Novel Research in Microbiology Journal (2019), 3(1): 258-270
Research Article

(Print) (ISSN 2537-0286)
(Online) (ISSN 2537-0294)
https://nrmj.journals.ekb.eg/
DOI: 10.21608/nrmj.2019.28112

Isolation, characterization, and detection of antibacterial activity of a bioactive compound produced by marine *Bacillus* sp. MH20 from Suez Bay, Egypt

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Received: 29 January, 2019; Accepted: 16 February, 2019; Published online: 27 February, 2019

Abstract

This study focused on isolating and identifying a new bacterial strain from marine water and sediments of Suez bay, Timsah Lake, Egypt; capable of producing a bioactive secondary metabolite. Of the 552 bacterial isolates recovered, only 40 of these isolates exhibited antagonistic activities against different bacterial pathogens. A promising bacterium isolated from Suez bay marine water was identified as *Bacillus* sp. MH20 using 16S rRNA sequencing, and deposited in GenBank with accession number KM374670. This isolate showed sequence similarity of 88% to *B. sonorensis* strain NBRC 101234. Well-cut diffusion assay was adopted to determine the *in vitro* antibacterial activity of the active compound produced by this bacterium; whereas, GC-MS analysis was used to identify the components of this bioactive substance. GC-MS analysis showed that the active principle might be Phthalic acid, octyl 2-pentyl ester with retention time of 23.13 min. Crude extract of *Bacillus* sp. MK20 at a concentration of 200 mg/l exhibited LC_{50} of 2.5 ppm against *Artemia salina*. The aims of the current work were; to isolate and identify one of the promising bacterial strain from marine water, capable of producing bioactive antibacterial compound and evaluating its bio-toxicity, for its potential use in the control of bacterial human, animal and fish diseases.

Keywords: *Bacillus* sp. MH20; GC-MS characterization; Bio-toxicity; Extracellular metabolite

1. Introduction

Production of antimicrobial compounds by most bacteria seemed to be a general phenomenon. Different ways for microbial defence have been reported including; broad-spectrum classical antibiotics, secondary metabolites such as organic acids, and lytic agents such as lysozymes. Moreover,

several types of proteinaceous exo-toxins and bacteriocins; which were biologically active peptide moieties with bactericidal activity, were described. At time being, marine bacteria were considered as important sources of microbial metabolites that displayed antibacterial and antifungal potential (Woo *et al*., 2002; El-Nagar and Barakat, 2008; Guedes *et al*., 2014).

Woo *et al*., (2002) pointed out that marine epiphytic bacteria associated with nutrient-rich algal surfaces and invertebrates; have also been shown to produce antibacterial secondary metabolites, which inhibited the settlement of potential competitors. This was in agreement with Kasanah and Hamann, (2004), who reported that finding of antibiotic producing bacteria, was highly increasing with the existence of more bacterial biofilms on the surfaces of marine organisms.

Frequent occurrence of multiple antimicrobial resistance patterns in some *Aeromonas hydrophila*, *A. veronii* biovar *sobria*, and *A. caviae* strains isolated from fish and marine water was reported by Radu *et al*., (2003). In the earlier study of Allen *et al*., (2010), they revealed that the origins of antibiotic resistance in the marine environment was relevant to human health; because of the increasing importance of zoonotic diseases, as well as the need for predicting the emerging resistant pathogenic microorganisms.

Antibiotic-susceptibility pattern was also significant for selective isolation of microorganisms. The Aeromonads have been regarded universally to exhibit resistance to the penicillins (penicillin, ampicillin, and carbenecillin) for quite a long time (Awan *et al*., 2009). Antibiotic susceptibility pattern of *A. hydrophila* isolates and other indicator bacteria was carried out on Mueller-Hinton agar medium, using agar disc diffusion method firstly described by Odeyemi *et al*., (2012). *Bacillus* spp. have been reported by Beri *et al*., (2012) to be promising microbes for producing secondary metabolites which have antagonistic effects against several bacterial and fungal pathogens.

Of the several hundred of naturally produced antibiotics that have been purified, only a few had sufficient non-toxicity to be used in medical purposes (Sawale *et al*., 2014). This study aimed to isolating and identifying a novel bacterial strain with high antagonistic potential against several human, animal and fish pathogens, with acceptable toxicity limit.

2. Materials and methods

2.1. Isolation of marine bacteria

Marine sediment; and marine water samples were collected from Suez bay and Lake Timsah, Egypt, and were immediately transferred to the laboratory for isolation. Approximately one gram of soil sediment was aseptically transferred into 9 ml of filtered seawater. 0.1 ml of the soil suspension and that of marine water were inoculated separately into plates of De Man, Rogosa and Sharpe (M.R.S) agar medium (Peptone 10.0 g - Lab Lemco-powder 8.0 g - Yeast extracts 4.0 g - Glucose 20.0 g - Sorbitan mono oleate 1ml - Di potassium hydrogen phosphate 2.0 g - Sodium acetate $3H_2O$ 5.0 g - Tri ammonium citrate 2.0 g - Magnesium sulphate $7H_2O$ 0.2 g - Manganese sulphate. $4H_2O$ 0.05 g - Agar 10.0 g, Sea water 1l - pH 6.2±0.2) by using filtration technique, and then plates were incubated at 37°C±2 for 24 h. After incubation, colonies which appeared on the agar medium were re-streaked into the nutrient agar (NA) medium (Peptone 5.0 g - Beef extract 3.0 g - Sodium chloride 6.0 g - Agar 12.0 g - Sea water 1l - pH 6.8 ± 0.2) for purification.

2.2. Preparation of bacterial extract

The isolated bacterial cultures were grown on Zobell medium (Sea water 800 ml - Distilled water 200 ml - Yeast extract (Oxoid) 1 g - Peptone (Oxoid) 5 g - Agar-agar (Oxoid) 15 g - $FeSO_4.7H_2O$ traces 0.005 g - pH 7.5 ± 0.2) for 24 h at 35°C under shaking (120 rpm) (Risa *et al*., 2012). Cell free broth

was adjusted to pH 5.0 using 1N ethyl acetate. The organic phase was collected and evaporated by rotary evaporator at 60-70°C, and then the residue was stored at -20°C until further use (Sudha and Masilamani, 2011).

2.3. *In vitro* antagonism between isolated marine bacteria and tested indicator bacterial pathogens

Tooth picking technique was used to test the ability of isolated marine bacteria to inhibit the growth of other indicator bacteria according to Ravikumar *et al*., (2011); in addition, well-cut diffusion assay was also used. 15 ml of NA medium seeded with indicator bacteria were poured into plates. After solidifying, wells were punched out using 0.5 cm cork borer. 100 µl of isolated bacterial filtrates were inoculated into each well. All plates were incubated at 37°C for 24-48 h. Three replicate plates were used for each treatment. After incubation, the diameters of inhibition zones were measured in cm for each treatment (El-Sersy and Abou-Elela, 2006).

Indicator bacteria used in well-cut diffusion assay include; *B. cereus*, *B. subtilis* 6633, *S. aureus* 5258, *S. aureus* 25923, *E. coli* 19404, *Klebsiella* sp. 53637, *P. aeruginosa* 9027, *P. aeruginosa* PA01 7/2, *P. aeruginosa* PA01 8/2, *P. aeruginosa* 27853, *Salmonella typhimurium* 14028, *Vibrio damsel* and *V. falvialis*. These strains were acquired from Microbiology Lab., National Institute of Oceanography and Fisheries, Alexandria, Egypt.

Different types of media such as; nutrient broth, Zobell medium, and peptone water were manipulated to determine the best medium, optimum conditions for bacterial growth, and antibacterial agent production. The bacterial suspension (200 ml) was added to 250 ml Erlenmeyer flasks each containing 100 ml of one of the media stated previously. The cultures were shaken (120 rpm) at 37°C. After 24 h, aliquots (1 ml) were taken from the cultures, and then centrifuged for 30 min. at 5000 rpm. The growth of isolate was recorded by measuring the optical density (OD) at 550 nm in reference to El-Sersy and Abou-Elela, (2006); Al-Qadiri *et al*., (2008). The effect of different pH in the ranges 0f 5-9 on antibacterial activity of the marine isolate against *E. coli* 19404 strain; was estimated according to the diameters of inhibition zones on Zobell medium. Moreover, different temperatures ranging from 25-44°C were also investigated against the same indicator strain, to determine the appropriate temperature suitable for bioactive compound production (Sharma and Saharan, 2016).

2.4. Thin layer chromatography and GC-MS analysis of promising bacterial filtrate

Thin layer chromatography (TLC) was used to separate the components of the bacterial crude extract. Different solvents such as; Ethyl acetate, Hexane, Acetone and Diethyl either; were used for estimating the most suitable solvent, and to determine the acceptable ratio of this solvent for analyzing the bioactive compound (Morlock *et al*., 2014). The crude extract exhibiting antibacterial activity was subjected to GC-MS analysis using GC column; at oven temp. 70°C, injector temp. 200°C, spilt mode ratio 40 with a flow rate of 1.51 ml/min. The MS was with ion source temp.: 200 °C, interface temp.: 240°C, scan range: 40-1 000 m/z, event time 0.5 sec., solvent cut time: 5 min., MS start time: 5 min., MS end time: 35 min., ionization: EI -70ev according to Sudha and Masilamani, (2011).

2.5. Identification of promising bacterial isolate using 16S rRNA

The bacterial isolate (no. 32) which showed potent antibacterial potential against most tested bacterial pathogens was identified using 16S rRNA. Gene coding for 16S rRNA was partially amplified using the universal primers; F: - AGA GTT TGA TCC TGG CTC AG, R: - GGT TAC CTT GTT ACG ACT T.

According to Skariyachan *et al*., (2014), the amplified products were purified using Gene Jet genomic DNA purification Kit. The derived 16S rRNA gene sequence was compared to the GenBank database (NCBI), to search for similar sequences using the basic local alignment search tool algorithm (BLAST).

2.6. Bio-toxicity of the bacteria crude extract

Artemia salinas (active Naupli free from egg shells) were used to determine the bacterial crude extract toxicity assay. By preparing different stock solutions of bacterial extract (100, 200, 500, 1000, 1500, 2000 and 4000 mg/ ml); ten *Artemia salinas* were added to each concentration. After 24 h, dead shrimps were counted using microscope, and the percentage of mortality was calculated. Data were transformed to the probit analysis for the determination of Lethal concentration LC_{50} of the crude extract (Ivanova *et al*., 2010).

3. Results

3.1. Bioactivity of isolated marine bacteria against indicator one's

From 552 tested marine bacteria; only 40 isolates were able to inhibit the growth of other tested indicator bacterial pathogens using tooth picking technique (Data not shown). Only 5 isolates out of these 40 were able to inhibit the growth of indicator bacteria using well-cut diffusion assay. Results presented in Table (1 a, b) as average diameters of inhibition zones of indicator's bacteria growth, showed that the highest inhibitory potential was obtained by the bacterial isolate no. 32, which was isolated from Suez bay. On the other hand, the other isolates showed lower activities against indicator bacteria.

3.2. Molecular identification of promising marine bacterium

Based on results of antagonistic interactions, the promising bacterial isolate (no. 32) was identified by Gene coding for 16S rRNA. Gene sequence was compared with those which gave the highest homology using Blast search computer based program. The resulting data demonstrated that the concerned isolate was similar to *B. sonorensis* strain NBRC 101234 by 88 %. The phylogenetic relationship for this isolate and the closely related ones were analyzed as clear in Fig. (1). Based on these results, the experimental marine bacterium has been identified as *Bacillus* sp. MH20 with accession number (KM374670).

3.3. Effect of different culture media on the growth of bacteria

The different culture media used were Zobell, peptone and nutrient broth media; to record the highest growth of the selected isolate no. 32, estimated by measuring absorbance of the cultures at 550 nm. Maximum growth of the selected isolate was recorded in Zobell medium (Fig. 2).

3.4. Effect of pH and temperature on the antibacterial activity

Different pH and temperature values were tested for promoting maximal antibacterial potential of isolate no. 32, against tested indicator *E. coli* 19404 strain. Results showed that optimum pH and temperature for antibacterial activity against tested strain were 7.5 and 35°C, respectively (Fig. 3a, b).

3.5. Determination of the appropriate solvent(s) for extraction of the active compounds

On using different solvents, the highest inhibition zone (35 mm) against the indicator strain was obtained using ethyl acetate. Moreover, a quality of 1.8 g of crude extract was obtained from one liter of selected bacterial culture. However, the extracted products obtained by other solvents showed lower antibacterial activity towards indicator bacterium *E. coli* ATTC 19404.

Table 1(a): Results of *in vitro* antagonism assay of isolated marine bacterium against indicator bacterial pathogens

Bacterial strains		**Inhibition zones (cm) of different marine bacterial isolates**																			
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
B. cereus	G+	-	-	-	0.5	0.5	0.5	0.3	-	0.8	-	-	-	-	-	0.5	-	-	-	-	0.3
***B. subtillus* 6633**	G+	-	-	1.4	0.4	0.5	0.4	0.5	-	0.3	-	-	-	-	-	-	0.4	-	-	1.6	0.6
***S. aureus* 5258**	G+	-	-	-	0.3	-	-	-	-	0.2	-	-	-	-	-	-	-	-	-	-	0.3
***S. aureus* 25923**	G+	-	-	-	-	0.4	0.5	-	0.5	-	-	0.4	-	-	-	-	-	0.4	0.3	-	0.4
***E. coli* 19404**	G-	1.5	0.5	0.4	0.4	0.6	0.5	0.6	0.5	0.5	0.5	0.5	0.6	0.5	0.5	0.5	0.5	0.5	0.6	0.6	0.5
***Klebsiella* sp. 53637**	G-	-	-	-	-	-	0.5	-	-	-	-	0.3	-	-	-	-	-	-	0.4	-	0.5
***P. aeruginosa* 9027**	G-	-	-	-	0.3	0.3	-	-	-	0.7	-	-	-	-	-	0.4	-	0.3	0.4	-	0.4
***P. aeruginosa* PA01 7/2**	G-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.4	-	-	-	0.3	0.5
***P. aeruginosa* PA01 8/2**	G-	-	0.5	-	-	-	-	-	0.4	-	-	-	-	-	-	-	-	-	0.5	-	0.3
***P. aeruginosa* 27853**	G-	-	-	-	0.4	-	0.3	-	-	0.3	-	0.3	-	-	-	-	-	-	-	-	0.4
***S. typhimurium* 14028**	G-	0.5	-	-	-	-	0.5	0.5	-	0.4	0.5	0.5	-	-	-	-	-	-	-	0.5	0.5
V. damsel	G-	-	-	0.6	0.5	0.5	-	0.4	0.5	0.7	0.7	-	-	-	-	0.4	0.6	0.5	-	-	0.5
V. falvialis	G-	-	0.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.4	-

-Results were averages of three replicate plates for each treatment.

Table 1(b): Results of *in vitro* antagonism assay of isolated marine bacterium against indicator bacterial pathogens (continued)

Bacterial strains		**Inhibition zones (cm) of different marine bacterial isolates**																			
		21	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**39**	**40**
B. cereus	G+	-	-	1.3	-	-	-	0.5	1.2	-	-	-	1.4	-	-	-	1.3	-	-	1.4	0.5
***B. subtillus* 6633**	G+	-	0.3	1.0	-	0.4	-	-	1.0	-	0.3	0.4	1.0	-	-	0.4	0.8	-	0.4	1.0	-
***S. aureus* 5258**	G+	-	-	1.3	-	0.7	0.4	0.5	1.3	-	-	-	1.4	0.2	-	-	1.3	0.5	-	1.1	0.5
***S. aureus* 25923**	G+	-	0.4	1.6	-	0.3	-	-	1.3	-	0.4	-	1.4	-	-	0.2	1.1	-	0.5	1.3	-
***E. coli* 19404**	G-	0.9	0.5	1.1	0.4	0.4	0.5	0.4	1.0	0.5	-	0.5	1.0	0.4	0.4	-	1.0	0.5	-	0.7	0.3
***Klebsiella* sp. 53637**	G-	-	-	1.0	-	-	-	-	1.0	-	0.4	-	1.0	0.5	-	-	0.8	-	-	0.8	-
***P. aeruginosa* 9027**	G-	-	-	1.6	-	-	-	-	1.5	0.3	-	-	1.6	0.4	-	0.5	1.3	-	-	1.6	-
***P. aeruginosa* PA01 7/2**	G-	0.5	-	1.4	-	-	-	-	1.4	-	0.2	-	1.3	-	0.4	-	1.4	-	0.4	1.1	-
***P. aeruginosa* PA01 8/2**	G-	-	-	1.3	-	-	-	0.2	1.3	-	0.3	-	1.6	-	0.4	-	1.5	-	0.3	1.5	-
***P. aeruginosa* 27853**	G-	-	0.3	1.6	-	0.5	0.2	0.3	1.6	0.4	-	-	1.6	-	-	-	0.9	-	-	1.6	-
S. typhimurium 14028	G-	0.3	-	1.1	-	0.3	0.4	-	1.0	-	-	-	1.0	-	0.5	-	0.7	0.5	-	0.5	-
V. damsel	G-	-	-	1.0	-	-	-	-	1.0	-	-	-	1.0	0.4	-	-	1.0	-	-	1.1	-
V. falvialis	G-	-	-	1.5	-	-	-	-	1.6	-	0.5	-	2.0	-	0.3	-	2.0	-	0.6	1.6	-

-Results were averages of three replicate plates for each treatment. The tables showed the five promising isolates no. (20, 23, 28, 32 and 39) which inhibited indicator's pathogenic strains activity. Isolate No. 32 was chosen for identification and production of bioactive compound

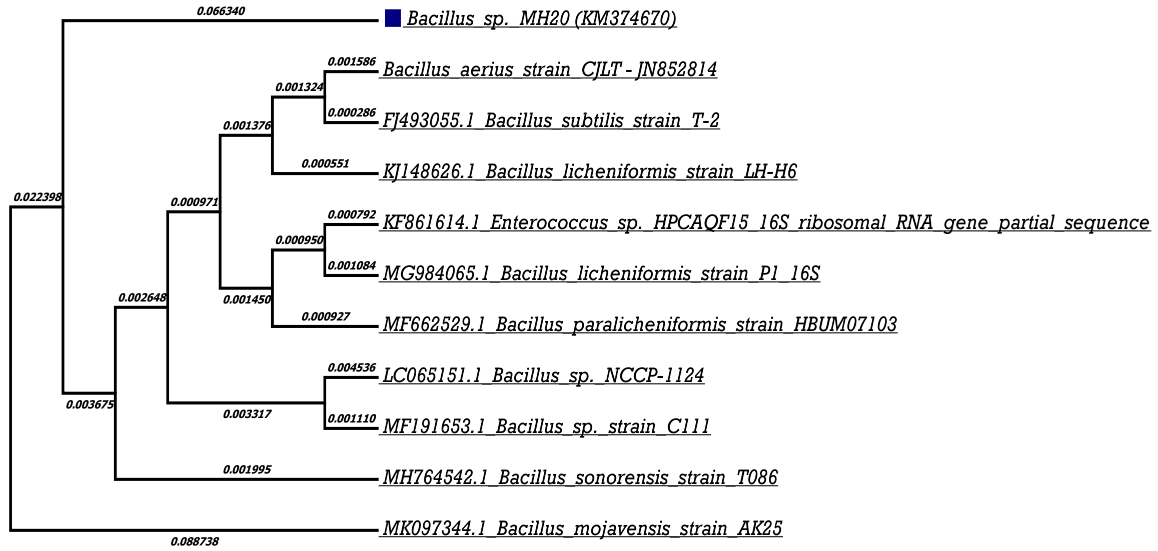


Fig. 1: Phylogenetic analysis of *Bacillus* sp. MH20 based on partial sequencing of 16S rRNA gene

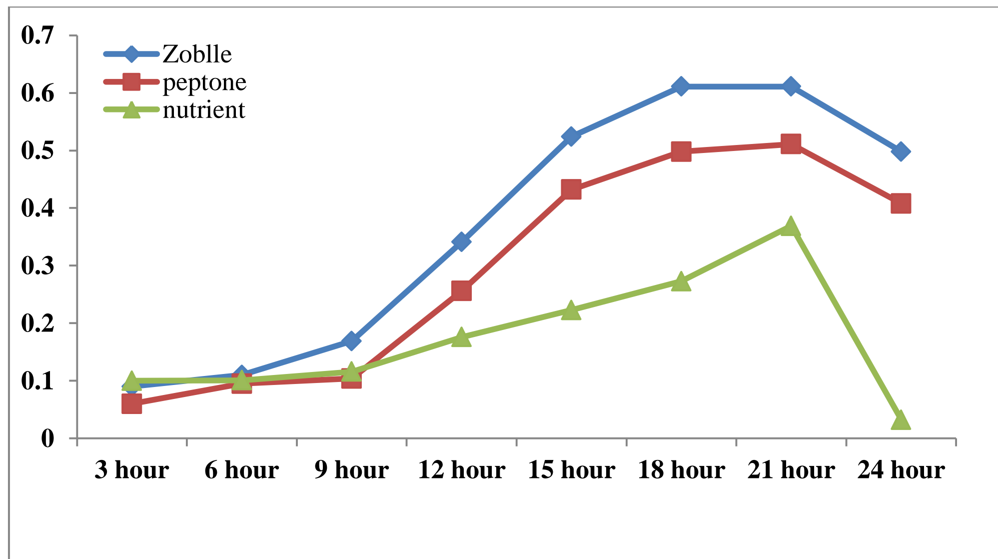


Fig. 2: Growth of *Bacillus* sp. MH20 on different media measured as optical density at 550 nm.

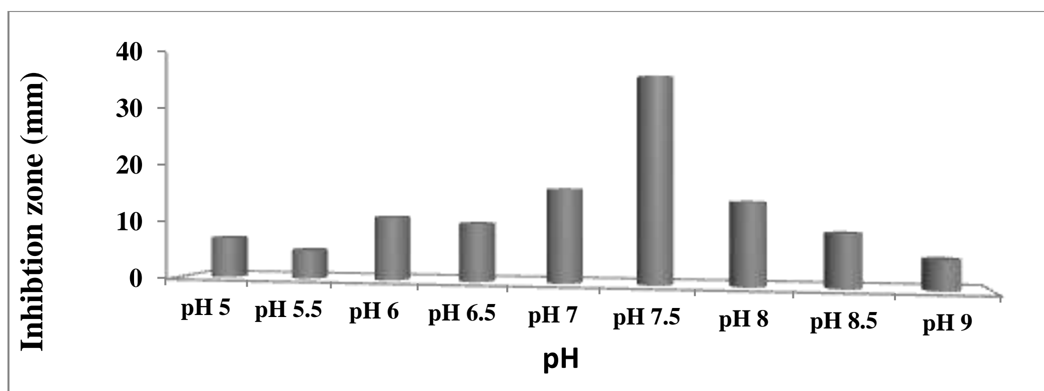


Fig. 3a: Effect of different pH on the antibacterial activity of *Bacillus* sp. MH20 isolate

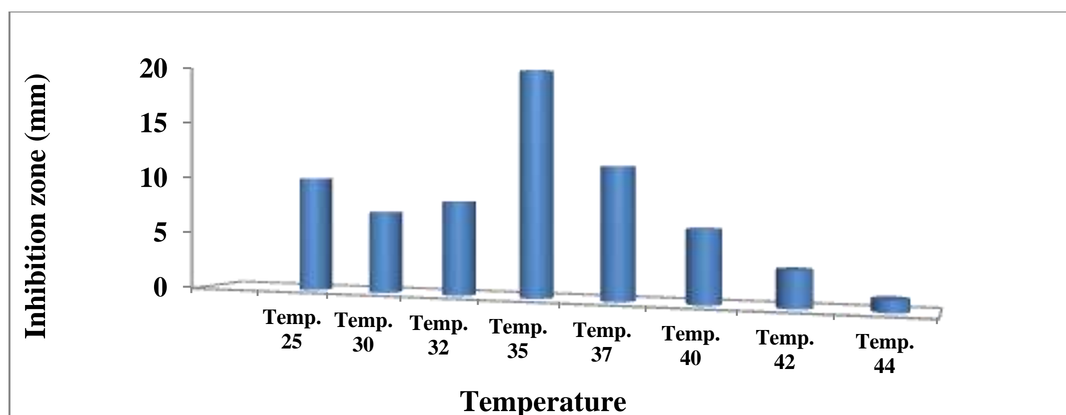


Fig. 3b: Effect of different temperatures on the antibacterial activity of *Bacillus* sp. MH20 isolate

3.6. Thin Layer Chromatography

Different solvents were used as stationary phase, and ethyl acetate was used as mobile phase to determine the best solvent for separating the components of the tested compound. Results showed that acetone: ethyl acetate (2:1) was best combination of solvents separating this bioactive compound.

3.7. Gas chromatography mass spectrum analysis (GC-MS)

The GC-MS analysis revealed that, the active principal components are presented in Table (2), Fig. (4-5). The most potent compound which showed antibacterial activity might be Phthalic acid, octyl 2-pentyl ester with Retention time = 23.13 min., relative abundance (compared with the other compounds within the crude) = 70%, odorless, colored band, and had detectable activities.

3.8. Bio-toxicity of different concentrations of the ethyl acetate crude extract of *Bacillus sp*. MH20 against *Artemia salina*

The bio-toxicity assay was carried out by testing different concentrations (ranging from 100 to 4000 µg/ml) of *Bacillus* sp. MH20 extract against *Artemia salina* as biomarker. The mortality percent was estimated. Results indicated that the crude bacterial extract has a low toxicity effect, where LC_{50} value was 2.5 ppm (Table 3, Fig. 6).

Table 2: Components of *Bacillus* sp. MH20 extract obtained by GC-MS analysis

Component	Relative abundance	Percentage %	Drug or other compound
4(1H)-Pyrimidinone, 6 amino-2-methyl-5-nitroso	18.69	12	Other compound
Loprazolam	18.69	61	Drug
Pyridine-3-carbonitrile,5-allyl-4,6-dimeethyl-2-mercapto	19.16	28	Other compound
Pyrrolo[1,2-a]pyrazine^{-1},4-dione, hexahydro-3-(2-methylpropyl)	19.69	34	Other compound
Pyrrolo[1,2-a]pyrazine^{-1},4-dione, hexahydro-3-(2-methylpropyl)	19.95	22	Other compound
Cyclotrisiloxane, 2,4,6-trimethyl-2,4,6-triphenyl	21.19	16	Other compound
Phthalic acid, octyl 2-pentyl ester	23.13	18	Other compound
Phencyclidine	24.97	66	Drug

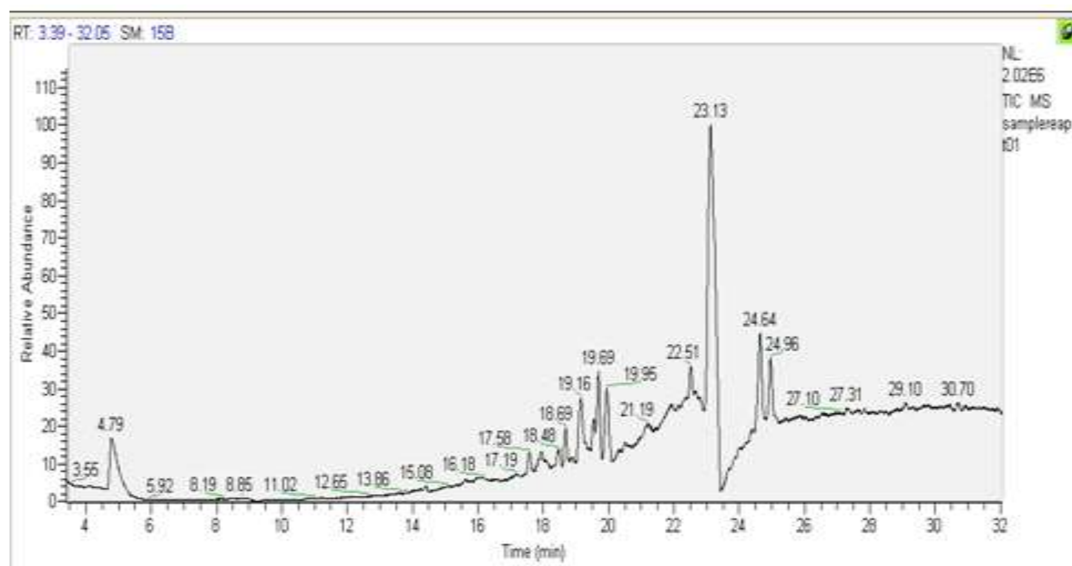
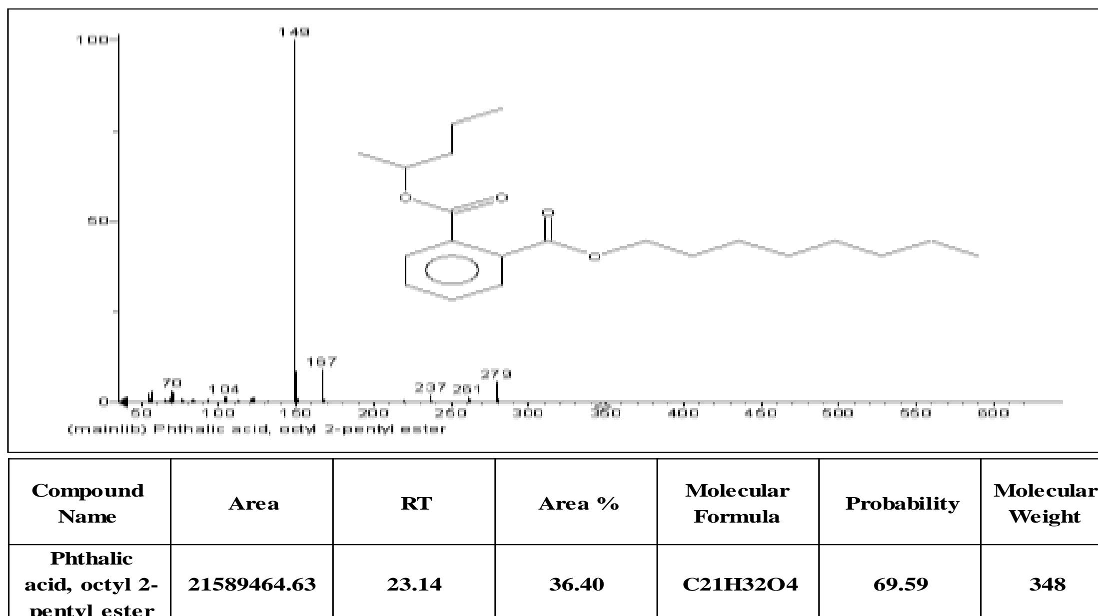


Fig. 4: GC-MS of the bioactive compound produced by *Bacillus* sp. MH20



Compound Name	Area	RT	Area %	Molecular Formula	Probability	Molecular Weight
Phthalic acid, octyl 2-pentyl ester	**21589464.63**	**23.14**	**36.40**	**C21H32O4**	**69.59**	**348**

Fig. 5: Mass spectra and features of bioactive compound produced by *Bacillus* sp. MH20

Table 3: Bio-toxicity of *Bacillus* sp. MH20 extract against *Artemia salina*

Crude extract conc. (mg/ml)	Log crude conc. (ppm)	Mortality % of *A. salina* after 24 h
100	2.0	**30**
200	2.3	**40**
500	2.7	**60**
1000	3.0	**70**
1500	3.2	**80**
2000	3.3	**90**
4000	**3.6**	**100**

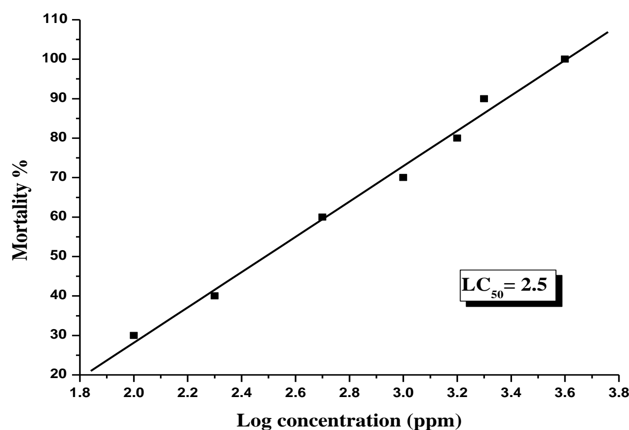


Fig. 6: Relation between mortality of *A. salina* and concentration of crude extract of *Bacillus* sp. MH20 with LC_{50}

4. Discussion

Marine bacteria have been recognized as important sources of novel bioactive compounds. Thus great development of marine biotechnology will produce novel compounds that may contribute significantly towards drug development (Kasanah and Hamann, 2004). The production of antimicrobial agent acting against some fish pathogenic bacteria was reported by earlier by El-Naggar and Barakat, (2008). Of 552 marine isolates, 5 tested isolates showed the ability to inhibit some clinical human, poultry and animal bacterial pathogens. The marine bacterium was identified as *Bacillus* sp. MH20, which showed a broad spectrum inhibitory potential against these tested indicator pathogens.

The present investigation showed that the optimum condition for the production of antibacterial agent was achieved at pH 7.5, and 35°C, where the highest inhibition zone was 35 mm. These results were in agreement with several authors; who showed that the maximum production of antimicrobial agents was obtained at pH ranged from 6.5-7.5 and temperature ranged from 25-35°C using different Pseudomonas strains (Lee, *et al*. 2003). Many authors reported the use of Placket Berman technique to adapt different samples grown on different media components (He *et al*. 2007; Li *et al*. 2008).

The ethyl acetate was proven to be the most potent organic solvent for extracting the bioactive compounds from the selected isolate. The crude extract showed inhibition zone of 30 mm against *V. falvialis*. Partially similar data were reported by Uzair *et al*., (2006). Earlier, Lee *et al*. (2003) reported that the dark oily brown ethyl acetate crude product (100-150 mg/l) obtained from marine *P. aeruginosa* CMG1030, showed inhibition zones of 15-33 mm against some bacterial pathogens using agar well diffusion assay. They added that a total of 41 mg of an antibiotic were yielded from 50 liters of *Pseudomonas* cultures. It showed antifungal activity against *Colletotricum orbiculare* (31 mm) using the disk-agar diffusion assay.

The GC mass analysis revealed that Phthalic acid, octyl 2-pentyl ester compound could be the antibacterial agent separated from the crude extract, according to previous studies of Sangappa and Thiagarajan, (2013); Sudha *et al*., (2016). They

extracted antimicrobial compounds from marine fungi and bacteria with extraction of phthalic acid compounds specifically. Ivanova *et al*., (2010) used *Streptomyces avidinii* to isolate phthalic acid, which has potential applications in a broad range of industrial, agricultural and medical processes. Also, Al-Bari *et al*., (2006); Chairman *et al*., (2012); Sudha and Masilamani, (2012) added that phthalate extracted from *Streptomyces bangladeshiensis* showed antimicrobial activity against Gram-positive bacteria and some pathogenic fungi.

The bio-toxicity of the produced ethyl acetate crude extract was estimated using *Artemia salina*. It showed low toxicity where the LC_{50} value was recorded to be 2.5 ppm. However, previous results obtained by Guedes *et al*., (2014) showed the bio-toxicity of some molluscicidal compounds using *Artemia salina*, where the LC_{50} value was found to be 11.460 µg\ ml.

Conclusion

This work emphasized the potential use of bioactive compounds produced by *Bacillus* sp. MH20, for enhancing the application of natural antibacterial agents worldwide. These marine active compounds thus represent alternative sources of antibacterial compounds; that may have future industrial applications.

Acknowledgement

The authors are thankful to Dr. Kholoud Barakat, Dr. of Microbiology, NIOF, Alexandria, for helping them with GC-MS analysis of the antibacterial compound.

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