academicJournals

Vol. 8(4), pp. 398-405, 22 January, 2014
DOI: 10.5897/AJMR2013.5457
ISSN 1996-0808 ©2014 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Evaluation of the probiotic properties of *Bacillus* spp. strains isolated from Tunisian hypersaline environments

Abderrahmen Merghni[1#], Nadia Leban[2*#], Abir Behi[1] and Amina Bakhrouf[1]

[1]Laboratory of Analysis, Treatment and Valorization of Pollutants of the Environment and Products (LATVPEP). Faculty of Pharmacy, University of Monastir, 5000. Tunisia.
[2]Biochemistry and Molecular Biology Laboratory, Faculty of Pharmacy, Monastir, Tunisia.

Accepted 22 May, 2013

Bacillus **species are ubiquitous and diverse both in the terrestrial and marine ecosystems. In this study,** ***Bacillus*** **species were isolated from Tunisian hypersaline environments and their probiotic properties were studied. In total, 13 strains were identified as a** ***Bacillus*** **spp. Antagonism assay revealed an inhibitory effect of these strains against pathogenic bacteria. Cell surface hydrophobicity values ranged between 0.33 and 30%. The tested strains were able to produce extracellular enzymes such as amylase, lipase, caseinase, and lecithinase. Qualitative analysis of biofilm results show that seven strains were able to produce slime on Congo red agar. Furthermore, the investigated strains were fairly adhesive to glass and polystyrene with values ranging from 0.07 to 0.70 at 570 nm.**

Key words: *Bacillus* spp., hypersaline, antagonism, extracellular enzymes, adhesion.

INTRODUCTION

The domain of bacteria contains many types of halophilic and halotolerant microorganisms, spread over a large number of phylogenetic groups (Ventosa et al., 1998). Within the lineages of gram positive bacteria (Firmicutes), halophiles are found both within the aerobic branches (*Bacillus* and related organisms) and also within the anaerobic branches (Oren, 2002).

Due to their ability to form spores and withstand a range of variable environ-mental conditions, *Bacillus* spp. adapt easily to diverse habitats. *Bacillus* species can be found in marine environ-ment and are part of the microflora of several marine species (Hovda et al., 2007). Perfectly adapted to harsh conditions, halophilic bacteria have the ability to grow in environments with hight salt concentration. Their particu-larities are manifested by stable proteins and enzymes. Besides, marine bacteria are known to produce wide range of compounds, which have potential applications as bioactive compounds, probiotics and nutritional supple-ments (Watanabe et al., 1996). The term probiotic, means “for life”, originating from the Greek words “pro” and “bios”. Today probiotics are quite commonplace in health promoting “functional foods” for humans, as well as therapeutic, prophylactic and growth supplements in animal production and human health (Sullivan and Nord, 2002; Senok et al., 2005; Abdelkarim et al., 2012).

Bacillus spp. have been shown to possess adhesion abilities, produce bacteriocins (antimicrobial peptides)

***Corresponding author. E-mail: nadia.leban@cpbs.cnrs.fr; lebannadia@yahoo.fr. Tel.: +216 97 20 81 55/ +33 64 20 98 68 2.

[#]Authors contributed equally to this work.

Abbreviations: NBSW, Nutrient broth sea water; **TBE,** Tris-borate-ethylenediaminetetraacetic acid; **TSB,** trypticase soy broth; **PBS,** phosphate buffered saline; **TSA,** trypticase soy agar; **CSH,** cell surface hydrophobicity; **DLVO,** Derjaguin, Landau, Verwey, and Overbeek; **CRA,** Congo red agar.

Table 1. Geographic location of bacteria isolation sites.

Site	Sebkhat El Meleh	Sebkhat Moknine	Sahline saltworks	Sebkhat Sidi El Heni	Bkalta saltworks	Sfax saltworks	Sebkhat El Adhibet
Geographic coordinates	33°19‘N 10°55’E	35°35‘N 10°56’E	35°45‘N 10°42’E	35°35‘N 10°30’E	35°35‘N 11°10’E	34°43‘N 10°44’E	33°06‘N 11°23’E

N, North; E, East.

and provide immunostimulation (Cherif et al., 2001; Barbosa et al., 2005). Several research articles demonstrate the benefits of using *Bacillus* to improve shrimp growth performance, survival, immunity, and disease resistance in aquaculture (Farzanfar, 2006). Systematic studies of *Bacillus* have always focused on the terrestrial *Bacillus,* although marine Bacilli are noted for their ability to produce different antibiotics, glucanases and cyclic acylpeptides (Oguntoyinbo, 2007).

Besides their enzyme production, marine Bacilli are also well known for the reduction of toxic heavy metals. Extremely halotolerant *Bacillus* strains were isolated from hypersaline environments (Oren, 2002). Their discovery could be of great biotechnological potential because many *Bacillus* isolates produce industrially important compounds.

In this study, halophilic *Bacillus* strains were isolated from Tunisian hypersaline environments and their probiotic properties were evaluated, in the aim to find more effective and environmentally friendly treatments for aquatic sector.

MATERIALS AND METHODS

Samples collection and bacterial characterization

Water samples were recovered from seven Tunisians hypersalines environments (Table 1). The samples were collected in 1 L sterile plastic bottles. The samples were immediately stored in a refrigerator for further microbial analysis. Bacterial strains were isolated according to the following procedure: 1 ml from samples were enriched 24 h at 30°C in nutrient broth sea water (NBSW) (salinity 34 g l^{-1} and pH 7.99) then speared on nutrient agar plate and incubated at 30°C. Only Gram and catalase positive rods were selected for further characterization.

Molecular identification of *Bacillus* strains

Total DNA was extracted from all the isolated strains according to the scheme described by Sambrook et al. (1989) and stored at -20°C. The two primers were (B-KF 5’-TCACCAAGGCRACGATGCG-3’ and B-KR: 5’CGTATTCACCGCGGCATG 3’) (Xi-Yang et al., 2006). Amplification was carried out using a 50-µl reaction volume: 2 µl DNA, 0.2 mM of each dATP, dGTP, dCTP and dDTP, 1x buffer solution, 1.5 mM $MgCl_2$, 1 mM of each primer (B-K1/F and B-K1/R1) and 1 unit of Taq DNA polymerase (Promega, USA). Each PCR program was conducted using a denaturation step of 3 min at 94°C, followed by 25 cycles of 94°C for 30 s, 63°C for 30 s and 72°C for 2 min, with an extension step of 72°C for 10 min. PCR products were separated by 1.5% agarose gel electrophoresis in Tris-borate-EDTA buffer (TBE) at 100 V. The sizes of DNA fragments were estimated using a 100-bp DNA ladder (Promega).

Antimicrobial activity using well diffusion agar assay (WDAA)

Potential probiotic strains were tested for their antagonistic activity using the well diffusion agar assay (WDAA) (Vaseeharan and Ramasamy, 2003) against four pathogenic strains: *Vibrio. alginolyticus* ATCC17749, *Vibrio. parahaemolyticus* ATCC17802, *Aeromonas hydrophila* ATCC7966 and *Salmonella typhymurium* ATCC 17802. The pathogenic bacteria were grown overnight in 10 ml of nutrient broth and then cultured for 24 h on nutrient agar at 30°C.

The common colonies from pure culture were suspended in 10 ml of physiological medium and well mixed during 5 min. 1 ml was spread over the agar plates. Potential probiotic strains were cultured in 10 ml nutrient broth for 24 h, 100 µl of the supernatant were introduced into the wells of the Muller Hinton MH agar medium and incubated for a period of 24 h at 30°C. Antibacterial activity was defined as the diameter (mm) of the clear inhibitory zone formed around the well.

Cell surface hydrophobicity

Hydrophobicity was measured by the hexadecane partitioning method of Van Loosdrecht et al. (1987). Bacterial cells grown overnight in trypticase soy broth (TSB) were washed with phosphate-buffered saline (7 mM Na_2HPO_4, 3 mM NaH_2PO_4 and 130 mM NaCl at pH 7.4) and resuspended in 4 ml of PBS, and the absorbance (OD_{600}) was determined (Abs1). One milliliter of hexadecane was added to each cell suspension and equilibrated for 10 min. Each suspension was re-incubated at 37°C for 30 min. The aqueous layer was removed and aerated to remove all traces of hexadecane, and absorbance (OD_{600}) was measured against a hexadecane-extracted PBS blank (Abs2). The hydrophobicity index (% adhesion to the solvent) was expressed as the ratio of absorbance of the hexadecane-extracted sample to absorbance of the sample before extraction. Percentage of adhesion was expressed as:

% adhesion = (1 - Abs2/Abs1)×100.

Phenotypic characterization of slime-producing bacteria

Qualitative detection of biofilm formation by tested strains was studied by culturing the strains on Congo red agar (CRA) plates as described previously (Chaieb et al., 2007). *Bacillus* strains were inoculated onto the surface of CRA plates, made by mixing 0.8 g Congo red with 36 g saccharose (Sigma) in 1 L of brain heart infusion agar, and were incubated for 24 h at 30°C under aerobic conditions and followed overnight at room temperature. Slime-producing bacteria appeared as black colonies, whereas non-slime

Table 2. Antagonistic activity of *Bacillus* spp. isolated strains in mm.

Strain	Pathogen			
	V. alginolyticus **ATCC 17749**	***V. parahemolyticus*** **ATCC 17802**	***S. typhimurium*** **ATCC 1408**	***A. hydrophila*** **ATCC 7966**
H1	12.1±0.36	10.1±0.12	*	*
H3	16.0±0.20	10.1±0.42	*	*
H4	*	12.2±0.25	*	*
M2	*	*	*	*
M5	14.1±0.10	12.1±0.12	22.0±0.06	16.4±0.21
Sa2	11.7±0.58	10.1±0.06	*	*
Sa3	*	10.0±0.00	*	*
SH1	*	12.1±0.31	10.1±0.42	17.0±0.00
SH3	*	10.2±0.20	18.0±0.06	*
BK2	12.2±0.25	*	18.0±0.00	*
SF1	12.1±0.12	10.1±0.06	*	*
CA	*	*	10.1±0.12	12.1±0.12
BC45	16.1±0.42	12.1±0.12	20.1±0.23	15.1±0.31

±, Standard deviation (n= 3); *, no activity.

producers remained non pigmented (Chaieb et al., 2007).

Biofilm formation in glass test tubes

For the biofilm formation assay, each *Bacillus* strain, was cultured in Subwoofer wireless transmitter (SWT) broth containing (per liter): 5 g of Bacto-Tryptone (Difco), 3 g of yeast extract (Difco), 3 ml of glycerol, 700 ml of filtered seawater, and 300 ml of distilled water, at 37°C with shaking and then transferred to glass test tubes. The cells were incubated without shaking for 10 h at 37°C, then stained with 1% crystal violet solution to visualize cells attached to the test tube (Wolfe et al., 2004). Thereafter, the tubes were rinsed with sterile distilled water. Biofilms formed at the air-liquid interface were stained purple. All the strains were tested in triplicate.

Semi quantitative adherence assay

Biofilm production by *Bacillus* spp. strains was determined using a semi-quantitative adherence assay on 96-well tissue culture plates, as described previously (Chaieb et al., 2007). Strains were grown in TSB supplemented with 1% (w/v) NaCl. Following overnight incubation at 30°C, the optical density at 600 nm (OD_{600}) of the bacteria was measured. An overnight culture, grown in TSB 1% at 37°C, was diluted to 1:100 in TSB supplement with 2% (w/v) glucose. A total of 200 µl of cell suspensions was transferred in a U-bottomed 96-well microtiter plate (Nunc, Roskilde, Denmark). Each strain was tested in triplicate. Wells with sterile TSB alone were served as controls. The plates were incubated aerobically at 37°C for 24 h. The cultures were removed and the microtiter wells were washed twice with PBS to remove non-adherent cells and dried in an inverted position. Adherent bacteria were fixed with 95% ethanol and stained with 100 µl of 1% crystal violet (Merck, France) for 5 min. The excess stain was rinsed and poured off and the wells were washed three times with 300 µl of sterile distilled water. The water was then cleared and the microplates were air-dried. The optical density of each well was measured at 570 nm (OD_{570}) using an automated Multiskan reader (Gio. De Vita EC, Rome, Italy). Biofilm formation was interpreted as highly positive ($OD_{570} \geq 1$), low grade positive ($0.1 \leq OD_{570} < 1$), or negative ($OD_{570} < 0.1$) (Knobloch et al., 2001).

Characterization of extracellular enzymes

The presence of exoenzymes was determined using the API Zym System (Bio-Mérieux) composed of 19 enzymatic substrates. The activities of various other enzymes were determined following inoculation of cultures onto trypticase soy agar (TSA) to which the following substrates had been added: 0.2% [wt/vol] strach for amylase, 1% [wt/vol] skim milk for caseinase, 1% Tween 80 for lipase, 5% [vol/vol] egg yolk for phospholipase (lecithinase) (Ben Kahla-Nakbi et al., 2006).

RESULTS

Bacterial characterization

Based on the results of the amplification of *Bacillus* 16s RNA gene, the thirteen tested strains yielded a PCR product with an amplicon sized around 1114 bp and were identified as *Bacillus* spp. (data not shown).

Antagonism assay

Potential probiotic strains have an inhibitory effect against pathogenic *Vibrio* strains *V. parahaemolyticus* ATCC17802, *V. alginolyticus* ATCC17749, *A. hydrophila* ATCC7966 and *S. typhymurium* ATCC 17802 (Table 2). The inhibitory zones were about 10-22 mm in diameter.

Cell surface Hydrophobicity (%)

The results of microbial adhesion to solvent are

Table 3. Qualitative and semi quantitative estimation of *Bacillus* spp. biofilm formation.

Strain	Hydrophobicity (%)	Phenotypes on CRA	Slime production	Glass surface	Polystyrene surface	
					Mean OD_{570} ± SD	Adhesion rate
H1	0.33	Orange	NP	A	0.14±0.10	++
H3	17.33	Orange	NP	A	0.13±0.06	++
H4	5.67	Black	P	A	0.07±0.04	-
M2	1.33	Orange	NP	A	0.07±0.02	-
M5	30.00	Pinkish red	NP	A	0.11±0.02	++
Sa2	4.00	Black	P	A	0.08±0.01	-
Sa3	26.00	Pinkish red	NP	NA	0.09±0.01	-
SH1	3.00	Black	P	A	0.17±0.01	++
SH3	3.67	Black	P	NA	0.09±0.02	-
BK2	19.67	Black	P	A	0.07±0.01	-
SF1	8.00	Pinkish red	NP	A	0.11±0.01	++
CA	1.33	Pinkish red	NP	NA	0.08±0.02	-
BC45	2.67	Black	P	A	0.70±0.01	++

NP, Slime non-producer; P, slime producer; NA, none adhesive; A, adhesive; -, non biofilm forming OD570≤0.1; ++, medium biofilm forming (0.1 ≤ OD570 < 1).

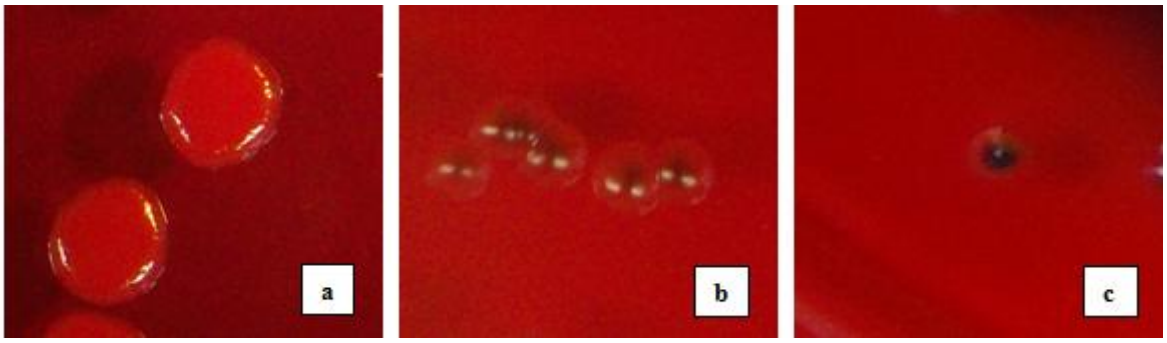

Figure 1. Morphotypes of *Bacillus* spp. strains based on the colorimetric scale obtained on Congo red agar: (a), orange colonies; (b), pinkish-red colonies with a darkening at the centre; (c), black colonies.

summarized in Table 3. Cells with hydrophobicity percentage greater than 70% as highly hydrophobic; from 70 to 30% as weakly hydrophobic and those with hydrophobicity lower than 30% as highly hydrophilic (Borges et al., 2008). We have found that the affinity to hexadecane was low suggesting a hydrophilic character for the majority of the studied strains. Only M5 strain was weakly hydrophobic.

Slime production

The ability of the tested strains to produce biofilm was assessed by culture on CRA plates. *Bacillus* strains (H1, H3 and M2) formed colonies with orange color. Other strains (M5, SA3, SF1 and CA) developed colonies with black center and red contour. H4, SA2, SH1, SH3, BK2 and BC strains were slime producing characterized by a black colonies (Figure 1).

Biofilm formation in glass surfaces

The majority of *Bacillus* spp. strains (10/13) were able to adhere to the glass giving a purple pellicule on the air-surface of the glass tube. We noted that both the intensity and the width of the pellicule differ from strain to strain. In fact, H3, M2 and SA2 strains are classified as faintly adherent with a very small purple pellicule. H1, H4 and SF1 strains showed a purple pellicule with medium width and were classified as moderately adherent. M5, SH1, BK2, and BC strains give a large purple pellicule and were classified as highly adherent. Other strains (SA3, SH3 and CA) were unable to adhere to the glass surface (Figure 2).

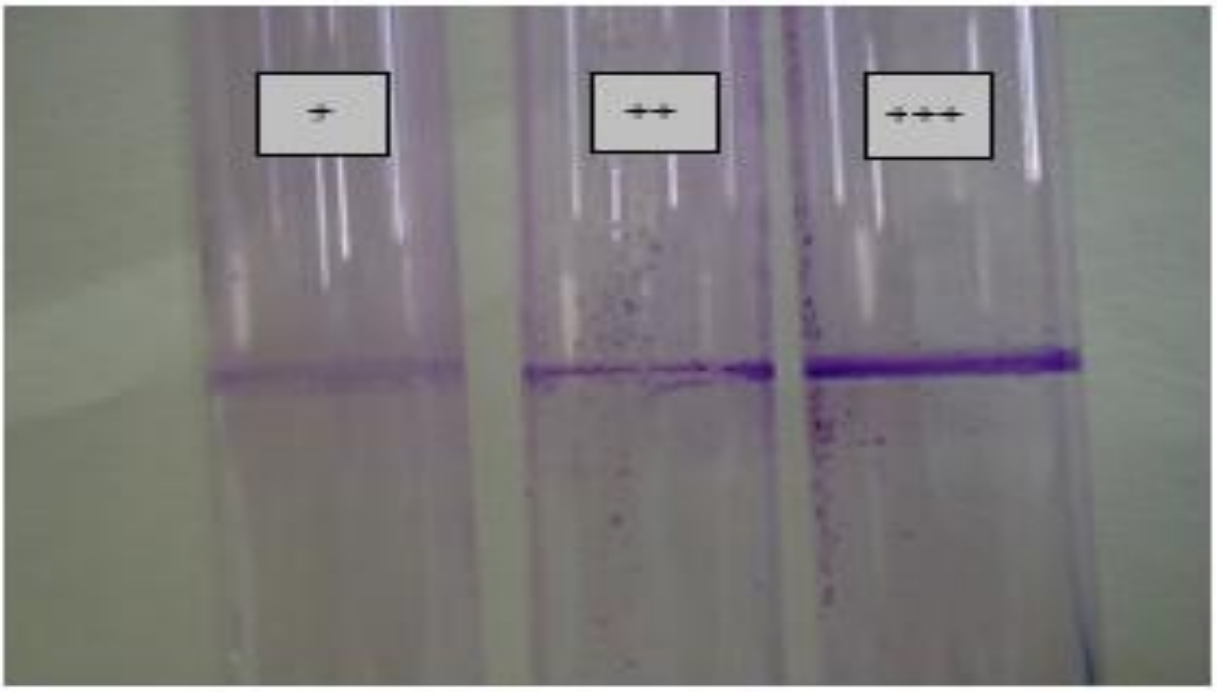

Figure 2. Adherence of *Bacillus* spp. strains on the surface of the glass tube. **+**, Slightly adherent; **++,** moderately adherent; **+++,** highly adherent.

Table 3. Qualitative and semi quantitative estimation of *Bacillus* spp. biofilm formation.

Strain	Hydrophobicity (%)	Phenotypes on CRA	Slime production	Glass surface	Polystyrene surface	
					Mean OD_{570} ± SD	Adhesion rate
H1	0.33	Orange	NP	A	0.14±0.10	++
H3	17.33	Orange	NP	A	0.13±0.06	++
H4	5.67	Black	P	A	0.07±0.04	-
M2	1.33	Orange	NP	A	0.07±0.02	-
M5	30.00	Pinkish red	NP	A	0.11±0.02	++
Sa2	4.00	Black	P	A	0.08±0.01	-
Sa3	26.00	Pinkish red	NP	NA	0.09±0.01	-
SH1	3.00	Black	P	A	0.17±0.01	++
SH3	3.67	Black	P	NA	0.09±0.02	-
BK2	19.67	Black	P	A	0.07±0.01	-
SF1	8.00	Pinkish red	NP	A	0.11±0.01	++
CA	1.33	Pinkish red	NP	NA	0.08±0.02	-
BC45	2.67	Black	P	A	0.70±0.01	++

NP, Slime non-producer; P, slime producer; NA, none adhesive; A, adhesive; -, non biofilm forming OD570≤0.1; ++, medium biofilm forming (0.1 ≤ OD570 < 1).

Biofilm formation in polystyrene surfaces

The results of the OD_{570} presented in the Table 3 showed that 46.1% of *Bacillus* spp. strains (6/13) were adhesive to polystyrene with values ranging from 0.11 to 0.70. The other strains were non- biofilm forming with an OD_{570} ≤1.

Enzymatic characterization

The tested *Bacillus* strains were able to produce exoenzymes such as amylase (92.3%) and lipase (84.6%). The caséinase was only produced by H1, M5, SA2, SA3 and SH1 strains. Excepting the H4 strains, all

Table 4. Extracellular enzymes products of *Bacillus* spp. strains.

Enzyme	H1	H3	H4	M2	M5	SA2	SA3	SH1	SH3	BK2	SF1	CA	BC45
Lipase	+	+	+	+	+	-	+	+	+	+	-	+	+
Caseinase	+	-	-	-	+	+	+	+	-	-	-	-	-
Lecitinase	-	-	+	-	-	-	-	-	-	-	-	-	-
Amylase	+	+	+	+	+	+	+	+	+	+	+	-	+
Phosphatase alcaline	*	*	*	*	+	*	*	+	*	*	*	*	+
Estérase(C4)	*	*	*	*	+	*	*	-	*	*	*	*	-
Estérase Lipase(C8)	*	*	*	*	+	*	*	+	*	*	*	*	-
Lipase(C14)	*	*	*	*	-	*	*	-	*	*	*	*	-
Leucine arylamidase	*	*	*	*	+	*	*	-	*	*	*	*	+
Valine arylamidase	*	*	*	*	-	*	*	+	*	*	*	*	-
Cystine arylamidase	*	*	*	*	+	*	*	-	*	*	*	*	+
Trypsine	*	*	*	*	+	*	*	-	*	*	*	*	-
α-chymotrypsine	*	*	*	*	-	*	*	-	*	*	*	*	-
Phosphatase acide	*	*	*	*	-	*	*	+	*	*	*	*	+
Naphtol-AS-BI-phosphohydrolase	*	*	*	*	+	*	*	+	*	*	*	*	+
α-galactosidase	*	*	*	*	-	*	*	-	*	*	*	*	-
β-galactosidase	*	*	*	*	-	*	*	+	*	*	*	*	-
β-glucuronidase	*	*	*	*	-	*	*	-	*	*	*	*	-
α-glucosidase	*	*	*	*	+	*	*	+	*	*	*	*	+
β-glucosidase	*	*	*	*	-	*	*	-	*	*	*	*	-
N-acétyl-β-glucosaminidase	*	*	*	*	-	*	*	-	*	*	*	*	-
α-mannosidase	*	*	*	*	-	*	*	-	*	*	*	*	-
α-fucisidase	*	*	*	*	-	*	*	-	*	*	*	*	-

+, Production; -, non production; *, non tested; N-AS-BI-P, naphtol-AS-BI-phosphohydrolase.

other tested bacteria were lecithinase negative (Table 4). Taking consideration of the previous results, only M5, SH1 and BC strains were selected for enzymatic characters studied on Api-ZYM system. Results showed that all investigated cells are able to assimilate the alkaline phosphatase, Naphtol-AS-BI-phosphohydrolase and α-glucosidase (Table 4).

DISCUSSION

In the present study, 13 bacteria strains have been isolated from different Tunisian hypersaline environments. The isolated strains were identified as *Bacillus* spp. according to Yang et al. (2006). In previous studies, eighty nine isolates were obtained from the sediments of four deep-sea, hypersaline anoxic brine lakes in the Eastern Mediterranean Sea and was dominated by representatives of the genus *Bacillus* (Terry et al., 2008). *Bacillus* spp. generally occur more frequently in sediments than in the water column, and *Bacillus* spores may account for up to 80% of the total heterotrophic flora (Hovda et al., 2007).

On diffusion agar assay only M5, SH1 and BC45 strains exhibited a zone of clearance against the tested pathogens. The inhibitory mechanism of the interaction was not characterized in this study. However, this result can be related to the ability of *Bacillus* to produce antibacterial compounds such as bacitracin, gramicidin S, polymyxin, and tyrothricin, which are active against a wide range of gram-positive and gram-negative bacteria (Ravi et al., 2007). On the other hand, other tested bacteria showed an antagonist effect but against all pathogens. It has been found that *Bacillus* species isolated from the soil are effective against Gram-positive and Gram-negative bacteria whereas their extensive inhibition effect is particularly against Gram-positive bacteria (Yilmaz et al., 2006).

Cell surface hydrophobicity (CSH) of the bacteria is one of the most important factors which govern the mechanism of bacterial adhesion to inanimate and biological surfaces. Furthermore, the surface charges and hydrophobicity of bacteria were influenced by the environmental condition (Vesterlund et al., 2005), explaining, in fact, the variation of bacteria capacity to adhere to substrates. All strains tested in this study grow on Congo red agar plates and gave after 18 to 24 h of incubation at 37°C three different morphotypes on the basis of the colour of the colonies obtained. The morphotype I is characterized by an orange colonies

(3/13 strains) morphotype II with pinkish-red colonies with a darkening at the centre (4/13 strains) and morphotype III which were considered as slime producers were characterized by black colonies (6/13 strains). Previous studies used this medium to study the phenotypic formation of biofilm for several bacteria including *Aeromonas* spp. and *Staphylococus* spp. (Saidi et al., 2011). On abiotic surfaces, the tested *Bacillus* strain were able to adhere to glass surface characterized by a purple pellicule on the air-surface of the glass tube, and most of them exhibit a high potential to adhere to polystyrene microplates with biofilms formation. This mechanism begins with the attachment of bacteria to abiotic surface, by means of pili, flagella or other materials, followed by the production of exopolysaccharides to form a glycocalyx (Wong et al., 2002). The DLVO (Derjaguin, Landau, Verwey, and Overbeek) theory of colloid stability has been used by several groups to try to explain attachment of micro-organisms to surfaces (Hermansson, 1999). Dan (2003) suggested that the DLVO approach to bacterial adhesion tended to treat bacterial cells as traditional colloidal particles, characterized by having an even surface and an evenly distributed surface charge. The problem remains that cells contain many complicated surface structures such as flagella, pili, fimbriae, glycoproteins, carbohydrates, teichoic acids and other biological materials composed of proteins in *Bacillus* species up to 9% of total cell proteins are associated with the cell wall. These complicated surface structures may exert their own localized cell surface charge at a microscopic cell surface level that could possibility mediate attachment through local electrostatic attraction despite the cell's having an overall electrostatic repulsion (Palmer et al., 2007). We noted that adhesion ability differ from strain to strain and from surface to another. In fact, there is no reliance between the slime producing ability on CRA plates and the adhesion power developed on polystyrene material. In fact, 4 strains characterized by orange colonies on Congo red agar (slime non producers), were adhesive to polystyrene microtiter plates.

Extracellular enzyme activity of the isolates revealed that the majority of the tested strains are able to produce amylase and lipase. Bal et al. (2009) found that all isolated marine *Bacillus* spp. possess extracellular enzymes amylase and protease. Some of other isolated *Bacilllus* spp. strains could able to produce caseinase. Previously studies reported that *Bacillus* bacteria secrete many exoenzymes, such as proteases, carbohydrolases and lipases, which are very efficient in breaking down a large variety of proteins, carbohydrates and lipids into smaller units (Ninawe and Selvin, 2009). The *Bacillus* strains (M5, H1 and BC45) analyzed in this study were heterogeneous on the basis of their exoenzymes profile tested on Api-ZYM system. However, α-glucosidase was produced by all tested stains. In fact, it is shown that members of the *Bacillus* genus are known to express a number of α-glucosidase exhibiting a diversity of substrate specificities (Sadler et al., 1984). As well, multiple research investigations were aimed on screening and purification of thermostable enzymes from *Bacillus* genus α-glucosidases from *Bacillus caldovelax* DSM 411, *Bacillus flavocaldarius* KP1228 (FERM-P9542), *Bacillus thermoamyloliquefaciens* KP1071 (FERM-P84776) and *Bacillus* sp. DG0303 have been well characterized (Ferrari et al., 1993; Kashiwabara et al., 2005). Similarly, alkaline phosphatase were produced by all tested stains. Thirty one strains of *Bacillus* spp. were isolated from soil sample, including 7 isolates of *Bacillus stearothermophilus* with one showing high production of alkaline phosphatase (Hamza and Hassan, 2005).

Conclusions

Halophilic bacterial communities isolated from Tunisian hypersaline environments have not been thoroughly studied. In this study, a great number of halophilic bacilli were isolated from distinct hypersaline environments of Tunisia in order to screen their antagonism effect against pathogens, adhesive ability and exoenzymes production capacities. The studied strains were identified as *Bacillus* spp. and were shown to have interesting properties, thus making them good candidates for biotechnological applications.

REFERENCES

Abdelkarim M, Maria Ángeles E, Zeineb H, Karima B, Fathi K, Amina B, Boubaker K (2012). Survival and retention of the probiotic properties of Bacillus sp strains under marine stress starvation conditions and their potential use as a probiotic in Artemia culture. Res. Vet. Sci. 93:1151-1159.

Bal S, Mishra RR, Rath B, Sahu HK, Thatoi HN (2009). Characterization and extracellular enzyme activity of predominant marine *Bacillus* spp. isolated from sea water of Orissa Coast, India. Malays. J. Microbiol. 2:87-93.

Barbosa TM, Serra CR, La Ragione RM, Woodward MJ, Henriques AO (2005). Screening for *Bacillus* isolatres in the broiler gastrointestinal tract. Appl. Environ. Microbiol. 71:968-978.

Ben Kahla-Nakbi A, Chaieb K, Besbes A, Zmantar T, Bakhrouf A (2006). Virulence and enterobacterial repetitive intergenic consensus PCR of *Vibrio alginolyticus* strains isolated from Tunisian cultured gilthead sea bream and sea bass outbreaks. Vet. Microbiol. 117:321-327.

Borges MT, Nascimento AG, Rocha UN, Tótola MR (2008). Nitrogen starvation affects bacterial adhesion to soil. Braz. J. Microbiol. 39:457-463.

Chaieb K, Chehab O, Zmantar T, Rouabhia M, Mahdouani K, Bakhrouf A (2007). In vitro effect of pH and ethanol on biofilm formation by clinicalica positive *Staphylococcus epidermidis* strains. Ann. Microbiol. 57:431-437.

Cherif A, Ouzari H, Daffonchio D, Cherif H, Ben Slama K, Hassen A, Jaoua S, Boudabous A (2001). Thuricin 7: a novel bacteriocin produced by *Bacillus thuringiensis* BMG1.7, a new strain isolated from soil. Lett. Appl. Microbiol. 32:243-247.

Dan N (2003). The effect of charge regulation on cell adhesion to substrates: salt-induced repulsion. Colloids Surf. B Biointerfaces 27:41-47.

Farzanfar A (2006). The use of probiotics in shrimp aquaculture. FEMS Immunol. Med. Microbiol. 48:149-58.

Ferrari E, Jarnagin AS, Schmidt BF (1993). Commercial Production of Extracellular Enzymes in *Bacillus subtilis* and Other Gram-Positive Bacteria. Biochemistry, Physiology, and Molecular Geneticis, Sonenshein AL, Hoch JA, Losick R (Eds.), Washington, DC: Am. Soc. Microbiol., ISBN 1-55581-053-5. pp 917-937.

Hamza HM, Hassan HG (2005). Local isolate of *Bacillus stearothermophilic* producing Alkaline phosphatase activity by two Fermentation systems. Natl. J. Chem. 17:143-150.

Hovda MB, Sivertsvik M, Lunestad BT, Lorentzen G, Rosnes JT (2007) Characterisation of the dominant bacterial population in modified atmosphere packaged farmed halibut (*Hippoglossus hippoglossus*) based on 16S rDNA-DGGE. Food Microbiol. 24:362-371.

Kashiwabara S, Azuma S, Tsuduki M, Suzuki Y (2000). The Primary Structure of the Subunit in *Bacillus thermoamyloliquefaciens* KP1071 Molecular Weight 540.000 Homohexameric α-Glucosidase II Belonging to the Glycosyl Hydrolase Family 31. Biosci. Biotechnol. Biochem. 64:1379-1393.

Knobloch JK, Bartscht K, Sabottke A, Rohde H, Feucht HH, Mack D (2001). Biofilm formation by *Staphylococcus epidermidis* depends on functional *Rsb*U, an activator of the *sig*B operon: differential activation mechanisms due to ethanol and salt stress. J. Bacteriol. 183:2624-2633.

Ninawe AS, Selvin J (2009). Probiotics in shrimp aquaculture: avenues and challenges. Crit. Rev. Microbiol. 35:43-66.

Oguntoyinbo FA (2007). Monitoring of marine *Bacillus* diversity among the bacteria community of sea water. Afr. J. Biotechnol. 6:163-166.

Oren A (2002). Diversity of halophilic microorganisms: Environments, phylogeny, physiology and applications. J. Ind. Microbiol. Biotechnol. 28:56-63.

Palmer J, Steve F, John B (2007). Bacterial cell attachment, the beginning of a biofilms. J. Ind. Microbiol. Biotechnol. 34:577-588.

Ravi AV, Musthafa KS, Jegathammbal G, Kathiresan K, Pandian SK (2007). Screening and evaluation of probiotics as a biocontrol agent against pathogenic *Vibrios* in marine aquaculture. Lett. Appl. Microbiol. 45:219-223.

Sadler DF, Ezzell JR, Keller KF, Doyle RJ (1984). Glycosidase activities of *Bacillus anthracis*. J. Clin. Microbiol. 19:594-598.

Saidi N, Snoussi M, Usai D, Zanetti S, Bakhrouf A (2011). Adhesive properties of *Aeromonas hydrophila* strains isolated from Tunisian aquatic biotopes. Afr. J. Microbiol. Res. 31:5644-5655.

Sambrook J, Fritsch EF, Maniatis T (1989). Molecular Cloning: A Laboratory Manual, 2nd edn, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. 1659 pp. ISBN 0-87969-309-6.

Senok AC, Ismaeel AY, Botta GA (2005). Probiotics: facts and myths. Clin. Microbiol. Infect. 12:958-966.

Sullivan A, Nord CE (2002). The place of probiotics in human intestinal infections. Int. J. Antimicrob. Agents 20:313-319.

Terry JM, Andrea MS, Boyd A, McK Henrik S, Jörg F, Kenneth NT (2008). Diversity of Bacillus-like organisms isolated from deep-sea hypersaline anoxic sediments. Saline Systems 4:8 doi:10.1186/1746-1448-4-8.

Van Loosdrecht MC, Lyklema J, Norde W, Schraa G, Zehnder AJ (1987). The role of bacterial cell wall hydrophobicity in adhesion. Appl. Environ. Microbiol. 53:1893-1897.

Vaseeharan B, Ramasamy P (2003). Control of pathogenic *Vibrio*. spp. by *Bacillus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*. Lett. Appl. Microbiol. 36:83-87.

Ventosa A, Nieto JJ, Oren A (1998). Biology of moderately halophilic aerobic bacteria. Microb. Mol. Biol. Rev. 62:504- 544.

Vesterlund S, Paltta J, Karp M, Ouwehand AC (2005). Adhesion of bacteria to resected human colonic tissue: quantitative analysis of bacterial adhesion and viability. Res. Microbiol. 156:238-244.

Watanabe K, Ishikawa C, Yozawa K, Kondo K, Kawaguch A (1996). Fatty acid and lipid composition of an licosapentaenoic acid-producing marine bacterium. J. Mar. Biotechnol. 4:104-112.

Wolfe AJ, Millikan DS, Campbell JM, Visick KL (2004). *Vibrio fischeri* σ^{54} controls motility, biofilm formation, luminescence, and colonization. Appl. Environ. Microbiol. 70:2520-252.

Wong HC, Chung YC, Yu JA (2002). Attachment and inactivation of *Vibrio parahaemolyticus* on stainless steel and glass surface. Food. Microbiol. 19:341-350.

Xi-Yang W, Mark JW, Michael H, James C (2006). Development of a group specific PCR combined with ARDRA for the identification of species of environmental significance. J. Microbiol. Methods. 64:107-119.

Yilmaz M, Soran H, Beyatli Y (2006). Antimicrobial activities of some *Bacillus* spp. strains isolated from the soil. Microbiol. Res.161:127-131.