Halotolerant *Bacillus* spp. strains isolated from the Great Sebkha of Oran (Algeria): a source of antimicrobial secondary metabolites

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Abstract

Bacillus species are Gram-positive bacteria found in abundance in nature, they could colonize different habitats even under extreme conditions and their secondary metabolites were found to possess various potential activities, notably antimicrobial. In this study, three-halotolerant *Bacillus* sp. LMB3051, LMB3073 and LMB3093 were isolated from water of the Great Sebkha of Oran, they exhibited interesting antimicrobial activities against a broad spectrum of reference bacterial and fungal strains. Their metabolites were extracted with chloroform and ethyl acetate solvents. Structural elucidation of actives compounds was carried out using gas chromatography–mass spectrometry (GC-MS). Fifty-six compounds were identified; they include tert-butyl phenol compounds, fatty acid methyl esters due to the methylation procedure, hydrocarbons, aldehydes, benzoquinones, pyrrols, and terpenes. Literature reports such compounds to have wide biological and pharmaceutical applications. Partial 16S rRNA gene sequencing of the three isolates showed very high similarity with many species of *Bacillus*. The comparison with 16S rRNA EzBioCloud database revealed that the isolates LMB3051 (529 bp) and LMB3073 (429 bp) showed a similarity of 99.81% and 98.60%, respectively, with *Bacillus licheniformis* ATCC 14,580, *B. aureus* 24 K, *B. paralicheniformis* KJ-16, *B. glicinifermentans* GO-13, *B. haynesii* NRRL B-41327, and *B. piscis*16MFT21. The isolate LMB3093 (400 bp) showed 98.75% of similarity *with Bacillus paralicheniformis* KJ-16 and *B. haynesii* NRRL B-41327. The findings suggest that the Great Sebkha of Oran is a valuable source of strains exhibiting variety of beneficial attributes that can be utilized in the development of biological antibiotics.

Materials and Methods

Phenotypical characterization

- morphological, cultural, biochemical, and physiological characterization was realized as described by **Logan *et al.* (2009)**

Molecular identification

- Genomic DNA was extracted using the standard phenol/chloroform method **(Ettoumi *et al.* 2013).**
- The amplification of the 16S rRNA gene was carried out using, the universal Primers 5'-S-D-Bact-0008-a-S-20–3' and 5'-S-D-Bact-1495-a-S-20–3'(**Daffonchio *et al.* 2000**).
- The amplified 16S rRNA fragments were sequenced and identified using EzBioCloud databases **(Yoon *et al.* 2017).**

Screening of antimicrobial activities

- The antagonistic properties of the isolates were screened by the agar plug method (**Balouiri *et al.* 2016**) against Gram positive bacteria (*Bacillus cereus* ATCC11778 and *Staphylococcus aureus* ATCC 25,923), Gram-negative bacteria (*Escherichia coli* ATCC 25,922 and *Acinetobacter baumannii* ATCC 19,606), and fungi (*Candida albicans* ATCC 444, *Candida albicans* ATCC 10,231, and *Aspergillus flavus* MNHN994294

Extraction of secondary metabolites

- Was performed from solid substrate fermentation (agar culture) with two organic solvents, ethyl acetate, and chloroform, using the method of **Kim *et al.* (2016)**

GC-MS analysis of bioactive crude extract

- The analysis was carried out using Agilent 7890B gas chromatography instrument coupled with an Agilent MS 240 Ion Trap with capillary column HP-5MS (5% phenyl methyl polysiloxane, 30 m, 250 µM, 0.25 µM)

References

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Results

Table 1. Morphological, physiological, and biochemical characteristics of LMB3051, LMB3073, and LMB3093 strains

Characteristics	LMB3051	LMB3073	LMB3093
Gram staining	+	+	+
Motility	Motile	Motile	Motile
Cell morphology	Long and straight rods	Short rods	Long big rods
Arrangement	Singly or in chains	Singly and in irregular clumps	Singly
Spore	Ellipsoidal subterminal	Ellipsoidal subterminal	Ellipsoidal subterminal
Colonies (24 h on nutrient agar at 15% of NaCl)	3 to 4 mm in diameter, cream, opaque, circular, smooth, shiny, slimy, convex, and odorous	3 to 4 mm in diameter, whitish, opaque, circular, smooth, shiny, slimy, convex, no smell	2 to 4 mm of diameter, cream, opaque, circular, smooth, slimy, shiny, flat, and odorous
NaCl tolerance	0 to 20% (w/v)	0 to 20% (w/v)	0 to 20% (w/v)
NaCl optimum (%)	0%	0%	0%
Range of temperature for growth	15 to 60 °C	15 to 60 °C	15 to 60 °C
Temperature optimum °C	30–40 °C	30–37 °C	30–37 °C
Range of pH for growth	5 to 11	5 to 11	5 to 11
pH optimum	6–7	6–7	6–7
Catalase	+	+	+
Oxydase	+	+	+
Respiratory test	Aero-anaerobic facultative	Aero-anaerobic facultative	Aero-anaerobic facultative

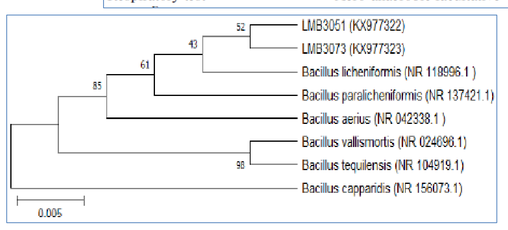

Fig 1. Phylogenetic tree constructed by performing bootstrap analysis of 1000 data sets using the MEGA 6 program, based on the analysis of 16S rRNA partial sequences showing the relationships between isolates LMB3051 and LMB3073 and related species. *Bacillus capparidis* was used as an outgroup The bar indicates 0.005 substitution per nucleotide position. Accession numbers are given in parentheses

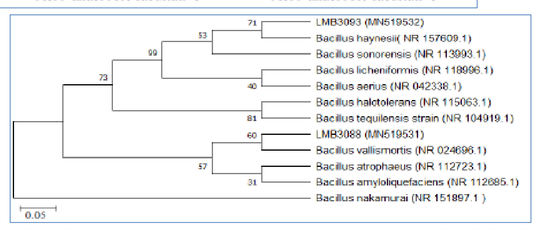

Fig 2. Phylogenetic analyses of the isolate LMB3093 basing on 16S rRNA partial sequences. Phylogenetic dendrogram was evaluated by performing bootstrap analysis of 1000 data sets using MEGA 6. 16S rRNA sequence. *Bacillus nakamurai* was used as an outgroup. The bar indicates 5 substitution per 100 nucleotide. Accession numbers of the reference strains were indicated in parentheses

Table 2. Inhibitory effects of the chloroform and ethyl acetate extracts of the isolates using disc diffusion method

Test microorganisms	Zone's diameter (mm)					
	LMB3051		LMB3073		LMB3093	
	Chloroform	Ethyl acetate	Chloroform	Ethyl acetate	Chloroform	Ethyl acetate
Bacillus cereus ATCC 11,778	15.33 ± 0.57[a]	14.33 ± 0.57[a]	17.33 ± 0.57[a]	22.33 ± 0.57[a]	13.33 ± 0,57[a]	12.66 ± 0.57[a]
Staphylococcus aureus ATCC 25,923	19.66 ± 0.57[a]	17.66 ± 0.57[a]	16 ± 1[a]	19.33 ± 0.57[a]	15.33 ± 1.52[a]	14 ± 1[a]
Escherichia coli ATCC 25,922	13.33 ± 0.57[a]	12.33 ± 0.57[a]	12.33 ± 0.57[a]	14 ± 1[a]	11.33 ± 1.15[a]	10 ± 0[a]
Acinetobacter baumannii ATCC 19,606	10 ± 0[a]	9.66 ± 1.52[a]	12.66 ± 1.15[a]	15.66 ± 0,57[a]	09.66 ± 1.52[a]	09 ± 1[a]
Candida albicans IP 444	13 ± 0[a]	12 ± 1[a]	11.66 ± 0.57[a]	16 ± 0[a]	10.33 ± 1.52[a]	10 ± 1[a]
Candida albicans ATCC 10,231	12.66 ± 0.57[a]	11.66 ± 0.57[a]	11.33 ± 0.57[a]	13.66 ± 1.15[a]	11 ± 1[a]	10 ± 0[a]
Aspergillus flavus MNHN994294	33.33 ± 0.57[a]	31.66 ± 0.57[a]	34.66 ± 1.52[a]	37.66 ± 0.57[a]	30.33 ± 0.57[a]	29 ± 1[a]

Values were expressed as mean ± standard deviation (n = 3). Values in same columns with superscript (a) were strongly significant different ($p < 0.001$)

Table 2. Chemical composition of the chloroformic and ethyl acetate extracts of the isolate LMB3051

Solvent	Compounds identified	CAS n	%
Chloroform	Hexadecanoic acid bis (2ethylhexyl) ester	103-23-1	100
Ethyl acetate	3-n-hexylthiolane, S, S, dioxid	71053-07-1	6,059
	1, 3- Di-tert-butyl benzene	1014-60-4	1.360
	2, 5- Di- tert- butyl- 1, 4- benzoquinone	2460-77-7	0.512
	2,6-Di-tert-butyl-1,4-benzoquinone	719-22-2	27.646
	2, 4- Di- tert- butyl phenol	96-76-4	8.310
	1-Nonyl cycloheptane	None	13,734
	3,5-Di-tert-butyl-4-hydroxybenzaldehyde	1620-98-0	5.963
	Hexadecanoic acid bis (2ethylhexyl) ester	103-23-1	35.957

Table 2. Chemical composition of the chloroformic and ethyl acetate extracts of the isolate LMB3073

Solvent	Compounds identified	CAS n	%
Chloroform	1, 3- Di-tert-butyl benzene	1014-60-4	1.548
	1- Fluorododecane	334-68-9	5.125
	1-Nonyl cycloheptane	None	0.767
	2,6-Di-tert-butyl-1,4-benzoquinone	719-22-2	0.597
	2,6-di-tert-butyl-4-(dimethylaminomethyl) phenol	88-27-7	24.741
	2, 4- di- tert- butyl phenol	96-76-4	4.649
	3, 5- di- tert- butyl -4-hydroxyacetophenone	14035-33-7	1.075
	2, 4, 6- Tri -tert- butylphenol,	732-26-3	0.874
	3,5-Di-tert-butyl-4-hydroxybenzaldehyde	1620-98-0	13.340
	4,4'-Ethylenebis(2,6-di-tert-butylphenol)	1516-94-5	2.756
	Pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-3-	5654-86-4	6.928
	2-Propenoic acid, 3-(4-methoxyphenyl)	830-09-1	2.969
	Tetracontane, 3, 5, 24, trimethyl	55162-61-3	3.335
	Carbonic acid eicosyl vinyl ester	None	5.74
	9- Octadeoenamide, (z)-	301-02-0	3.728
Ethyl acetate	1-Nonyl cycloheptane	None	11.205
	1, 3- Di-tert-butyl benzene	1014-60-4	1.449
	2 (3H)- Naphtalenone,4, 4a, 5, 6, 7,8-hexahydro-4a-5-dimethyl-3-(1-methylethyldiene)-4ar-cis	19598-45-9	10.539
	2, 4- di- tert- butyl phenol	96-76-4	4.667
	Cyclo pentane acetic acid, 3-oxo-2 pentyl-methyl ester	24851-98-7	4.066
	Tridecanoic acid, 12-methyl-, methyl ester	5129-58-8	0.788
	3,5-Di-tert-butyl-4-hydroxybenzaldehyde	1620-98-0	3.382
	7-Acetyl-6-ethyl-1,1,4,4-tetramethyl-1,2,3,4-tetrahydronaphthalene	88-29-9	3.103
	Hexadecanoic acid methyl ester	112-39-0	5.807
	Heptadecanoic acid, 16 methyl, methyl ester	5129-61-3	4.806
	Hexadecanoic acid bis (2 ethyl-hexyl) ester	103-23-1	36.781

Table 2. Chemical composition of the chloroformic And ethyl acetate extracts of the isolate LMB3093

Solvent	Compounds identified	CAS n	(%)
Chloroform	1, 3- Di-tert-butyl benzene	1014-60-4	1.955
	1- Fluorododecane	334-68-9	8.089
	1-Nonyl cycloheptane	None	1.459
	2,6-Di-tert-butyl-1,4-benzoquinone	719-22-2	0.735
	2,6-di-tert-Butyl-4-(dimethylaminomethyl) phenol	88-27-7	19.297
	2, 4- di- tert- Butyl phenol	96-76-4	5.059
	3, 5- di- tert- butyl -4-hydroxyacetophenone	14035-33-7	1.433
	2, 4, 6- Tri -tert- butylphenol	732-26-3	1.188
	3,5-Di-tert-butyl-4-hydroxybenzaldehyde	1620-98-0	11.839
	Propanohydrazide, N2-(3,5-di-tert-butyl-4-hydroxybenzyl) -N2-phenyl	304870-86-8	2.687
	Pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	5654-86-4	10.012
	Tritetracontane	7098-21-7	1.818
	4,4'-Ethylenebis (2,6-di-tert-butylphenol)	1516-94-5	1.270
	2-Propenoic acid, 3-(4-methoxyphenyl)	830-09-1	2.185
	Z -8-methyl -9- tetradecenoic acid	None	1.018
	9- Octadecenamide, (z)-	301-02-0	0.509
	Hexadecanoic acid bis (2ethylhexyl) ester	103-23-1	1.579
	13-Docosenamide, (Z)	112-84-5	5.160
Ethyl acetate	1-Undecyne	2243-98-3	0.840
	3-n-hexylthiolane, S, S, dioxide	71053-07-1	5.467
	1-Tetradecyne	765-10-6	1.189
	2, 5- di- tert- butyl- 1, 4- benzoquinone	2460-77-7	0.464
	2,6-Di-tert-butyl-1,4-benzoquinone	719-22-2	4.103
	2, 4- di- tert- butyl phenol	96-76-4	6.031
	1-Nonyl cycloheptane	None	10.564
	2-Propenoic acid, 3-(4-methoxyphenyl)	830-09-1	4.75
	Oleyl alcohol, trifluoro acetate	None	1.004
	Benzoic acid, tridecyl ester	None	2,966
	Hexadecanoic acid bis (2ethylhexyl) ester	103-23-1	3.609
	Benzoic acid, pentadecyl ester	None	1.805
	Z, 5 Methyl-6-Heneicosen-11-one	None	1.254