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Isolation and Antagonistic Interactions of *Bacillus hwajinpoensis* HMA123 as anti-*Vibrio* Active Producer

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About 110 seawater and sediment samples were collected from Alexandria Western Harbor, Egypt, in order to study the antagonistic effect towards different *Vibrio* sp. (*V. fluvialis*, *V. harveyi* and *V. damasi*). In addition, the occurrence of the *Vibrio* sp. during five seasons (2012-2013) was also estimated. The results indicated that 31 % of marine isolates inhibited at least one of the tested *Vibrio sp*. while, 10 % of tested marine isolates showed activity against the tested *V. fluvialis*, *V. harveyi* and *V. damasi*. The highest percentage of antagonistic activity was detected in summer and the Marine sediment samples showed relatively higher antagonistic activity and *Vibrio* counts compared to the seawater samples. The anti-*Vibrio* activities were 33 % and 29 %, respectively. While, the occurrences of *Vibrio* sp. were ranged from 1.92 % to 32.38 % in sediment and from 0.23 % to 13.21 % in seawater samples. The most potent anti-*Vibrio* isolate was detected as a new marine Bacillus strain with an accession number of KP050555, it identified as *Bacillus hwajinpoensis* HMA123 using the 16S rRNA gene. The obtained anti-*Vibrio* activities were 27.50±1.51, 21.36±1.23 and 9.00±0.82 AU against *V. fluvialis*, *V. harveyi* and *V. damasi*, respectively. Also, this new strain showed a broad antibacterial spectrum against *Aeromonas sp.*, *Pesudomonas aeruginosa, Streptococcus faecalis* and *Bacillus subtilis*, with activities 23.00±1.53, 15.26±1.18, 8.36±0.93 and 11.16±1.32 AU, respectively. The GC-Mass spectrophotometry of *B. hwajinpoensis* HMA123 crude extract revealed the existence of fatty acids methyl esters (25.27%), tetracosane (16.06%), hentriacontane (26.65%) and eicosane (32.02%).

Key words: Antagonistic activity, *Bacillus hwajinpoensis*, crude extract, pathogens, *Vibrio fluvialis*.

The bacterial communities have been frequently studied, recent studies show that lots of bacterial communities need to be still characterized in marine environment[1]. Some marine bacteria are inhibitory to other bacteria, many report suggesting that bacterial interactions could play an important role in marine ecology[2,3]. The control of unwanted microorganisms is essential in all aspects of life, and microbial diseases must be treated in humans, animals, and plants[4].

Bacteria of the Vibrio genus are ubiquitous members of the normal microbiota of coastal marine environments, and are frequently the cause of disease (vibriosis) in almost all cultured marine aquatic animals such as crustaceans, molluscs and fishes causing mass mortalities worldwide (V *harveyiV., anguillarum, V. alginolyticus, V. cholerae, V. fischeri, V. furnissii, V. harveyi, V. ordalii, V. salmonicida, V. splendidus, V. vulnificus and V. wodanis*)[5-8]. *Vibrio* sp. is among the most studied marine heterotrophic bacteria. Factors such as temperature and salinity that regulate its distribution and abundance have been elucidated at the meso-scale and ocean basin levels. At smaller scales, its attachment to pelagic particles is well

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documented. To successfully colonize in the marine environment, Vibrio sp. must compete against the other 10 to 1,000 million phylogenetically diverse bacteria that reside within acubic centimeter of a particle[9].

The oceans cover more than 70 % of the earth's surface, and little is known about the antimicrobial active marine bacteria[4]. The diversity of marine environments has exerted a driving force on bacteria selection leading to new adaptive strategies and the synthesis of new metabolites. Microbial secondary metabolites have been recognized as a major source of compounds endowed with ingenious structures and potent biological activities[10]. Marine microorganisms are also the focus of attention due to their production of secondary metabolites that may have a range of pharmaceutical and biotechnological applications[4,11,12,13,14]. One technique to alleviate this condition is the use of microorganisms as biocontrol agents, either by antagonistic exclusion or by direct inhibition. This technique has been gaining popularity recently with an increase in the use of probiotic bacteria. For this purpose, several bacteria that may be potential biocontrol agents have been identified, and these bacteria are a potential source for new antibacterial substances[5].

Since the 1960s there has been increasing research on marine natural products[15]. Studies of the chemistry and biological activity of marine organisms have shown that they contain a large amount and variety of secondary metabolites with chemical structures different from those found in land organisms[16]. Since 1990, the bioactive metabolites discovered in marine bacteria have increased exponentially[17]. Recently, several biologically active substances have been isolated from marine bacteria[18-19]. Taking these into consideration, and trying to look for potential agents that can control the growth of fish pathogenic bacteria, including *Vibrio* sp., several antagonistic strains were isolated. Some of these strains were studied for bacteriolytic activity resulting from the production of proteolytic enzymes[20]. In addition, the same strains were found to produce low molecular weight compounds that inhibit the growth of *Vibrio* strains[5].

This study aims to estimate the heterotrophic marine bacteria and the occurrence of *Vibrio* sp. in seawater and sediment samples from the Western Harbor of Alexandria, during five seasons. The strategy was to screen and test the bacterial isolates with antagonistic activity against *Vibrio* sp. pathogens. Also, to isolate, identify and evaluate the marine bacteria which have potentiality to produce anti-*Vibrio* compounds.

MATERIALS AND METHODS

Sampling

Water and sediment samples were collected from eleven sits along Alexandria Western Harbor, Egypt. Samples were collected seasonally from winter 2012 to winter 2013. Sampling was performed according to the World Health Organization manual for recreational water and beach quality monitoring and assessment[21]. Temperature was monitored on sits using thermometer.

Counting of heterotrophic marine bacteria and *Vbrio* sp.

Total heterotrophic marine bacteria (THB) were counted using standard pour plate method into Marine agar 2216 (MA), (Oxoid LTD, England). Estimation of *Vibrio sp*. was detected using filtration method onto thiosulphate Citrate Bile Salt agar (TCBS), (Oxoid LTD, England), Plates were incubated at 30°C and final counts of colony forming units (CFU) taken after 24-48 h[22]. Triplicates were used for each.

Bacterial indicators with growth conditions

The antagonistic bacteria used as target strains in the current investigation were *Vibrio harveyi* ATCC14126, *Vibrio fluvialis, Vibrio damasi, Pseudomonas aeruginosa ATCC8739, Staphylococcus aureus* ATCC6538, *Streptococcus faecalis, Escherichia coli, Aeromonas sp.* and *Bacillus subtilis*. Various antagonistic bacteria were previously isolated from several marine sources used in previous paper by the aid of National Institute of Oceanography and Fisheries, Egypt. The bacterial strains were stored at -20°C in marine nutrient broth medium (MB), (1.5% Bacto Peptone and 0.5 % yeast extract in 100 ml filtered seawater) containing 20% glycerol and sub-cultured to the same agar (1.5%) slants and maintained at -4°C. Target strains used for experiments were seeded from the agar slants and

grown in 10 ml of MB at 30°C for 24h.

Primary screening for anti-*Vibrio* activity

Colonies with different morphological characterization were selected from seawater and sediment cultures. The antibacterial activity of the isolates was assayed on solid medium according to the spot method[7]. The previous marine pathogens (*Vibrio harveyi* ATCC14126, *Vibrio fluvialis* and *Vibrio damas*) were used as target strains. Each was prepared as a 24-h culture in MA medium and resuspended in sterile seawater (SW, aged natural seawater, filtered through a 1.0-mm filter and then autoclaved). The suspensions were adjusted to tube 1 of the MacFarl scale and spread on appropriate plates. A small amount of the isolates to be tested, cultured on MA, were deposited in spots (2–3 mm diameter) onto the surfaces of seeded plates. Plates were incubated at 30°C overnight. Antagonistic interactions were scored when zones of inhibition were observed. Inhibition areas around the spot of at least 1mm were considered positive for antibacterial activity[23].

Assays were performed in triplicate, and only when inhibition was observed in all three assays were isolates scored as positive for inhibition[9].

Preparation of culture supernatant

The marine bacterial isolates were grown in marine nutrient broth medium at 30ºC, and 150 rpm. The culture broth was centrifuged at 10000 rpm for 15 minutes to remove bacterial cells. The inhibition of pathogenic bacteria by these culture supernatant were tested by the agar well-cut diffusion method[24].

The antagonism assay

The antagonistic activity of bacterial supernatant was detected using well-cut diffusion technique in which, five-millimeter-diameter wells were punched in marine nutrient agar plates (using a sterile gel puncher) inoculated with bacterial pathogenic strains. Fifty microliter (µl) of tested cell free supernatant was pippitted into each well. The plates were incubated at 30ºC for 24h. Each set was prepared in triplicate. After incubation, the radius of clear zone around each well (Y) and the radius of the well (X) were linearly measured in mm to calculate the activity unite (AU). Which was calculated according the following equation: $AU=Y^2/X^2$

Where, Y is the radius of the clear zone around the zone and X is the radius of the well itself [25, 26].

Molecular identification

The molecular identification of the most potent bacterial isolate was carried out at Sigma Scientific Services Company, El Giza, Egypt.

Extraction of the active substances

A three-day culture (300 ml) of antagonistic strains was extracted with equal volume of methanol or ethyl acetate. After soaking, the crude extract was evaporated until complete dryness to concentrate the material and re-suspended in the appropriate solvent[5]. The crud extract was used for determination of antagonistic activity using the well-cut diffusion method.

Characterization of the anti-bacterial agents

The effect of proteolytic enzymes

To prove the protein nature of inhibitory agents, the isolated supernatant substances were exposed to several proteolytic enzymes (proteinase K,20 mg ml^{-1}; trypsin,5 mg ml^{-1} and subtilisin, 100 mg ml^{-1}) and assessed using the agar well diffusion technique according to Deraz *et al*.(2005)[27].

Chemical Composition Analysis:

Identification of the chemical constituents of the crude extract of the selected isolate was estimated using (Hewlett Packard) HP (High performance) 5890 gas liquid chromatography (GLC) coupled with 5989 B series mass spectrometer (MS). The percentage of each compound was calculated as the ratio of the peak area to the total chromatographic area. The GC-MS peaks were identified[27] by comparison with several data reported and the profiles from the Wiley 275 libraries.

Statistical analysis

Three replicates were used in each experiment, unless otherwise stated. All results were presented as means ± their standard deviations. The Pair-wise correlation coefficients between temperature and bacterial counts were calculated at p-value < 0.05. Statistical analysis was performed by using MINTAB[16].

RESULTS

Cultural bacterial load in marine environment

Cultural bacterial load in seawater

A total of 55 seawater samples from 11 different sits were cultured, during 5 seasons (Figure 1). Seawater samples exhibited high viable count of total heterotrophic ($5.7 \times 10^4 \pm 1.9$

Table 1: The occurrence percentage of *Vibrio* sp. with respect to total heterotrophic bacteria

Seasons	% Occurrence of *Vibrio* sp.	
	Seawater	Sediment
Winter 2012*	13.21	4 .34
Spring	1.02	1.92
Summer	0.32	19.28
Autumn	0.47	32.38
Winter 2013	0.23	11.35
Total** (n=55)	0.39	17.38

*(n=11 for each season);
**Total (n=55 for seawater and 55 for sediments)

$x10^3$CFUml^{-1}) in summer and low count (2.6 $x10^2$±1.2 $x10^2$ CFUml^{-1}) in winter 2012. The highest count 1.8$x10^2$±1.1$x10^2$CFU ml^{-1} of *Vibrio* sp. was detected in summer and the lowest count 1.6 $x10^1$±0.7$x10^1$CFU ml-1 was in spring. Also it was noticed that the viable counts of total heterotrophic and *Vibrio* sp. in seawater were increased to 1.9$x10^4$±6.1$x10^3$ CFU ml^{-1} and 4.6 $x10^1$±1.3$x10^1$ CFU ml-1, respectively, in winter 2013 comparing with winter 2012. The positive and significant correlation coefficient (r=0.65 at p<0.05 and n=55) between the counts of *Vibrio* sp and the temperature of seawater improved

Table 2: Primary screening for antagonistic activity of the marine bacterial isolates against the members of genus Vibrio

Season	Isolate code	Target strain		
		V. fluvialis	*V. harveyi*	*V. damasi*
Winter 2012	4w-1	±	+	±
	5w-1	-	+	-
	5w-3	±	+	-
	9w-1	-	+	-
	3s-1	±	+	+
Spring	3w-1	+	+	-
	5w	+	-	-
	7w	±	+	-
	5s	±	±	±
	7s-2	+	+	-
Summer	4w-2	-	+	+
	5w-1	+	+	+
	5w-2	++	+	+
	3s-4	++	+	±
	4s-1	+	++	-
	5s-1	±	+	+
	7s-2	-	-	+
Autumn	3w-1	+	+	±
	4w-2	±	+	-
	9w-1	±	+	-
	5s-2	-	+	-
	6s-1	+	+	-
	7s-1	+	-	-
Winter 2013	3w-1	±	-	-
	7w-2	±	±	±
	9w-2	+	±	-
	5s-4	++	+	-
	6s-1	++	++	+
	7s-1	++	-	-
	7s-2	±	+	-

s = Isolated from sediment samples w= Isolated from seawater samples
± Presence of hesitate inhibition zone - Absence of inhibition zone on agar plate
++ Good antagonistic activity against pathogen

the effect of seawater temperature on the counts of *Vibrio* sp.

Cultural bacterial load in sediment

A total of 55 marine sediment samples from 11 different sits were cultured, during 5 seasons (Figure 2). Sediment samples exhibited higher load of bacterial communities comparing with seawater samples in the different five seasons. Marine sediment samples exhibited high viable counts of total heterotrophic and *Vibrio* sp. ($1.4x10^5 \pm 7.9x10^4$ CFU g^{-1} and $2.7x10^4 \pm 1.1x10^4$CFU g^{-1}, respectively) in summer; and low counts ($4.6 x10^3 \pm 1.2x10^3$CFU g^{-1} and $2.0x10^2 \pm 9.0x10^1$ CFUg^{-1}, respectively) in winter 2012. On the other hand, the viable counts of total

Table 3. The percentage of antagonistic activity during different seasons

Seasons	Temperature (°C)		Number of representative bacteria		%Antagonistic activity	
	Seawater	sediment	Seawater	Sediment	Seawater	Sediment
Winter 2012	16.7	16.84	14	3	28	33
Spring	-	-	12	9	25	22
Summer	29.19	29.05	8	11	37	36
Autumn	23.61	23.58	15	9	20	33
Winter 2013	17.17	17.51	7	10	42	40
Total			56	42	29*	33**

* 16 active isolates of total 56 representative isolates from seawater
** 14 active isolates of total 42 representative isolates from sediments

Table 4. The characteristics of *B. hwajinpoensis* HMA123

Characteristic	*B. hwajinpoensis*	Characteristic	*B. hwajinpoensis*
Morphological characters		Acetoin production	+
Colony color	Yellow	Gelatinase production	+
Gram reaction	+	Oxidase production	-
Presence of spores	+	Catalase production	+
Motility	+	Nitrate	+
Physiological characters		Utilization of	
²-galactosidase production	+	D-glucose	-
Arginine dihydrolase	-	D-mannitol	-
Lysine decarboxylase	-	Inositol	-
Ornithine decarboxylase	-	D-sorbitol	-
Citrate utilization	-	D-rhamnose	-
H_2S production	-	D-sucrose	-
Urease production	-	D-melibiose	-
Tryptophanedeaminase	-	Amygdalin	-
Indoleproduction	-	L-arabinose	-

Table 5. Chemical composition of the crude extract of *B. hwajinpoensis* HMA123 using GC-MS.

Compound	Molecular formula	Molecular weight	Retention Time	Area %	Similarity %
Hexadecanoic acid, methyl ester	$C_{17}H_{14}O_2$	270.45	51.839	16.76	98
10-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296.49	57.671	3.84	95
Octadecenoic acid, methyl ester	$C_{19}H_{34}O_2$	294.47	58.438	4.67	95
Tetracosane	$C_{24}H_{50}$	338.65	77.801	16.06	97
Hentriacontane	$C_{31}H_{64}$	436.85	81.216	26.65	97
Eicosane	$C_{20}H_{42}$	282.54	83.399	32.02	98

heterotrophic and *Vibrio* sp. in sediment were increased to $2.1x10^4 \pm 8.4x10^3$ CFUg^{-1} and 1.9x1046.1x10^3 CFU g^{-1}, respectively, in winter 2013 comparing with winter 2012. The positive and significant correlation coefficient ($r=0.69$ at $p<0.05$ and $n=55$) between the counts of *Vibrio* sp. and the temperature of sedimentim proved the effect of sediment temperature on the counts of *Vibrio* sp.

The occurrence of *Vibrio* sp

In terms of percent of THB, the occurrence of *Vibrio* sp. in seawater ranged from 0.23 to 13.21%. In sediment samples the occurrence of *Vibrio* sp. ranged from 1.92% to 32.38 %.The occurrence of *Vibrio* sp. attached to sediment

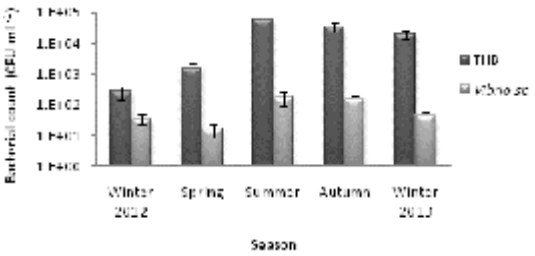

Fig. 1. The viable counts of total heterotrophic bacteria (THB) and *Vibrio* sp. in seawater samples from Alexandria Western Harbour

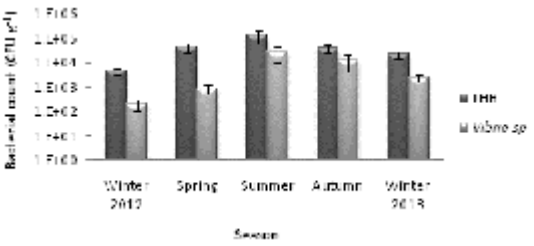

Fig. 2. The viable counts of total heterotrophic bacteria (THB) and *Vibrio* sp. in marine sediment samples from Alexandria Western Harbour

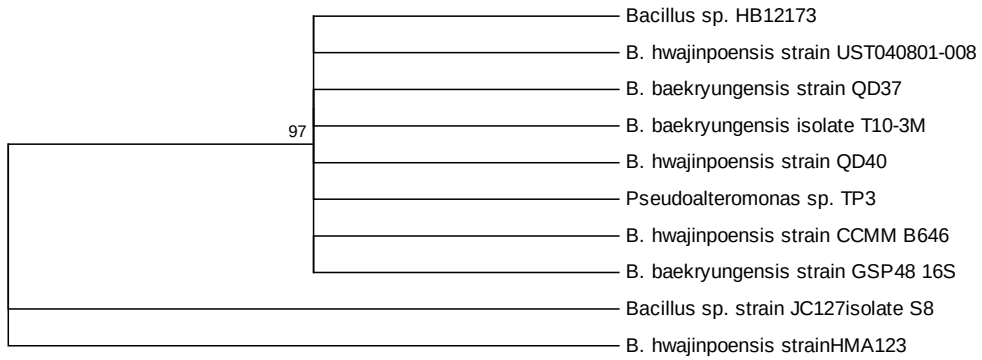

Fig. 3. Phylogenetic relationship of the new strain (*B.hwajinpoensis* HMA123) and the most closely related strains presented in the Genbank database

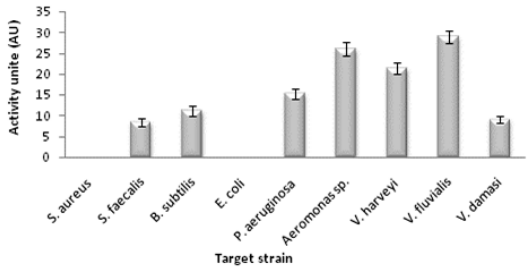

Fig. 4. Antagonism activity of *B. hwajinpoensis* HMA123 supernatant against different bacterial pathogens

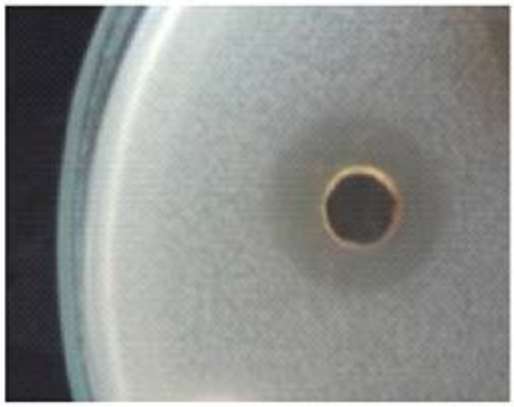

Fig. 5. The antagonistic activity of the marine strain *B. hwajinpoensis* HMA123crude extract against pathogen *V. fluvialis* using the well diffusion method

particles(17.38 %) is very higher comparing with free cells in seawater (0.39 %) (Table 1).

Screening for anti-*Vibrio* activity

Marine bacteria were enumerated for the isolation of antagonistic marine bacteria. During the five seasons a total of ninety eight bacterial colonies with different morphological characterization on marine agar were selected and tested for antagonistic activity. Thirty bacterial isolates from them (31%) inhibited at least one of the target strains (Vibrio sp.). The screening revealed that 25% of the tested isolates exhibited antagonistic activity against *V. fluvialis V. harveyi* and only 12% of isolates exhibited antagonistic activity against *V. damasi*. Spectra of antagonistic activity (represented about 10%) resulting in the inhibition of all *Vibrio* sp. assayed (*V. fluvialis*, *V. harveyi* and *V. damasi*). Isolate 6s-1 (isolated during winter 2013) displayed good activities against the three target strains V. *fluvialis, V. harveyi* and *V. damasi.* Therefore, it was selected and subjected to further examination (Table 2).

Antagonistic interactions

During 2012, the highest percentage of antagonistic activity (37% and 36% for seawater and sediment isolates, respectively) was detected in summer. During the overlap season (comparison between winter 2013 and winter 2012) when the temperature of seawater increased about 0.5°C, the antagonistic activity of marine bacterial isolates increased to 42%. Also when the temperature of sediment increased about 0.67°C the antagonistic activity increased to 40%. It has been found that the higher incidence of antagonistic activity (33 %) was detected in sediment attached bacteria comparing with free cells from seawater samples (29 %), (Table 3).

Molecular identification

The obtained sequence of the rRNA gene indicated the isolation of a new bacterial strain belong to *Bacillus hwajinpoensis* with an accession number of KP050555 compared with the data base presented in the Gen bank, it was identified as *Bacillus hwajinpoensis HMA123*, Figure 3.

Characterization of *B.hwajinpoensis HMA123*

The physiological and biochemical characters of *B. hwajinpoensis* is strain HMA123 are represented in Table 4. The stain was morphology gram-positive (gram-variable in old culture) isolated from marine sediment.

Antagonism assays of *B. hwajinpoensis* HMA123 against different bacterial pathogens

The supernatant of strain *B. hwajinpoensis* HMA123 showed a wide spectrum of antibacterial activities against *Vibrio* sp. and several others gram-positive and Gram-negative bacterial pathogens (Figure 4). *Bacillus hwajinpoensis* HMA123 had antibacterial activities against the three *vibrio* sp. tested. The highest activity was against *V. fluvialis* (Figure 5) with an activity unite of 27.50±1.51 AU, followed by *V. harveyi* (21.36±1.23 AU) and *V. damasi* (9.00±0.82AU). *Bacillus hwajinpoensis* HMA123 had antibacterial activity against other Gram negative bacteria as *Aeromonas sp.* (23.0±1.53 AU) and P. *aeruginosa* (15.26±1.18 AU); and also member of Gram-positive bacteria as where the activity units were 11.16±1.32 AU against *B. subtilis* and 8.36±0.93 AU against *S. faecalis*.

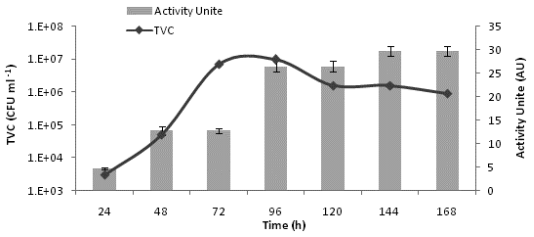

Fig. 6. Evaluation of the growth and the production of antibacterial substance of *B. hwajinpoensis* HMA123. The curve indicates the bacterial growth(Total viable count, CFU ml^{-1}) and bars indicate the antagonistic activity (AU) against *V. fluvialis*

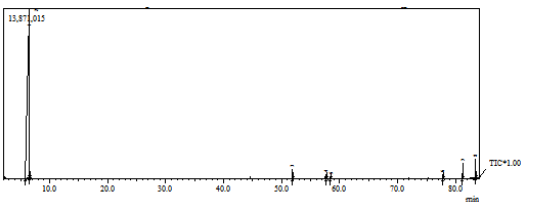

Fig. 7. The gas liquid chromatography mass spectrometer of *B. hwajinpoensis* HMA123 extract

Effect of culture age on the production of anti-vibrio compounds

Data obtained from the experimental growth of *B.hwajinpoensis HMA123* and their bioactive products showed that the organic production increased significantly between 3 and 4 days compared to 1, 2 and 3 days. The best antagonistic activity was recorded when bacteria entered the stationary phase. *Bacillus hwajinpoensis* HMA123 showed maximum growth also showed maximum antagonistic activity of 29.74±1.14 AU against *V.fluvialis* after 144 h Also *B.hwajinpoensis* HMA123 showed 89% of the maximum activity after only 96 h (Figure 6).

Characterization of the anti-bacterial agents of *B. hwajinpoensis*HMA123

Effect of proteolytic enzymes

The cell free culture of *B. hwajinpoensis*HMA123 supernatantwas exposed to different proteolytic enzymes (proteinase K, 20 mg ml^{-1}; Trypsin, 5 mg ml^{-1} and subtilisin, 100 mg ml^{-1}). it was found that the used enzymes showed no inhibition effect on the bioactivity of the tested supernatant of *B. hwajinpoensis*HMA123 compared to the untreated supernatant this indicates the obtained bioactive product hasn't protein nature**.**

Chemical characterization of crud bacterial extract

The GC-MS of *B. hwajinpoensis* HMA123 crude extract determined the main constituents and the relative percentage of the identified compounds. Six compounds were detected (Table 5, Figure 7). The gas chromatography revealed high amount (25.27%) of fatty acids (hexadecanoic acid methyl ester**,** 10-octadecenoic acid methyl ester and octadecenoic acid methyl ester**)** and other compounds such as tetracosane (16.06%), hentriacontane (26.65%) and eicosane (32.02%).

DISCUSSION

Marine particles are hot spots for microbial activity, and molecular phylogenetic analysis has established that the dominant species of particle-attached bacteria are different from those free living in the surrounding seawater[9].

The total bacterial load in marine environment was enumerated for the isolation of antagonistic marine bacteria. Also select potential probionts from marine environment, like seawater and sediment as biocontrol against pathogenic *Vibrio sp*.[28].

Sediment samples from Alexandria Western Harbor, exhibited higher load of total bacterial communities(10^4- 10^5CFU g-1) comparing with seawater samples (103-10^4CFU ml^{-1}) during the different five seasons and affected by environmental temperature (r=0.69). Also the occurrence of *Vibrio* sp. in sediment (1.92%- 32.38 %) is higher than in seawater samples (0.23 % - 13.21%), Saravanakumar et al. (2011)[4] reported that the total bacterial load of sediment ranged from 10^5 -10^6 CFU g^{-1}. The bacterial composition of marine sediment predominantly consisted of *Bacillus* sp. (40%), followed by *Vibrio* sp. (31.42%), *Flavobacterium* sp. (8.57%), *Alteromonas sp*. (5.71%), *Staphylococcus* sp. (5.71%), *Micrococcus* sp. (5.71%) and *Pseudomonas* sp. (2.85%).

Change in temperature is often a major environmental factor in triggering waterborne disease outbreaks. Previous research has revealed temporal and spatial patterns of bacterial population in several aquatic ecosystems[29]. Changes in global climate have raised concerns about the emergence and resurgence of infectious diseases. *Vibrio* sp. a reemerging pathogen that is proliferates and transported on marine particles. Patterns of disease *Vibrio* sp. outbreaks correlate with sea surface temperature increases, but the underlying mechanisms for rapid proliferation of *Vibrio* sp. during ocean warming events have yet to be fully elucidated[9]. Long *et al*. (2005)[9] suggested that marine bacterium bacterium antagonism is a contributing factor in regulating the proliferation of *Vibrio* sp. on particles. Importantly, autochthonous bacteria appear to become less inhibitory against *Vibrio* sp. at elevated temperatures. Hence, as sea surface temperatures increase due to changes in global climate, reduced competitiveness from other autochthonous microbes may contribute to increasing abundance and geographic spread of this and other pathogens.

The screening for anti-*Vibrio* activity in the present study revealed that 31% of marine bacteria inhibited at least one of the target strains. (*V. fluvialis*,*V. harveyi* or *V. damasi*).

Jayanth *et al*. (2002)[30] reported that antagonistic marine bacteria, which were tested for their ability to inhibit the growth of tested organisms showed that out of 62 antagonistic marine bacteria 18 (29.03%) had the ability to inhibit at least any one of the tested organism.

The marine sediment associated bacteria have been extraordinary significance in sever areas of science and medicine. The marine represent an under explored environment for microbial discovery[4].

The study explained that the higher incidence of antagonistic activity (33 %) was detected in sediment attached bacteria comparing with free cells from seawater samples (29 %).

Particle-associated bacteria also aggressively employ antagonistic interactions against other bacteria, perhaps to limit competition in these nutrient-rich microenvironments[9]. Research was centered on secondary metabolites that are produced when cellular growth stops and synthesized as mixtures of chemically related compounds, with a huge variety of chemical structures, as a consequence of the diversification and branching of their biosynthetic routes. The factors that trigger the production of secondary metabolites are not well known, but they can be produced when some nutrient in the environment is in limited supply, altering the production of primary metabolites, giving rise to enzyme inductors that lead to secondary metabolites[31].

Considering the pervasive nature of antibiosis, it was reported that the antagonistic interactions between marine bacteria and *Vibrio* sp. impede the latter from colonizing and proliferating on the surfaces and the elevated water temperatures affect such interactions. The *Vibrio cholerae* strains were sensitive to far fewer antagonistic interactions at 30°C. The relationship between antagonism and temperature is particularly intriguing in view of the increased occurrence of *Vibrio* sp. outbreaks. It has been proposed that climate changes are expanding the range of pathogenic organisms both spatially and temporally[9]. The seasonal variation during 2012 explained that, the highest percentage of antagonistic activity was detected in summer. In overlap season (winter 2012) when the temperature of sediment increased about 0.67°C the antagonistic activity increased to 40%.

Long et al.(2005)[9] tested the hypothesis that autochthonous marine bacteria impede the spread of *V. cholerae* in the marine environment. It was found that some marine bacteria are capable of inhibiting the growth of *V. cholerae* on surfaces and that bacterial isolates derived from pelagic particles show a greater frequency of *V. cholerae* inhibition than free-living bacteria. *Vibrio cholerae* was less susceptible to antagonism at higher temperatures. Bacillus species exhibit a wide range of physiologic abilities that allow the organism to flourish in every environment and compete favorably with other organisms within the environment, due to its ability to form spores produce metabolites that are heat stable, and have antagonistic effect on other microorganisms[32].

Systematic studies of the *Bacillus* group have been biased towards terrestrial and pathogenic isolates, and relatively few studies have examined Bacillus species from marine environments. In previous study, twenty Bacillus strains from diverse marine environments and sequenced their 16S rRNA. Using molecular comparisons, the strains were separated into thirteen Bacillus genotypes and identified 9 species: *B. hwajinpoensis, B. aquaemaris. B. badius, B. cereus group, B. firmus, B. halmapalus, B. litoralis, B. sporothermodurans and B. vietnamensis*[33]. *Also* Yoon et al. (2004)[34] reported that *B. hwajinpoensis*, novel members of Bacillus rRNA group 6 isolated from seawater of the East Sea and the Yellow Sea in Korea.

The antagonistic activity of Bacillus against a number of pathogens has been reported in many literatures[35, 36, 37] In the present communication, the anti-*vibrio* activity of a marine *B. hwajinpoensis* HMA123 strain is reported. The gram-positive bacterium *Bacillus subtilis* produces a large number of antibiotics, which are classified as ribosomal or nonribosomal[35].

The bacterial strain *Bacillus subtilis* UTM 126 produced antimicrobial activity against pathogenic *Vibrio* species, including *V. alginolyticus, V. parahaemolyticus, and V. harveyi*[36].

In the present study the most potent strain which identified as *B.hwajinpoensis* HMA123 displayed a wide spectrum of antagonistic activities against V. *fluvialis ,V. harveyi* and *V. damasi.* And other Gram negative

bacteria as *Aeromonas sp.* and *P. aeruginosa*, also against member of Gram-positive bacteria as *S. faecalis* and *B. subtilis*.

Phylogenetic analysis of the sediment associated producer strains showed that 12 strains are clustered within the Firmicutes group belonging to several *Bacillus* sp. and *Halobacillus sp*. with 94 – 98% similarity between them. Of the 35 isolates tested, 42% showed antagonistic action against pathogens. The marine sediment associated bacteria shall therefore be evolved as putative probiotic bacteria to counteract fish/shrimp disease problems[4].

In previous study, by screening for antibacterial substance producing (twenty nine) Bacilli isolated from Lonar lake, all the bacillus species showed that the antimicrobial activity against the *E. coli* (100%), out of twenty nine, twenty seven (93%) Bacillus species showed antimicrobial activity against *K. pneumoniae*, seven (24%) were antimicrobial against to *P. vulgaris* and nine (31%) were found antimicrobial against *E. aerogenes* and twelve (41%) Bacillus species showed antimicrobial activity against *S. aureus*[37].

Data obtained from the experimental growth of the environmental marine strain and extraction of their bioactive products showed that the organic production increased significantly between 96 and 120 h compared to 24, 48 and 72 h. The best inhibition effect was recorded when bacteria entered the stationary phase[31]. Also, Isnansetyo et al. (2009)[38] reported that strain S2V2 produced extracellular non proteinaceous antibacterial substances. The highest antibacterial activity was found when strain S2V2 was cultured for 96 h in ZoBell broth medium.

Most of the bioactive agents have been isolated from *Bacillus*, *Streptomyces*, *Alteromonas*/*Pseudoalteromonas*, *Pseudomonas*, and *Cytophaga* obtained from seawater, sediments, marine algae, and invertebrates that produce quinones, polyenes, macrolides, alkaloids, peptides, and to a smaller extent terpenoids. It has been determined that some products obtained from marine bacteria have bioactive effects against other marine bacteria[31, 39, 40].

The genus Bacillus has been in use in the biotechnology industry for a very long time with a number of new cultures exhibiting a variety of benefits to humans. Members of the Bacillus genus are often considered microbial factories for the production of a vast array of biologically active molecules potentially inhibitory for pathogens growth[10]. These bacteria in general represent a new and rich source of secondary metabolites that need to be explored [37]. It is therefore necessary for isolation and purification of the chemical substances (Metabolites) for detail chemical studies in order to determine their composition and structures[32].

Representative of the Bacillus genus and biologically active metabolites produced by them in addition to their practical application in various branches of the economy have been widely studied in the scientific literature. This confirms that continued comprehensive recherché in them advisable[41].

The GC-Mass spectrophotometry of *B. hwajinpoensis* HMA123 crude extract revealed the existence of high amount of fatty acids methyl esters (25.27%) and other compounds such as tetracosane (16.06%), hentriacontane (26.65%) and eicosane (32.02%). The fatty acids methyl esters are known to have potential antibacterial and antifungal principle for clinical application[42]. Fatty acids are nontoxic compounds that show bactericidal effects and have been investigated for many years. These fatty acids have been incorporated in foods with the purpose of preventing the action of pathogens like *Salmonella*, *Listeria* and *Staphylococcus.* Also previous study isolated a fatty acid from the cyano bacteria *Lyngbya majuscula* with activity against *Candida albicans*[31]. *The* phytochemical profile and the protective effects of *Ceratonia siliqua* pods essential oil (CsEO), a food and medicinal plant exhibited moderate to strong antimicrobial activity against the tested species (13 bacteria and 8 fungal strains) strains using agar diffusion and broth micro dilution methods. Twenty five different components were identified in the CsEO. Among them, the major detected components were: nonadecane, heneicosane, naphthalene, 1,2-benzenedicarboxylic acid dibutylester, heptadecane, hexadecanoic acid, octadecanoic acid, 1,2-benzenedicarboxylic acid, phenyl ethyl tiglate, eicosane, farnesol 3, camphor, nerolidol and n-eicosane[43].

CONCLUSION

This study explained that the density of *Vibrio* sp. and the antagonistic activity of marine bacteria affected by seasonal variation and elevated temperature due to climatic change. Also this study provides primary evidence that *B. hwajinpoensis HMA123* strains are novel promising sources for antibacterial substance. These bacteria in general represent a new and rich source of secondary metabolites that need to be explored, but the challenge remains to purify and elucidate the chemical structure of its inhibiting metabolites. These active products might be useful for controlling *V. fluvialis* and other pathogenic bacteria.

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