Global Veterinaria 17 (6): 495-504, 2016
ISSN 1992-6197

DOI: 10.5829/idosi.gv.2016.495.504

Production of Antimicrobial Pyrrol-Derevatives Acting Against Some Fish Pathogens from Marine *Bacillus pumilus*mmm

[1]*Mona A. Malash,* [1]*Manal M. El-Naggar,* [1]*Hala E. Abou El Hassayeb and* [2]*Madiha Salah Ibrahim*

[1]Microbiology Laboratory, Marine Environmental Division,
National Institute of Oceanography and Fisheries, Alexandria, Egypt
[2]Department of Microbiology, Faculty of Veterinary Medicine, Damanhour University, Egypt

Abstract: The need for the development of new antimicrobials to overcome drug resistance in bacterial pathogens has been stressed by various researchers worldwide. Working on marine microbial resources may lead to the discovery of an alternative antimicrobial agents added to the commercial used ones. In this study several marine bacterial isolates were screened for their ability to produce anti-microbial agent(s) against *Aeromonas Hydrophila* using agar well diffusion method. The potent antimicrobial producer was isolated from the western harbor of Alexandria and identified as *Bacillus pumilus* MMM using the 16S rRNA gene sequence, it showed inhibition zone of 20mm. The optimization process was performed using the Plackett –Burman experimental design. The optimized luria broth culture medium increased the antimicrobial activity, the inhibition zone was 30 mm. The extraction was proceeded using ethyl acetate, it led to more antimicrobial activity the inhibition zone was 33 mm. Partial characterization of the ethyl-acetate extract of *Bacillus pumilus* MMM was performed using the GC Ms spectrophotometry, this analysis indicated the most probable effective compound as anti-microbial agent according to the obtained data was pyrrolo [1, 2-a] pyrazine-1, 4-dione derivatives. It showed also inhibitory effect against *Vibrio parahemolyticus*, *Pseudomonas aeruginosa* and *Staph aureus*. The mode of action of this pyrrolo- derivatives secondary metabolite on the *Aeromonas hydrophila* cells was estimated though the Transmission Electronic Microscope (TEM) examination it led to a rupture in the bacterial cell wall, intracellular granulation and a sever damage for the nucleus compared to the untreated cells.

Key word: Antimicrobial-*Aeromonas hydrophila* · *Bacillus pumilus*-Pyrrol · Fish pathogen

INTRODUCTION

Bacterial diseases in cultured fish are considered the main problem to aquaculture system. *Aeromonas hydrophila* is among the most common bacteria in freshwater and sea water habitats throughout the world. Genus *Aeromonas* includes prominent microbiota in freshwater reservoirs where they together with other microorganisms act as natural bio-filters and promote self-purification of the water body. They are necessarily present in normal microflora and hydrobionts inhabiting fish reservoirs [1]. They frequently cause problems in both feral and cultured fish [2]. Motile *Aeromonas septicemia* (MAS) is a more dramatic bacterial disease affecting various species of fish and shellfish, feral as well as farmed in both fresh and seawater, cause a serious problem for the fish farming industry in Egypt as well as in other countries and is responsible for heavy economic losses caused with high mortality [3]. The course of the disease usually runs in an acute manner. Clinical conditions associated with systemic infection result in mortality within 24–48 hours. In more chronic types of clinical conditions, eroded fins occur as well as skin lesions and sluggish swimming [4]. Antibiotic resistance of *Aeromonas hydrophila* isolated from marketed fish and prawn was reported [5]. *Aeromonas* species are responsible for wide range of human diseases that vary from self-limiting gastroenteritis to severe fatal septicemia and traveler's diarrhea, in addition to extra intestinal symptoms as meningitis and endocarditis [6].

Corresponding Author: Madiha Salah, Department of Microbiology, Faculty of Veterinary Medicine, Damanhour University, Egypt. E-mail: madihasalah@yahoo.com.

A number of valuable antimicrobial and metabolites have been derived from terrestrial microorganisms (99% of the known microbial compounds) although efforts in this area have diminished since the late 1980s because of the feeling that this resource has been exhaustively studied [7]. In this respect, researchers switched over to new environments for novel pharmaceutical compounds. Over 70% of the earth's surface is covered by oceans and it is believed that life originated in the oceans. Additionally, the oceans are considered as a source for natural products mainly accumulated in marine living organisms. Several bioactive compounds of therapeutic interest have been isolated from marine invertebrates and some of them were reported to be of microbial origin. The marine microorganisms are widely distributed in oceans all over the earth and acting as a great source for the discovery of different natural products [8]. Numerous of these compounds show pharmacological activities and are helpful for the invention and discovery of bioactive compounds have antimicrobial activity such as bacteriocins [9]. Further studies were developed to obtain therapeutic agents as antibacterial [10-11], antifungal [12], antitumor [13, 14] and anti-parasitic, insecticidal, antiviral and anti-inflammatory and antioxidant [7,15-17]. From genus Bacillus several antimicrobial compounds were developed [16 – 19]. this study aims to screening for marine antimicrobial agent(s) against *Aeromonas hydrophila*, using bacteria isolated from seawater and sponge collected from different stations of the Western Harbor of Alexandria (Egypt), Identification of the most potent isolate by 16s rRNA, optimization of production anti-microbial agent(s) to reach the ideal conditions for maximum bioactivity using a Plackett-Burman experimental design [20], extraction of the crude material using ethyl acetate [21], characterization of antimicrobial component to determined most probable anti-microbial component by using GC Ms [22] and finally determine mode of action by observing change in the cell wall by using transmission electronic microscope TEM [23].

MATERIALS AND METHODS

Isolation and Identification: Thirty five marine bacterial isolates were obtained from Seawater and sponge samples from Western Harbor, Alexandria, Egypt. These strains were cultured in nutrient agar slants for 24 h. screening for the anti-microbial activity was carried out by using the agar well diffusion method [24], in this method all marine strains were inoculated into 100 ml of nutrient broth individually. The inoculated strains were broth cultured in a rotary shaker at 120 rpm for 2 days at temperature (30°C). Then the broth culture was centrifuged on two run first on 12000 rpm for 15 minute then 10000 rpm for 10 minute to obtain supernatant free from cells, 50 µ of tested fish pathogen (*Aeromonas hydrophila*) (OD =0.8) (*Aeromonas hydrophila* obtained from Animal Health Research Institute, Alex, Egypt) were seeded in 50 ml nutrient agar and poured into petri dish (9 cm in diameter) and wells 10 mm in diameter were punched in the agar with a sterile tip, 75 µ of each extract were put in each well then incubated for 24-48 hour to observe zone of inhibition and measure diameter. The most potent isolate was identified using microscopic examination, biochemical examination and PCR detection using 16S rRNA sequencing molecular technique. This process was carried out at NIOF (National Institute of Oceanography and Fisheries in Alexandria, Egypt).

Effect of Different Cultural Media: Five different culture media were tested for the production of the anti-microbial activity. They were Nutrient broth, Zobell medium, Tryptone soya broth, Horikoshi medium and LB medium. This study was carried out in 250ml conical flask using 100 ml of each culture medium inoculated with the most potent anti-microbial isolate (M-6) then incubated in shaker incubator at 120 rpm and 30°C for 48 h were used to determine the best media for growth and production of the anti-microbial activity.

Optimization the Production of the Anti-Microbial Agent Using

Plackett-Burman Experimental Design: The Plackett-Burman experimental design, a fractional factorial design [20] was applied in this study to reflect the relative importance of various nutritional and environmental factors on the production of anti-microbial agent(s). Seven independent variables representing components of LB medium plus some of physiological factors were screened in nine combinations organized according to the Plackett-Burman design matrix. For each variable of the basal medium, a high (+) and low (-) level was tested. The factors tested were (LB medium; tryptone (TY) 10gm; yeast extract (YE) 5gm and Sodium chloride (NaCl) 10gm; Inoculum size (IS) 2ml; pH level (pH) =7, temperature (Temp) 30°C and Incubation period (IP)=48h.

The main effect of each variable was determined using the following equation

Exj= (Mi+ – Mj-) /N

where Exj is the variable main effect, Mi and Mj are the + - anti-microbial activity in trials were the independent variable (Xj) was present in high and low levels, respectively and N is the number of trails divided by 2. A main effect figure with a positive sign indicates that the high level of this variable is nearer to optimum. Using Microsoft excel, statistical t-values for equal unpaired samples were calculated for determination of variable significance [25].

Verification of Plackett-Burman Experiment: In order to validate the obtained results and to evaluate the accuracy of the applied Plackett-Burman statistical design, a verification experiment was carried out in triplicates. According to the main effect results, the predicted near optimum and far from optimum levels of the independent variables were examined and compared to the basal condition settings [26].

Extraction of Antimicrobial Agent(s) Using Ethyl Acetate: The *Bacillus pumilus* MMM cultured on the optimized LB medium was centrifuged and the supernatant was extracted using equal amount of ethyl acetate (1:1 vol/vol). The extract was completely dried using a rotary evaporator then the antimicrobial activity was estimated [21].

The Broad Spectrum of Ethyl Acetate Extract of *Bacillus pumilus* MMM: The effect of ethyl acetate extract of *Bacillus pumilus* MMM on different bacterial fish pathogens (*Aeromonas hydrophila, Staph. Aureus, Pseudomonas aeruginosa, Vibrio parahemolyticus and E.coli*) were tested using the agar well diffusion method according to Sharma *et al*. [24].

Determination of the Mode of Action: The mode of action of the *B. pumilus* MMM supernatant and ethyl acetate extract on *A. hydrophila* was detected and compared with the normal activated cells. (1ml) of *Aeromonas Hydrophila* was incubated separately for 24h in presence of both the supernatant and the ethyl extract of *Bacillus pumilus* MMM (1:1 vol/vol). These cells were harvested using centrifugation for 5min at 5000rpm and then examined using transmission electronic microscope (TEM) (at the Central lab, National Institute of Oceanography and Fisheries, Alexandria, Egypt), compared to the normal cells according to Padmavathy and Vijayaraghavan [23].

Partial Characterization of Anti-Microbial Extract of *Bacillus pumilus* MMM: For a partial chemical characterization of the anti-microbial ethyl acetate extract of *B. pumilus*MMM a gas chromatography mass spectrometry (GC-Ms) (Trace DSQII MS) was used at the Marine pollution lab, National Institute of Oceanography and Fisheries, Alexandria, Egypt according to Jasim *et al*. [22].

RESULTS

Isolation and Identification Process: Thirty five marine microbial isolates were screened for their ability to produce anti-microbial agent(s) using agar well diffusion. The results showed that only seven bacterial isolates were able to produce antimicrobial agent(s) against *Aeromonas hydrophila*, four of them were Gram positive, three Gram negative and inhibition zones ranged from (7 to 15) mm. The Microscopic examination showed the most potent isolate (M-6) was a Gram positive rod shape bacilli, spore forming bacteria. While, the Biochemical examination using of VITIK® systems at Mabaret El- Asafra lab in Alex, Egypt, indicated the isolation of *Bacillus pumilus* with probability 99%.

Genotypic Characterization: Bacterial isolate (M-6) which isolated from seawater samples collected from Western Harbor of Alexandria (Egypt) was identified using 16S rRNA. Genomic DNA of bacterial isolate was extracted and the gene coding for the 16S rRNA was partially amplified using the primer F: AGA GTT TGA TCC TGG CTC AG and R: GGT TAC CTT GTT ACG ACT T. The amplified PCR fragment of the potent bacterial isolate was purified and detected using agarose gel electrophoresis. The full sequencing to the PCR product was carried out using ABI 3730xl DNA Sequencer. The obtained sequence was sent to the Genbank to be compared with other well-known bacterial strains. It was found the most potent isolate (M-6, antimicrobial producer) was identified as *Bacillus pumilus* strain MMM with association number KX610081.

Effect of Different Culture Media: The influence of different culture media on the production of anti-microbial agent (s) by *B.pumilus* MMM was recorded. Five different recommended culture media were tested. It was observed that LB medium increased the anti-microbial activity where the resulted inhibition zone was 20 mm compared to the other tested culture media; nutrient broth medium (15 mm), TSB (15 mm), Horikoshi medium (10 mm) and Zobell medium (10mm).

Optimization of Antimicrobial Agent(s) Production Using Plackett-Burman Design and the Verification Experiment: The components of the best culture medium (LB medium) for *B. pumilus*MMM in addition to some of the physiological conditions were optimized to maximize the anti-microbial activity using the Plackett-Burman statistical design. The main effect of each variable upon the anti-microbial activity of *B. pumilus* MMM was estimated and the determination of variable significance, statistical t-values for equal unpaired samples were also calculated with respect to observations and presented graphically in Fig. (1). It was showed that high level of sodium chloride, inoculum size, yeast extract affected positively on the anti-microbial activity. While the use of the low levels of tryptone, temperature, pH and incubation period resulted in increasing the anti-microbial activity. From the obtained data it can observed the most critical tested factor was the incubation period where on examining the high level (72h) it led to nil anti-microbial activity. So, the results indicated the anti-microbial activity with an inhibition zone of 30mm can be obtained through this optimized medium composed of; (TY) 5 gm, (NaCl) 15 gm, (YE)7gm, (IS) 3ml, (IP) 24 h, (pH) 6 and (Temp.)25°C.

Antimicrobial Activity of the Ethyl Acetate *B. Pumilus* MMM Extract: The results indicated that the obtained antimicrobial activity was increased where the inhibition zone was 33mm compared to the crude supernatant (30mm) (Fig. 2).

Moreover, the bioactivity of ethyl acetate *B. Pumilus* MMM extract was tested using other fish pathogens. It showed inhibition zones as follow; 15 mm against *Vibrio Parahemolyticus,* 15mm against *Staph Aureus* and 10 mm against *Pseudomonas Parahemolyticus.* On the other hand, it showed no bioactivity against *E. coli.*

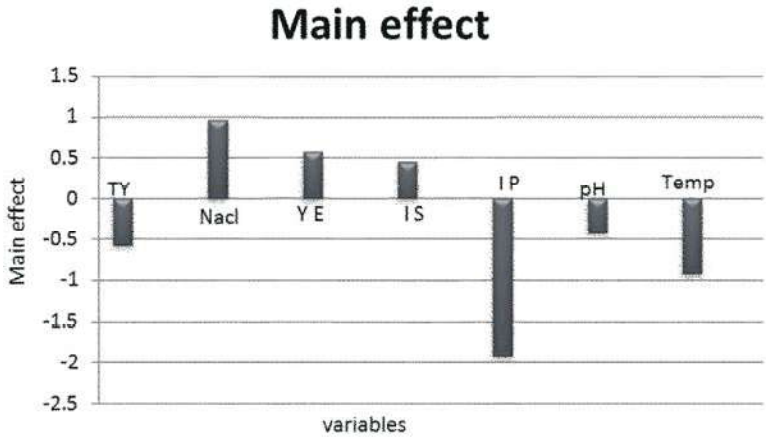

Fig. 1: The main effect of the tested cultured variables affecting the production of anti-microbial agent(s) by *B. pumilus* MMM using Plackett-Burman experimental design.
* TY (Tryptone), NaCl (Sodium Chloride), YE (Yeast Extract), IS (Inoculum Size), IP (Incubation Period), Temp (Temperature).

Table 1: Verification of Plackett- Burman experimental design for anti-microbial activity of *B. pumilus*MMM

Level	TY (gm)	NaCl (gm)	YE (gm)	IS (ml)	IP (h)	PH	Temp°C	Inhibition zone (mm)
Optimized medium	5	7.5	15	3	24	8	25	30
Basal medium	10	5	10	2	48	7	30	20
Anti-optimized medium	15	2.5	5	1	72	6	40	0

* TY (Tryptone), NaCl (Sodium Chloride), YE (Yeast Extract), IS (Inoculum Size), IP (Incubation Period), Temp (Temperature).

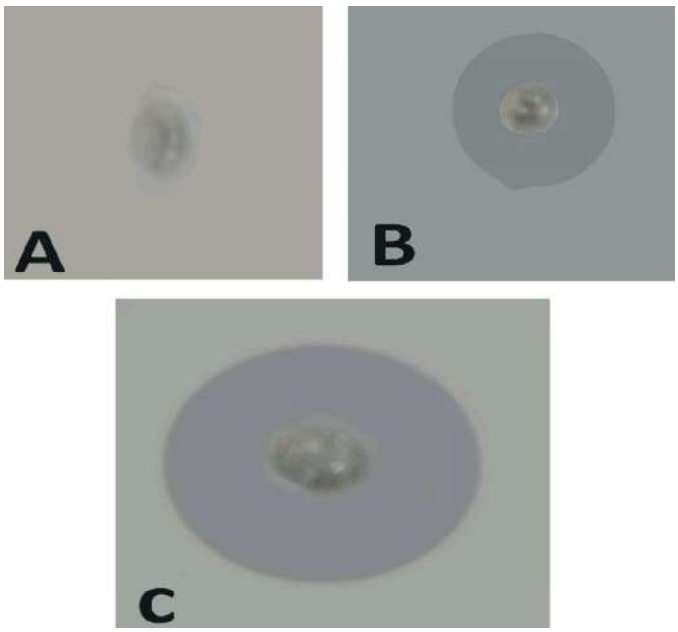

Fig. 2: Plate photographs show results of modified agar well diffusion method, untreated *A. hydrophila* cells (control) (A), inhibition zone of *A. hydrophila* cells caused by the secondary metabolites of *B pumilus*MMM before physiological optimization (B), after optimization process (c, inhibition zone caused by optimized secondary metabolites of *B. pumilus* MMM)

Table 2: Integration Peak List analysis of *B. pumilus*MMM ethyl acetate extract using GC-Ms.

Suggested Compound	Retention time (min)	Area (%)	Probability (%)
Ethanol, 2-(2-butoxyethoxy)-, acetate	11.307	1.5	85.9
Acetic acid, 4-(1H-indol-4-yl)-2-methyl-but	11.908	5.7	13.2
Hexadecane	15.269	1.7	18.0
*Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	19.583	5	41.2
1-Eicosanol	20.172	3.1	2.42
Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	21.316	20	70.1
: Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	21.783	15	58.2
Hexadecanol	23.886	1.9	6.14
Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	29.719	11.08	80
Phenol, 3-[4-(2,3-dihydro	34.945	35.02	2

Partial Characterization of *Bacillus pumilus* MMM Ethyl-acetate Extracts Using Gas Chromatography-Mass Spectrophotometer (GC-Ms): The GC-Ms analysis of ethyl-acetate fractions indicated the presence of different chemical components as shown in table (2). It was obtained that the probability of the total pyrrol- derivatives was ranged from 41.2 to 80% compared to the indexed data and they resampling 51.8 % from the total obtained area. This result may indicated the most probable effective anti-microbial agent obtained from the marine *Bacillus pumilus* MMM and acting against *A. hydrophila* cells was Pyrrolo [1,2-a]pyrazine-1,4-dione] derivatives.

Mode of Action Using Transmission Electronic Microscope (TEM) Examination: The action of both the supernatant secondry metabolits and the ethyl acetate crude extract of *B.pumilus*MMM on *A. hydrophila* cells was estimated compared to the normal untreated cells. It was showed these tested secondry metabolits led to a sever rupture in the cell wall, granulation inside the bacterial cell and massive destruction of nucleus especially on using the ethyl-acetate extract Fig. (3).

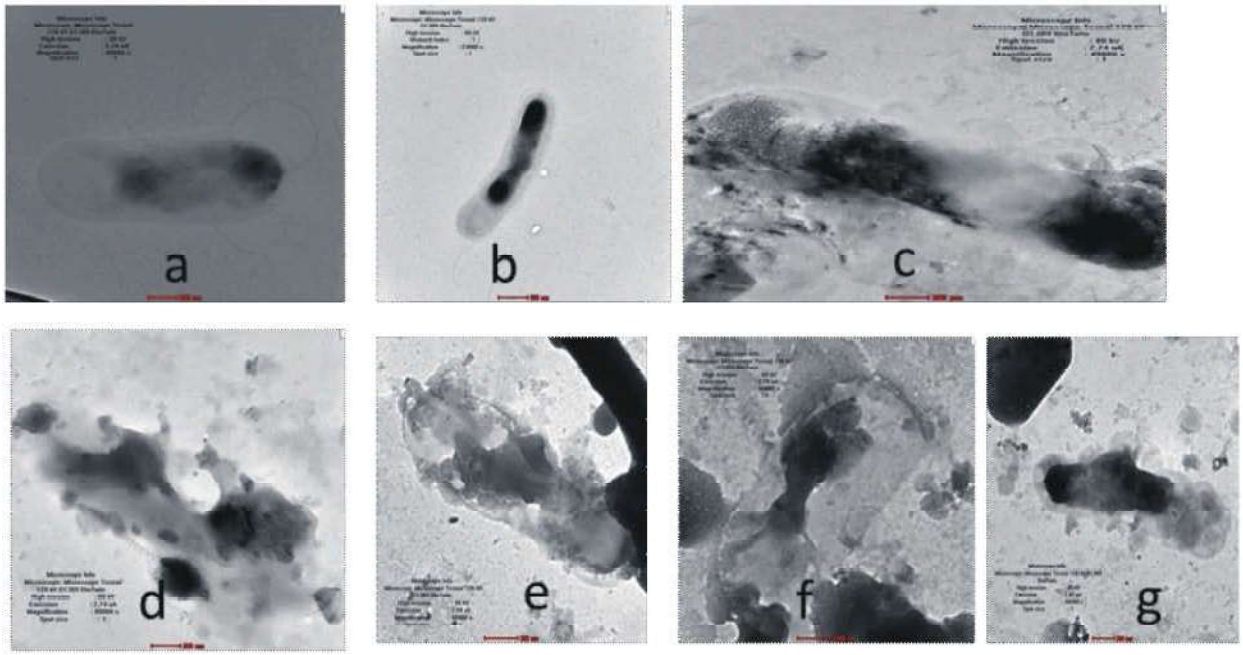

Fig. 3: TEM micrographs showing; normal and untreated *A. hydrophila* cells (a & b), *A. Hydrophila* cells treated with *B.pumilus* MMM supernatant (c & d) and *A. hydrophila* cells treated with *B. pumilus* MMM ethyl acetate crude extract (e, f & g)

DISCUSSIONS

In the present study agar well diffusion method was used to assay anti- microbial activity against *Aeromonas hydrophila*. Agar well diffusion method is widely used to evaluate the antimicrobial activity of plants or microbial extracts [20]. In present study 35 marine bacterial isolates were screened against *A.hydrophila* using Agar well diffusion method. It was found that seven isolates showed antimicrobial effect against *A. Hydrophila* and the most potent isolate acting against *A. hydrophila* was identified as *Bacillus pumilus* MMM strain using the 16s rRNA gene. Similarly, many authors used Bacillus species for producing several pharmaceutical products since they have a wide range of biological activities as anti-fungal agents [27] , anti-viral agents [28] anti-ameobocytic agents [29] and anti-mycoplasma agents [30].

Although it had been observed that strains of *Bacillus pumilus* produce substances with antibacterial activity, very little work has been done to isolate these substances. Gilliver [31] observed the antibacterial activity of strains of *B. pumilus*, but made no attempt to isolate the active principles. Dvonch and Benedict isolated an antibacterial agent from a strain of *B. pumilus* which they showed to be similar to subtenolin, an antibiotic from *B. Subtilis* [32]. Pumilicin 4, a novel bacteriocin with anti-MRSA and anti-VRE activity produced by newly isolated bacteria *Bacillus pumilus* strain WAPB4 [33].

The present study showed that culture media can enhance the anti-microbial activity, where the LB medium gave inhibition zone 20 mm against *Aeromonas hydrophila* and NB medium gives 15 mm In agreement with the results of this study Thakur *et al*. [34] showed variations in the type of nutritional sources such carbon and nitrogen sources besides changes in pH, temperature, incubation period, shaking and inoculum size of the antagonistic fungal strain which can greatly influence antibiotic biosynthesis. Statistical experimental designs were applied here as powerful tools for searching the key factors rapidly from a multi-variable system and minimizing the error in determining the effect of parameters and the results are achieved in an economical manner. Plackett-Burman Design, an efficient technique for medium component optimization, was employed to identify significant variables that enhance anti-microbial compound(s) production and to find out their probable optimal levels in a limited number of experiments [25].

Development of an efficient production process of secondary metabolites by microorganisms requires examination of a diverse array of species-specific features, including physical and chemical factors. Nutritional and

environmental factors play key roles in cell metabolism. Also several cultivation parameters like pH, incubation period and temperature play a major role in the production of bioactive metabolites [35]

In this study response to placket burmen design showed wide variation ranging from 33 mm zone of inhibition to nil activity corresponding to combined effect of seven parameters. The results indicate high anti-microbial activity inhibition zone of 30mm through the optimized medium (TY) 5 gm; (NaCl) 15 gm; (YE)7, 5 gm; (IS) 3ml; (IP) 24 h ;(pH) 6 and (Temp)25 °C.It was found that critical factor affect ing production of antibiotic is incubation period 24 h. In agreement with the results of the present study Awis *et al*. [36] Found that incubation period effect on production of antibiotic from *Bacillus Subtilis*, where free cell synthesis of peptide antibiotics, maximum activity (14mm was seen during 24 to 48 hours of incubation, which abruptly decreased at 72 hours, also it Sharon *et al* found that incubation period effect on production of antibiotic from marine actinomycetes, where maximum inhibition was achieved for cultures incubated for 7 days. However after 7 days of incubation there was a decline phase in the diameter of inhibition zone [37].

Results of the present study indicated that, the highest anti-microbial activity had obtained when crude ethyl-acetate extract used was 33 mm Similarly, Beriæ *et al* [38] used the ethyl acetate as a solvent to extract antimicrobial lipopeptide from *Bacillus* sp. In this study it was found that crude ethyl acetate extract have also antimicrobial activity against *Vibrio parahemolyticus* (15 mm), *Staph. Aureus* (15mm) and *Pseudomonas parahemolyticus* (10 mm) while it gave no activity against *E. coli*.

Padmavathy proved that ZnO nanoparticles have high bactericidal effect due to damage of the cell membrane of bacterial cell by using TEM [23], The present study showed that *Bacillus pumilus* extract have sever effect on *Aeromonas Hydrophila* bacterial cell, it showed lysis in cell wall, intracellular granulation and damage in nucleus so extract had bactericidal effect on bacteria [39].

By analysis of ethyle acetate crude extract with GC Ms, it was found that there are four peaks correspond to pyrrol compound Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), pyrrolo[1,2-a]pyrazine-1,4-dione and (phenylmethyl)-pyrrolo[1,2-a]pyrazine-1,4-dione.

It was obtained that the probability of the total pyrrol- derivatives was ranged from 41.2 to 80% compared to the index data and they resampling 51.8 % from the total obtained area. Pyrrol derivatives compounds had been reported to possess promising antimicrobial activity [40, 41], nematicidal activity [42] and antioxidant activity [43].

Another study conducted by Devi and Wahab [44] illustrated that hexahydro 3-(2-methylpropyl) - pyrrolo [1, 2-a] pyrazine-1, 4-dione in endophytic fungi isolated from Camellia sinensis possess strong antimicrobial activity.

The first marine bacterial metabolite to be reported was the highly brominated pyrrole antibiotic. This was isolated by Burkholder and co-workers [45] through fermentation studies of a Gram-negative bacterium obtained from the surface of the Caribbean Sea grass Thalasia.

On the other hand, Sathiyanarayanan *et al.* [46] reported that the active compound produced by pyrrolidone as a major part of crude extract was found to have prominent antimicrobial potency. The NMR data confirms the presence of pyrrole derivative. Pyrrole is an organic heterocyclic compound of five-membered diunsaturated ring structure composed of four carbon atoms and one nitrogen atom. Pyrrolidone is a keto pyrrole, which is a 5-membered lactam structure compound (gamma-butyrolactam). Also, Mithun and Ramachandra Rao [47] isolated a bacterial strain Micrococcus luteus that is capable of producing an anti-cancer agent which was identified as pyrrolo (1, 2-alpha) pyrazine1, 4 Dione hexahedron 3-(2-methylpropyl). Production of Anti-Inflammatory Pyrrol Compound from Marine Bacillus baekryungensis AMHSU was reported [48].

CONCLUSIONS

Bacillus species are known for production of many bioactive products, *Bacillus pumilus* MMM which isolated from sea water from western harbor of Alexandria, Egypt. *Bacillus pumilus* are able to produce anti-microbial agent against *Aeromonas hydrophila* isolated from fish, The results indicate high anti-microbial activity with inhibition zone of 30 mm through the optimized LB medium (TY) 5g, (NaCl) 15 gm, (YE) 7,5gm, (IS) 3ml, (IP) 24 h, (pH) 6 and (Temp) 25oC. It was found that ethyl acetate extract increase inhibition zone from 30 to 33mm, by analysis of ethyl-acetate extract of *Bacillus pumilus* with GC-Ms it was found that it is mainly pyrrol derivatives compounds which had been approved to have anti-microbial effect this compound make sever damage in *Aeromonas hydrophila* compared with normal cell.

ACKNOWLEDGMENT

Shimaa El-gebaly, Researcher of Fish Microbiology at Animal Health Research Institute,Alex,Egypt for providing us with fish pathogen

REFERENCES

1. Kompanets, E.V., N.M. Isaeva and I.A. Balakhnin, 1992. Bacteria of genus Aeromonas and their role in aquaculture. Microbiol. Zh, 54(4): 89-99.
2. Zhang, X., W. Yang, H. Wu, X. Gong and A. Li, 2014. Multilocus sequence typing revealed a clonal lineage of *Aeromonas hydrophila* caused motile Aeromonas septicemia outbreaks in pond-cultured cyprinid fish in an epidemic area in central China. Aquaculture, 432: 1-6.
3. Edward Noga, E.J., 2010. Text Book of Fish Disease: Diagnosis and treatment. 2nd ed. USA, Wiley-Blackwell, pp: 519.
4. Roberts, M.C., 1996. Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility and distribution. FEMS Microbiol. Rev., 19: 1-24.
5. Viswanatan, S., S. Manikandan and A. Haniffa, 2015. Evaluation of resistance against Antibiotics, Antiseptics and Disinfectansts in *Aeromonas hydrophila* isolated from Marketed Fishes. Pharmaceutical and Biological Evaluations, 2(2): 40-46.
6. Tsai, M., C. Kuo, M. Wang, H. Wu, C. Chein and J. Liu, 2006. Clinical features and risk factors for mortality in Aeromonas bacteremic adult with hematologic malignancies. J. Microbiol., Immunol. Infect., 39: 150-154.
7. Hunter, Pamela A., G.K. Darby and N.J. Russell, 0000. The need for new antibiotics: possible ways forward. Fifty-third symposium of the society for general microbiology bath, Vol. 1. No. 53. Cambridge University Press.
8. Olano, C., C. Méndez and J.A. Salas, 2009. Antitumor compounds from actinomycetes: from gene clusters to new derivatives by combinatorial biosynthesis. Nat. Prod. Rep., 26(5): 628-660.
9. lee, H.J., 2011. Review: Lantibiotics, Class I bacteriocins from the genus Bacillus. Journal of microbiology and biotechnology, 21(3): 229-235.
10. El-Gendy, B.E.D.M. and M.E. Rateb, 2015. Antibacterial activity of diketopiperazines isolated from a marine fungus using t-butoxycarbonyl group as a simple tool for purification. Bioorganic and medicinal chemistry letters, 25(16): 3125-3128.
11. Biswas, K., D. Paul and S.N. Sinha, 2016. Marine Bacteria: A Potential Tool for Antibacterial Activity. Journal of Applied and Environmental Microbiology, 4(1): 25-29.
12. El-Sheekh, M.M., S.M. El-Shafay, E.M. El-Ballat, 2015. Production and characterization of antifungal active substance from some marine and freshwater algae. international-journal, 6: 85-92.
13. Kumar, D. and S.R. Diwan, 2011. Marine natural alkaloids as anticancer agents. Opportunity. Challenge and Scope Nat. Prod: 213-268.
14. El-Naggar, M.M., S.A. El-Assar and A.M.A. Shata, 2015. Production of Antitumor Agents from Novel Marine Actinomycetes Isolated from Alexandria, Egypt. Single Cell Biology.þ thesis, Alex Univ, Egypt.
15. Tareq, F.S., M.A. Lee, H.S. Lee, Y.J. Lee, J.S. Lee, C.M. Hassan and H.J. Shin, 2014. Gageotetrins A–C, noncytotoxic antimicrobial linear lipopeptides from a marine bacterium *Bacillus subtilis*. Organic letters, 16(3): 928-931.
16. Prieto, M.L., L. O'Sullivan, S.P. Tan, P. McLoughlin, H. Hughes, M. Gutierrez and G.E. Gardiner, 2014. *In vitro* assessment of marine Bacillus for use as livestock probiotics. Marine drugs, 12(5): 2422-2445.
17. Chopra, L., G. Singh, V. Choudhary and D. K. Sahoo, 2014. Sonorensin: an antimicrobial peptide, belonging to the heterocycloanthracin subfamily of bacteriocins, from a new marine isolate, *Bacillus sonorensis* MT93. Applied and environmental microbiology, 80(10): 2981-2990.
18. Bacon, C.W., E.R. Palencia and D.M. Hinton, 2015. Abiotic and biotic plant stress-tolerant and beneficial secondary metabolites produced by endophytic bacillus species." Plant Microbes Symbiosis: Applied Facets. Springer India: 163-177.
19. Valgas, C., S.M.D. Souza, E.F. Smânia and A. Smânia Jr, 2007. Screening methods to determine antibacterial activity of natural products. Brazilian Journal of Microbiology, 38(2): 369-380.
20. Plackett, R.L. and J.P. Burman, 1946. The design of optimum multifactorial experiments. Biometrika, 33(4): 305-25.
21. Abu-Mejdad, N.M.J.A., F.L. Aaiz and O.T. Jassim, 2013. Antifungal activity OF ethyl acetate extract of four species Bacillus isolated from soil. Journal of American Science, 9:10.
22. Jasim, H., A.O. Hussein, I.H. Hameed and M.A. Kareem, 2015. Characterization of alkaloid constitution and evaluation of antimicrobial activity of Solanum nigrum using gas chromatography mass spectrometry[GC-MS]. Journal of Pharmacognosy and Phytotherapy, 7(4): 56-72.

23. Padmavathy, N. and R. Vijayaraghavan, 2008. Enhanced bioactivity of ZnO nanoparticles—an antimicrobial study. Science and Technology of Advanced Materials, 9(3): 035004.
24. Sharma, S.C.D., M.S. Shovon, M.S. Jahan, A.K.M. Asaduzzaman, M.A. Rahman and K. Biswas and Roy, 2013. Antibacterial and cytotoxic activity of *Bacillus methylotrophicus*-SCS2012 isolated from soil. The Journal of Microbiology, Biotechnology and Food Sciences, 2(4): 2293.
25. El-Sharouny, E.E., N.M. El-Toukhy, N.A. El-Sersy and A.A.E.A. El-Gayar, 2015. Optimization and purification of mannanase produced by an alkaliphilic-thermotolerant Bacillus cereus N1 isolated from Bani Salama Lake in Wadi El-Natron.Biotechnology, Biotechnological Equipment, 29(2): 315-323.
26. El-Naggar, M.M., U.M. Abdul-Raouf, H.A.H. Ibrahim and W.M.M. El-Sayed, 2014. Saccharification of Ulva lactuca via *Psoudoaltromonas piscicida* for biofuel production. Journal of Energy and Natural Resources, 3(6): 77-84.
27. Milner, J.L., S.J. Raffel, B.J. Lethbridge and J. Handelsman, 1995. Culture conditions that influence accumulation of zwittermicin A by *Bacillus cereus* UW85. Applied Microbiology and Biotechnology, 43(4): 685-691.
28. Steller, S., D. Vollenbroich, F. Leenders, T. Stein, B. Conrad, J. Hofemeister and J. Vater, 1999. Structural and functional organization of the fengycin synthetase multienzyme system from *Bacillus subtilis* b213 and A1/3.Chemistry & biology, 6(1): 31-41.
29. Gálvez, A., M. Maqueda, P. Cordovilla, M. Martínez-Bueno, M. Lebbadi and E. Valdivia, 1994. Characterization and biological activity against Naegleria fowleri of amoebicins produced by *Bacillus licheniformis* D-13. Antimicrobial agents and chemotherapy, 38(6): 1314-1319.
30. Peypoux, F., J.M. Bonmatin and J. Wallach, 1999. Recent trends in the biochemistry of surfactin. Applied Microbiology and Biotechnology, 51(5): 553-563.
31. Gilliver, K., 1949. The antibacterial properties of some species of aerobic spore-forming bacilli. British journal of experimental pathology, 30(3): 214.
32. Dvonch, W. and R.G. Benedict, 1953. Elaboration of a subtenolin-like antibiotic by *Bacillus pumilus*. Antibiotics and chemotherapy (Northfield, Ill.), 3(2): 192-194.
33. Aunpad, R. and K. Na-Bangchang, 2007. Pumilicin 4, a novel bacteriocin with anti-MRSA and anti-VRE activity produced by newly isolated bacteria *Bacillus pumilus* strain WAPB4." Current microbiology, 55.4: 308-313.
34. Thakur, D., T.C. Bora, G.N. Bordoloi and S. Maiumdar, 2009. Influence of nutrition and culturing conditions for optimum growth and antimicrobial metabolite production by Streptomyces sp. 201. J. Mycol. Med., 19: 161-167.
35. Kiranmayi, M.U., P. Sudhakar, K. Sreenivasulu and M. Vijayalakshmi, 2011. Optimization of culturing conditions for improved production of bioactive metabolites by Pseudonocardia sp. VUK-10. Mycobiology, 39: 174-181.
36. Awais, M., A. Pervez, A. Yaqub and M.M. Shah, 2010. Production of antimicrobial metabolites by *Bacillus subtilis* immobilized in polyacrylamide gel.Pakistan J. Zool., 42(3): 267-275.
37. Sharon, S.F.B., R.R. Daniel and R. Shenbagarathai, 2014. Optimization of antibiotic production by marine actinomycetes Streptomyces sp. KOD10 S.International Journal of Pharmaceutical Sciences, 6(2): 506-510.
38. Beriæ, T., M. Kojiæ, S. Stankoviæ, L. Topisiroviæ, G. Degrassi, M. Myers and D. Fira, 2012. Antimicrobial activity of *Bacillus* sp. natural isolates and their potential use in the biocontrol of phytopathogenic bacteria. Food Technology and Biotechnology, 50(1): 25-31.
39. Kohanski, M.A., D.J. Dwyer and J.J. Collins, 2010. How antibiotics kill bacteria: from targets to networks. Nature Reviews Microbiology, 8(6): 423-435.
40. Dashti, Y., T. Grkovic, U.R. Abdelmohsen, U. Hentschel and R.J. Quinn, 2014. Production of induced secondary metabolites by a co-culture of sponge-associated actinomycetes, Actinokineospora sp. EG49 and Nocardiopsis sp. RV163. Marine drugs, 12(5): 3046-3059.
41. Manimaran, M., J.V. Gopal and K. Kannabiran, 2015. Antibacterial activity of Streptomyces sp. VITMK1 isolated from mangrove soil of Pichavaram, Tamil Nadu, India. Proceedings of the National Academy of Sciences, India Section B: Biological Sciences: 1-8.
42. Wang, Y., K. Chen, Z. Li, Y. Wu, K. Guo, J. Li and H. Yang, 2014. Isolation and identification of nematicidal active substance from Burkholderia vietnamiensis B418. Plant Prot, 40: 65-69.

43. Ser, H.L., U.D. Palanisamy, W.F. Yin, S.N.A. Malek, K.G. Chan, B.H. Goh and L.H. Lee, 2015. Presence of antioxidative agent, Pyrrolo [1, 2-a] pyrazine-1, 4-dione, hexahydro-in newly isolated Streptomyces mangrovisoli sp. nov. Frontiers in microbiology: 6.
44. Devi, N.N. and F. Wahab, 2012. Antimicrobial properties of endophytic fungiisolated from medicinal plant Camellia sinesis. Int. J. Pharma. Bio. Sci, 3: 420-427.
45. Burkholder, P.R., R.M. Pfister and F.H. Leitz, 1966. Production of a Pyrrole Antibiotic by a Marine bacterium, Appl. Microbiol., 14: 649-653.
46. Sathiyanarayanan, G., R. Gandhimathi, B. Sabarathnam, G. Seghal Kiran and J. Selvin, 2014. Optimization and production of pyrrolidone antimicrobial agent from marine sponge-associated Streptomyces sp. MAPS15. Bioprocess Biosyst Eng., 37: 561-573.
47. Mithun, V.S.L. and C.R.C. Rao, 2012. In vitro studies on anti-cancer activity of anti-cancerous compound producing marine bacteria against on cancer cells by MTT assay. Journal of Chemical, Biological and Physical Sciences (JCBPS), 3(1): 276.
48. El-Naggar, M.M., H.M. Abd-Elnaby, S.A. Abou-Shousha, U.M. Abdul-Raouf and A.E. Abouelwafa, 2016. Production of Anti-Inflammatory Pyrrol Compound from Marine *Bacillus baekryungensis* AMHSU. World Journal of Fish and Marine Sciences, 8(2): 74-84.