</i>	HBSO20	++	++	-	-	-	++	++	-	++	+++
	HBSO2	+++	++	-	-	-	+++	++	-	+++	+++
	HBFW20	++	++	-	-	+	++	-	-	++	+++
<i>B.halotolerans</i>	HBSO19	+++	++	-	-	+	++	++	-	++	++
	HBSW29	++	-	-	-	-	++	-	-	++	+
<i>B.aquimaris</i>	HBFO12	++	++	-	-	-	++	-	+	++	-

Symbols: + growth; - no growth.

3.3 Hydrolytic activities of halophilic bacteria strains

The enzymatic activity results revealed that all identified strains were capable of producing at least one extracellular hydrolytic enzyme. Indeed, nine strains produce amylase, six strains produce cellulase, thirteen strains produce DNase, eleven strains produce lipase, and eight strains produce protease. The results of the combined hydrolytic enzyme activities show that seven strains (HBFO12, HBSO20, HBSW4, HBSO12, HBFW4, HBFW17, HBFO11) were able to produce four hydrolytic activities, four strains (HBSO2, HBFW2, HBFW7, HBSW27) showed three hydrolytic activities, three strains (HBSO19, HBFW20, HBSO7) displayed two hydrolytic activities and one strain (HBSW29) had one hydrolytic activity (Table 3).

The current study indicated that DNase was the most frequent activity, followed by lipase, amylase, protease and cellulase activities respectively. In a similar study, Moreno et al. [21]investigated the ability of halophilic bacteria to produce various extracellular hydrolytic enzymes (lipase, protease, DNase, amylase, pullulanase and xylanase) in the Atacama Desert, and also found that DNase producers were the most abundant isolates.In contrast, Kaitouni et al.[22] found that cellulase was the most represented enzyme activity among halophilic bacteria isolated from hypersaline environments of the pre-Rif region (Morocco). Indeed, the difference in bacterial diversity between the hypersaline environments of northern [22]and northwestern Morocco (current study) could be attributed to the fact that the two studies have only one species in common(*B. subtilis*).

Table 3: Hydrolytic activity of halophilic bacteria strains.

Species	Strain Number	Hydrolytic activity (mm)				
		Lipase	Cellulase	Protease	Amylase	Dnase
<i>V.salariaius</i>	HBFO11	1	8	0	4	2
<i>C.oceanisediminis</i>	HBSW27	0	4	0	1	14
	HBFW7	0	6	0	4	10
<i>B. subtilis</i>	HBFW4	3	0	5	4	3
	HBFW17	3	0	4	4	1
	HBSO12	0	8	8	8	4
	HBFW2	12	0	1	0	8
<i>B.spizizenii</i>	HBSO7	4	0	0	0	4
<i>B.mojavensis</i>	HBSW4	10	4	0	1	8
	HBSO20	6	12	0	4	2
<i>B.licheniformis</i>	HBSO2	3	0	1	0	4
	HBFW20	0	0	2	0	1
<i>B.halotolerans</i>	HBSO19	4	0	1	0	0
	HBSW29	2	0	0	0	0
<i>B.aquimaris</i>	HBFO12	1	0	6	2	12

Note: After incubation, the hydrolytic activity of each strain was measured as follows: Colony diameter - Halo diameter (mm).

The enzyme profiles for each species are presented in Table 3. All four strains belonging to *B.subtilis*were capable of producing protease and DNase, three were lipase and amylase producers, and only one showed cellulase activity. Furthermore, *B. subtilis* HBSO12 showed great biotechnological potential, due to its high capacity to produce all the enzymes studied except lipase, and it is also the most protease-producing strain. Moreover, several studies have already proven that *B. subtilis* is a source of extracellular protease [23-26]. In addition, *B. subtilis*has been previously reported as an excellent producer of different extracellular hydrolytic enzymes, including cellulase, amylase, lipase and protease, with satisfactory characteristics for many industrial applications such as industrial fermentation of sugars, soybeans and tobacco[27-30]. Indeed, the *B. subtilis* strains isolated in this study were able to produce all the extracellular enzymes already mentioned.

B. licheniformis, the second most represented species in this study, displayed low enzymatic activity, except for strain HBSO20 which showed the highest productivity of cellulase. This strain is a promising producer of other enzymes besides cellulase compared to previous studies [31, 32]. It also showed the ability to grow significantly at temperatures of 50°C and tolerance to alkaline pH.Accordingly, these characteristics give the strain HBSO20 the potential to produce thermostable and halo-alkaline enzymes for use in industries that require extreme conditions.

C.oceanisediminis,*B.aquimaris*, *B.halotolerans*, *B.mojavensis*, and *B.spizizenii* are among the species identified in this study and have already demonstrated technological interest in previous studies. Firstly,*C.oceanisediminis* the species that produced the most Dnase in this study has been previously identified as a source of these extracellular enzymes.[33, 21].secondly,the *B.aquimaris* species, which was shown to be capable of producing amylase, protease, Dnase, and lipase in this study, has previously demonstrated its biotechnological importance in several studies[34, 35]. Thirdly, both strains identified as *B.halotolerans*were capable of producing a lipase. Moreover, in a recent study, a lipase from this species was characterized and showed properties suitable for various industrial applications [36]. Fourthly, *B.mojavensis* HBSW4, the second most lipase-producing strain in this study, has also shown the ability to secrete Dnase, cellulase and amylase.Similarly,Moreno et al [21] had previously reported the potential of this species to

produce lipase and Dnase. Fifthly, *B.spizizenii* has shown the ability to produce only lipase and Dnase.On the other hand,a recent study reported the ability of this species to produce cellulase and protease, which has not been demonstrated by our strain [37].Lastly, HBFO11 identified as a member of *V.salarius* showed the ability to produce all hydrolases studied, except protease. As well, according to a similar study, this species was reported as a producer of cellulase and nuclease[38].

The species isolated in this study were previously isolated from several environmental extremes and showed high enzymatic potential as listed in table 4.

Table 4: Previous studies that isolated the same species as the present study with hydrolase activities

Isolation Site	Number of identified strains	Species similar to the species isolated in this study. (Number of strains)	Hydrolytic Activity	References
Debagh Hot Spring (Algeria)	41	<i>B.mojavensis</i> (16) <i>B.subtilis</i> (3) <i>B.licheniformis</i> (11)	Not determined	[39]
Mahala salt lake in Fars province (southern Iran)	13	<i>B.aquimaris</i> (1) <i>B.subtilis</i> (2)	Lipase	[40]
Maharloo hypersaline lake located in the south of Shiraz, Iran.	16	<i>B. subtilis</i> (3)	Protease	[23]
saline lakes ecosystems (Algeria)	74	<i>C.oceanisediminis</i> (1) <i>B.subtilis</i> (1) <i>B.spizizenii</i> (1) <i>B.halotolerans</i> , (3) <i>V.salarius</i> (4)	Amylase Cellulase Esterase Gelatinase Inulinase , Nuclease Pectinase Protease Xylanase .	[41]
Natural and artificial hypersaline environments in the pre-Rif region (northern Morocco)	56	<i>B. subtilis</i> (6)	Cellulase Amylase Protease Pectinase Inulinase	[22]
Marsh and two salterns from Lower Loukkos(west of Morocco)	122	<i>C.oceanisediminis</i> (8) <i>B.licheniformis</i> (1) <i>B.aquimaris</i> (32)	Protease Cellulase Amylase DNase	. [33]
Atacama Desert in northern Chile	25	<i>C.oceanisediminis</i> (1) <i>B.mojavensis</i> (1) <i>B.licheniformis</i> (1) <i>B.aquimaris</i> (1)	Protease Lipase Amylase DNase Pullulanase Xylanase	[21]
This study: The Sidi Moussa-Oualidia complex, Morocco	15	<i>V.salarius</i> (1) <i>C.oceanisediminis</i> (2) <i>B.subtilis</i> (4) <i>B.spizizenii</i> (1) <i>B.mojavensis</i> (1) <i>B.licheniformis</i> (3) <i>B.halotolerans</i> (2) <i>B. aquimaris</i> (1)	Protease Lipase Amylase DNase Cellulase	

4 Conclusion

The study of the biodiversity of halophilic bacteria producing extracellular enzymes from the salines of Sidi Moussa and Sidi El Abed allowed the identification of 15 strains belonging to eight species (*V. salarius*, *C. oceanisediminis* *B. subtilis*, *B. spizizenii*, *B. mojavensis*, *B. licheniformis* *B. halotolerans* and *B. aquimaris*) and showed the presence of higher hydrolase activity in all identified halophilic species. Furthermore, the growth of bacterial strains under different physicochemical conditions showed a great diversity within the same species. Therefore, these properties make our isolated strains potentially useful in industrial processes where extreme conditions would inhibit ordinary enzymes. Indeed, to explore the diversity of halophilic bacteria in Morocco, other research is currently underway to screen halophilic bacteria producing extracellular hydrolytic enzymes in other Moroccan hypersaline environments.

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