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Antibacterial, Anticoagulant and Anti-inflammatory Activities of Bioactive Compounds Produced by a Marine *Bacillus cereus*

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The marine isolate S1 isolated from shrimp surface was screened for its potentiality in producing antibacterial activity against some Gram negative and Gram positive pathogens. It showed promising antibacterial activity with the highest antagonistic action against *Aeromonas hydrophila* (16 mm). It was identified as *Bacillus cereus* S1 using 16 S rRNA sequence analysis. Maximization of the productivity with 1.3 fold increase was achieved using Plackett Burman experimental design. The optimized medium was formulated as (g/l): peptone, 7; beef extract, 1.5; sea water concentration (50%) with pH 5 and inoculum size (0.5ml for each 25 ml medium) and incubated for 12 h. Immobilization using adsorption on pumice improved the productivity by 1.6 fold compared to the basal medium while loss of antibacterial activity was up on using entrapment technique. The bioactive compounds were characterized by thermal stability even at 100 ºC while they were inactivated up on exposing to UV for 20 min. Moreover, the anticoagulant activity of *B. cereus* S1 was tested using PT and PTT tests. It succeed to prolong the clotting time to 40 sec and 253 sec respectively which represents about 3.3 fold and 7.2 fold compared to the control. Hexane extract was compared with other standard antibiotics and it was superior in its antibacterial effect. The extract was analyzed using gas chromatography–mass spectrometry (GC–MS). One major compound was identified as phthalic acid. Antibacterial activity of Phthalate was evidenced in previous studies.

Keywords: Antibacterial, anticoagulant, antiinflammatory activity, Bioactive compounds, *Bacillus* sp.

The resistance of pathogenic bacteria became the upcoming challenge in therapeutic agents, which is increasing with time due to misuse of antibiotics[1,2]. Gram-negative bacteria such as *Aeromonas*, *Vibrio*, *Flavobacterium*, *Pseudomonas*, and *Francisella* and Gram-positive bacteria from the genera *Streptococcus* and *Lactococcus*[3,4,5,6,7,8,9,10], are some of the pathogens responsible for different diseases and economic losses[11, 12].

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Recently, side effects of new therapeutic agents which have entered the clinical area, have been reported[13]. The challenge for scientists is to explore new antimicrobial agents from natural sources including bacterial strains with broad antimicrobial activity against different pathogens[14, 15].

Most of the biological activities have borne from oceans. Every year, marine organisms produce hundreds of new compounds [16] such as photo protective, anti-microtubule, anti-proliferative, anti-tumor, cytotoxic, and antifouling properties. Isolation of new microbes from marine environment has been reported[17, 18]

Marine bacteria associated to invertebrate surface often produce secondary

metabolites with amazing antagonistic activities including antivirulence, anticancer and antibacterial[19]. Search of secondary metabolites produced *Bacillus* species isolated from diverse groups of invertebrates has under laid objectives by the scientific community In the past two decades and obtained therapeutic enzymes, novel metabolites and bactericidal agents[20,21,22]. All these natural compounds with broad biological activities enable the bacterium to survive in its natural environment.

The environmental and nutritional conditions have a great influence on production of the antimicrobial substances. In order to develop an efficient product of antimicrobial substances, knowledge regarding the environmental factors affecting this process needs to be well identified[23,24]. Optimal production is achieved by using experimental designs as an excellent tool for optimization of culture conditions.

Thus the purpose of the present study is to isolate a marine invertebrate associated bacteria, evaluation of the antibacterial activity against different bacterial pathogens. Moreover the study would extend to optimization of the fermentation conditions using statistical design. Improvement of the production was also carried out. Furthermore, extraction and characterization of the bioactive compounds will be carried out and also identified by gas chromatography–mass spectral (GC–MS) and infrared spectroscopic analysis. Other different applications such as anticoagulation and anti-inflammation will be studied.

MATERIALS AND METHODS

Microorganism and culture conditions

The marine bacterial isolate was isolated from the surface of shrimp. It was isolated upon seawater agar medium and was maintained on nutrient agar slants[25]

Pathogenic indicators

Different Gram positive and Gram negative pathogens and including *Staphylococcus aureus* ATCC 6538, *Streptococcus faecalis*, *Pseudomonas aeruginosa* ATCC 8739, *Vibrio anguillarum* and *Vibrio fluvialis* were used as target strains for detecting the antagonistic properties. These indicator bacteria were kindly provided from the Department of Poultry and Fish Diseases, Faculty of Veterinary Medicine, Alexandria University

Antibacterial activity

B.cereus S1was precultured in marine nutrient broth at 30 ^{o}C on a rotary shaker incubator until the absorbance of the culture at A 550=1. The culture broth was centrifuged at 10000 rpm for 15 minutes to remove bacterial cells[26]. The ability of the cell free supernatant to inhibit the growth of the indicator bacteria was performed using the well-cut diffusion technique. Briefly, five-millimeter-diameter wells were punched in agar plates (using a sterile gel puncher) inoculated with bacterial pathogenic strains. 50 µl of each tested compound was added in each well. After incubation at 30^{o}C for 24h, the radius of the clear zone around each well was linearly measured in mm[27.]

Bacterial identification

DNA was isolated, purified using standard procedures[28] and the region of 16S rDNA was amplified using universal primers (27F (5'-AGAGTTTGATCCTGGCTCAG-3'), 1492R (5'-GGTTACCTTGTTACGACTT-3').16S sequence analysis was used to perform the genotypic characterization. Multiple alignments with the most closely members sequences similarity levels were carried out using Blast program (http://www.ncbi.nlm.nih.gov/blast). Sequences of rRNA genes, for comparison, were obtained from the NCBI database. A phylogenetic tree was reconstructed by Bioedit software .

Optimization of fermentation conditions using Plackett Burman experimental design

The Plackett -Burman experimental design[29,30] was applied to reflect the relative importance of various factors on the production of the bioactive compounds by *B.cereus* S1. High (+) and low (-) levels for each variable were tested. The examined variables in this experiment and their levels are shown in Table 1. Duplicate of the eight different trials were performed. The different trials (Row no.9 represents the basal control) are presented in Table 1. The main effect of each variable was determined with the following equation:

$$Exi = (Mi+ - Mi-) / N$$

Where Exi is the variable main effect, and Mi+, Mi- are the inhibition zone (mm) in the trials, each independent variable was used in high and low concentrations, respectively,

and N is the number of trials divided by 2. Microsoft Excel was used to calculate t-values for equal unpaired samples to determine the variable significance.

Effect of immobilization technique on production of antimicrobial agents

Immobilization was carried out by using adsorption of *B.cereus* S1 cells on different solid porous supports including luffa pulp, pumice, sponge and clay, 1.5 ml of bacterial suspension were added to 250 ml sterile flasks containing five grams of porous support materials in 50 ml of optimized culture medium. Luffa pulp and sponge pieces were around 0.5 cm in diameter. The flasks were then shacked slowly at 120 rpm under the optimized conditions. The antibacterial activity was estimated using well cut diffusion technique and compared with the free cells[32].

Preliminary characterization of the bioactive compounds

Effect of temperature

Ten millilitres each of the cell free culture supernatant of *B.cereus* S1 were dispensed in various 50 ml screw capped conical flasks. The flasks were subjected to different temperature treatment (30, 60, 100 ^{o}C) for 20 min[33]. The antibacterial activity was estimated using well cut diffusion technique.

Effect of UV

The effect of UV light on antimicrobial activity was determined as follows: 10 mL of filter-sterilized cell-free supernatant was exposed to UV irradiation at a distance of 25 cm for 15, 30, 45 and 60 minutes[34]. After each time interval, antimicrobial activity was analyzed by well-cut diffusion technique.

Scanning electron microscopy

The bacterial isolate was grown on optimized medium. A plug of cells was removed and prepared for fixation and dehydration procedures according to Bozzola and Russell[35]. The samples were dried completely in a critical point dryer and finally coated with gold in SPI-MODULE sputter coater. Then the specimens were viewed with a JEOL–JSM 5500LV.

Anticoagulant activity

Evaluation of Activated Partial Thromboplastin Time (APTT)

All clotting assays were carried out using normal citrated human plasma according to the manufacturers' specifications. For this, 50 µL of citrated normal human plasma was mixed with 50 µL of a supernatant solution before adding 50 µL of APTT reagent. The mixture was then incubated at 37°C for 3 min. Then, 50 µL of 0.025 M calcium chloride reagent was added to the mixture to trigger the coagulation cascade. The clotting time was recorded in seconds. Heparin was used as the standard[36].

Evaluation of Prothrombin Time (PT)

All clotting assays were carried out using normal citrated human plasma according to the manufacturers' specifications. For this, 50 µL of human plasma was mixed with 50 µL of a supernatant and incubated at 37°C for 3 min. Then, 50 µL of 0.025 M PT reagent was added to the mixture to trigger the coagulation cascade. The clotting time was again recorded in seconds[36].

Anti-inflammatory activity

Inhibition of albumin denaturation

Anti-inflammatory activity of the supernatant was estimated using methods of Sakat *et al* (2010)[37]. The reaction mixture was consisting of test extracts and 1% aqueous solution of bovine albumin fraction, pH of the reaction mixture was adjusted using small amount at 37°C HCl. The sample extracts were incubated at 37°C for 20 min and then heated to 51°C for 20 min after cooling the samples the turbidity was measured spectrophotometrically at 660 nm. The experiment was performed in triplicate. Percent inhibition of protein denaturation was calculated a s follows:

$$\% \text{ inhibition} = [\{\text{Abs control} - \text{Abs sample}\}/\text{Abs control}] \times 100$$

Extraction of bioactive compounds

Different organic solvents including ethanol, chloroform, methanol and hexane were used for extraction of the antimicrobial agents. 50 ml of each solvent was added to 50 ml fermented broth in a 250 ml separating funnel. The mixture was shaken vigorously for 20 min and kept to separate the solvent from aqueous phase. The organic phase was collected and evaporated by using a rotary evaporator and then dissolved in appropriate solvent[38]. Antibacterial activity was determined each time using well cut diffusion technique.

Antibiogram

Sensitivity of *A.hydrophila* to different standard antibiotics including Streptomycin (10µg),

Chloramphenicol (30 µg) and Ampicillin sulbactam (20 µg), Tetracyclin (30 µg) and Amoxixicillin (10 µg) was tested . The antagonistic effect of these antibiotics were compared to that of the active fraction using disk diffusion method[39] .

Spectral analysis

The active extract was analyzed using GC–MS. The peaks were identified by comparing with WILEY MASS SPECTRAL DATA BASE Library[40.]

Infrared

Pellets for infrared analysis were prepared by grinding a mixture of 1mg sample with 200 mg dry KBr, followed by pressing the mixture into a 16 mm diameter mold. The Fourier transform-infrared (FT-IR) spectra were recorded on a Bruker Vector 22 instrument with a resolution of 4 cm^{-1} in the 4000-400 cm^{-1} region.

RESULTS

Screening for antibacterial activity

The marine isolate S1 isolated from the surface of shrimp was tested for its potentiality in production of antimicrobial agents against some fish pathogens including *V. damsel*, *P.aeruginosa*, *S. faecalis*, *S.aureus* and *A. hydrophila* with maximum zone of inhibition comparatively to others. Results in Figure 1 showed that the highest antimicrobial activity was against *A. hydrophila* (16mm) followed by *P. aeruginosa* and *S. aureus* (12 mm), while the lowest antimicrobial activity was against *P. florescence* (11 mm). On the other hand there was lack of activity against *S. faecalis* and thus *A. hydrophila* was chosen to complete the study as it is the most susceptible bacterial pathogens. Results concluded that Gram negative bacteria was the most susceptible for the antagonistic compounds which is coincide with Telesmanich *et al*. (2000)[40]. The difference in the susceptibility may be due to physiological characteristic and metabolism of each strain, cell wall structure and degree of contact [41].

Antimicrobial activity by *Bacillus* spp. was proven in previous studies [42, 43, 44]. The results obtained in the present study indicated the potential production of bioactive compounds by marine *Bacillus* sp. Anand *et a l*(2006)[45] reported the production of a highly active metabolites by marine *Bacillus* sp. Mohan *et al* (2016)[46] stated that *Bacillus* sp. isolated as sponge endosymbiotic bacteria showed antimicrobial activity with broad range against virulent marine fish pathogens such as *Vibrio alginolyticus,Vibrio vulnificus*, *Vibrio parahaem*olyticus, *Aeromonas salmonicida*, *Flavobacterium* sp., *Edwardsiella* sp., *Proteus mirabilis* and *Citrobacter brackii* with zones of inhibition (16-23 mm). Many scientists isolated *Bacillus* sp. from diverse group of invertebrates and confirmed the production of novel active metabolites against aquatic fish pathogens [20, 21, 47].

Molecular identification of the marine isolate S1

DNA of the promising *Bacillus* sp. was extracted and the extracted 16SrRNA gene was

Table 1. The applied Plackett–Burman experimental design for seven cultural variables

Trial	Factors							
	Peptone (P)(g/l)	Beef (B)(g/l)	pH	Inoculum size(IS)(ml)	Incubation period(IP)(ml)	Seawater concentration (SW) (%)	Culture Volume (CV)(ml)	Diameter of inhibition zone (mm)
1	- [3]	-[1.5]	-[5]	+[1.5]	+[48]	+[150]	-[25]	13
2	+[7]	- [1.5]	“[5]	-[0.5]	-[12]	+[150]	+[75]	13
3	“[3]	+[4.5]	-[5]	“[0.5]	+[48]	-[50]	+[75]	15
4	+[7]	+[4.5]	“[5]	+[1.5]	“[12]	-[50]	-[25]	17
5	“[3]	_[1.5]	+[9]	+[1.5]	-[12]	“[50]	+[75]	13
6	+[7]	“[1.5]	+[9]	“[0.5]	+ [48]	-[50]	“[25]	13
7	“[3]	+[4.5]	+[9]	_[0.5]	“[12]	+ [150]	_[25]	14
8	+[7]	+[4.5]	+[9]	+[1.5]	+[48]	+ [150]	+[75]	0
9	0[5]	0[3]	0[7]	0[1]	0[24]	0[100]	0[50]	16
Main effect	3.75	-1.5	-4.5	-3	-4	-4	-4	
t-value	-0.8	-0.39	-1.1	0.1	-1.12	-1.3	-1.12	

Standard t-values are obtained from statistical methods (Cochran &Snedecor, 1989)[31].

amplified using the universal primers. The produced amplicons was analyzed using agarose gel electrophoresis as shown in Figure 2a. The amplified DNA was partially sequenced. This sequence was compared with those which gave the highest homology using Blast search computer based program. The resulting data indicated that the isolate under study was identified as *B.cereus* S1.The obtained similarity was 99% with session number KX683220. The phylogenetic relationships of this experimental isolate and the closely related relatives were analyzed as shown in Figure 2b.

Optimization of fermentation conditions using Plackett- Burman experimental design

Two phases of the application of Plackett-Burman statistical design was carried out. The first step was to screen for important factors and their levels that affect production process in shake flasks (Table 1). The second was the verification experiments to validate the results under specific optimized medium. All experiments were carried out in duplicates. Table 1 shows results of the experimental design. It was observed that the production of the antimicrobial agent was negatively affected by beef extract, inoculum size, incubation period, sea water (%), culture volume and positively affected by peptone concentration, which means that increasing levels of peptone cause increase in the antagonistic activity which in accordance with other previous studies[48] , while decreasing levels of the other variables cause increase in the antagonistic activity. The effect of some of these factors on the production was similar to that reported by Ali (2012)[49] who stated that beef extract, culture volume, inoculum size and incubation period had negative effect on antimicrobial agents production by marine *Pseudomonas piscicida* B12. The pH optimum for antimicrobial agent production was 6 which is in accordance with Wen Zhou et al (2010)[24] who stated that optimum pH for production of bioactive compounds by *B. ce*reus S1 was in the acidic range as was also reported by Sana *et al* (2008)[50]. Regarding effect of NaCl, Balakrishnan *et al* (2014)[44] observed that effect of NaCl is dependent

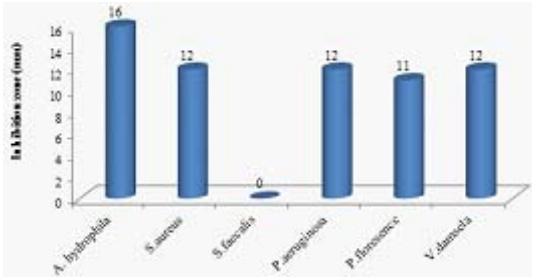
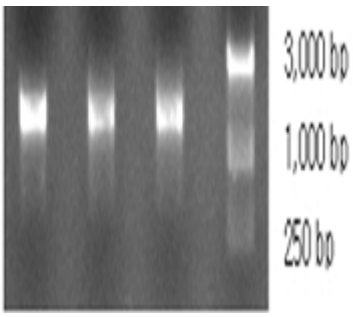


Fig. 1. Antibacterial activity of *B.cereus* S1 against some bacterial pathogens



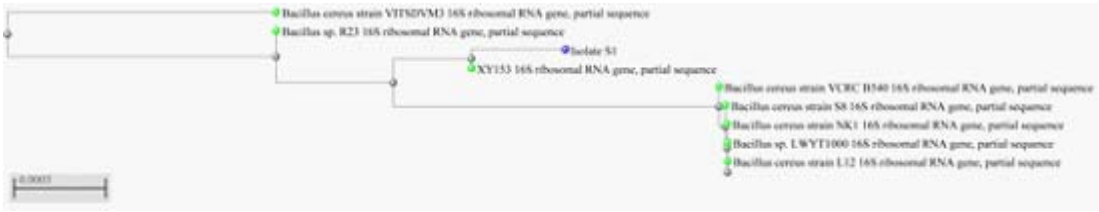


Fig. 2. 1 6S agarose gel electrophoreses of the extracted and amplified DNA. Lanes 1, 2, 3 are the purified PCR products. Lane 4 is molecular weight marker **(a)**; Phylogenetic relationships among the representative experimental strain and the most closely related *Bacillus* species **(b).** The dendogram was generated using tree view program

on the mechanism of expression of the bacterium to the particular salt concentration.

The main effect of each variable on the production of the bioactive compounds as well as t-values were estimated for each independent variable as shown in Table 1 and graphically presented in Figure 3. Results in this Figure indicated that the main effect of all variables were negative on the production of the antagonistic agents except for peptone which exhibited positive effect on the production. Results of t-test indicated that variations in sea water (%), culture volume and incubation period in the tested ranges had the most considerable effects on production of the antimicrobial agents by *B.cereus* S1. The interacting effect of sea water (%), culture volume and incubation period in three -dimensional representation is illustrated in Figure 4.

Abd-Elnaby *et al* (2016)[51] reported that increasing levels of pH, inoculum size caused an increase in the antagonistic activity by about 1.3 fold for *Streptomyces parvus*. Conversely to the present study, some of this finding was reported by Wefky *et al*. (2009)[48]

According to the obtained results, the predicted medium for cultivation of *B.cereus* S1 to enhance maximum production of the bioactive compounds was formulated as follows: (g /l) :peptone,7; beef extract, 1.5; concentrated seawater (50%), adjusted pH 5 and inoculum size (0.5ml for each 25 ml medium) all of which are incubated for 12 h.

Verification experiment

A verification experiment was carried out in order to evaluate the accuracy of the applied Plackett-Burman statistical design, predict the near optimum levels of independent variables. The obtained data were examined and compared to the basal and anti-optimized medium. Data revealed that antagonistic activity produced by *B.cereus* raised to 21 mm and realized 1.3 fold increase when growing in optimized medium (Figure 5).

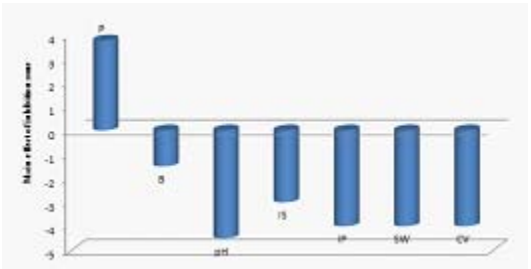


Fig. 3. Elucidation of fermentation conditions affecting the production of the antagonistic agents by *B.cereus* S1

Effect of immobilization on production of the bioactive compounds

Immobilization on different support materials was investigated to enhance the production of the antibacterial agents. As shown

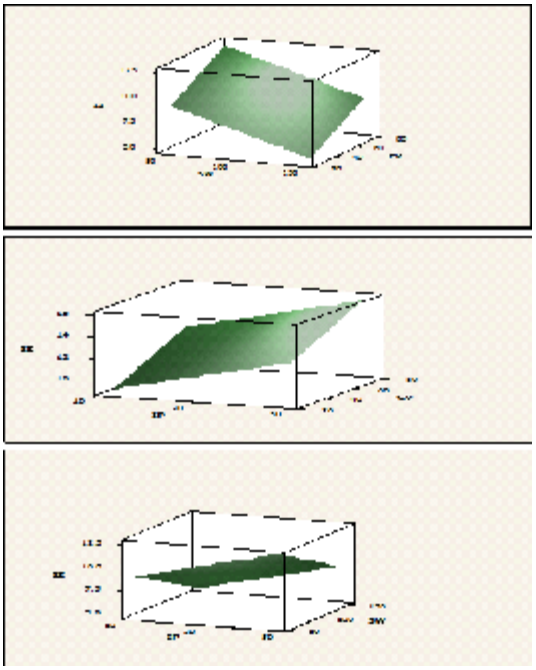


Fig. 4. Interaction effect between culture volume (CV) and sea water (%) (SW) (a); culture volume (CV) and incubation period (IP) (b) and seawater (%) (SW) and incubation period (IP) (c) Levels, with respect to inhibition zone (IZ)(mm) based on Plackett-Burman results

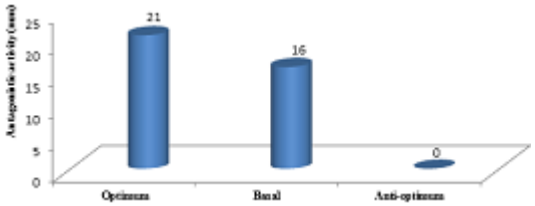


Fig. 5. Verification experiment of the applied Plackett-Burman statistical design by comparing the antagonistic activity of *B.cereus* S1 growing on the resulting optimized medium, the basal medium and the anti-optimized medium

Table 2. Antibacterial activity using free and immobilized cells

Response	Antagonistic activity using different support materials					
	Free cells	Luffa pulp	Sponge	Pumice	Clay	Ca alginate beads
IZ.(mm)	21	0	11	25	0	0

in Table 2, the antagonistic activity was increased by 1.2 fold compared to the free cells, while adsorption on the other support materials caused complete disappearance of the antagonistic activity. On the other hand, antagonistic activity was also disappeared up on using entrapped cells. These results may be due to poor mechanical stability of the support. Diffusion limitation of the bioactive compounds is an important factor affecting this process too[52]. Abd-Elnaby *et al*. (2016) [51] also reported the potentiality of the immobilized cells in raising the antimicrobial activity of *Streptomyces parvus* compared to free cells.

Preliminary characterization of the antibacterial metabolites in cell free supernatant

Stability of antimicrobial compounds produced by *B.cereus* toward temperature and UV was examined. Screening the effect of different temperature on stability of the bioactive components (Figure 6) showed that the antibacterial compounds produced by *B.cereus* were relatively stable even after boiling at 100 °C. Extreme temperatures, boiling for 10 min, had no affect on the antimicrobial agents produced by *B. subtilis* as was reported by Sabaté and Audisio (2013)[53]. The same finding was observed by Risøen *et al*. (2004)[54] who stated that the antimicrobial compound retained activity over a wide range of temperatures even up to 100°C. in other study by Chalasani *et al* (2015)[55], the antimicrobial compound was also stable at different temperatures 80% activity was retained a 80°C for 1 h, 75% at 100°C for 30 min and 60% at 121°C for 20 min. On the other hand, exposing the cell free supernatant to UV radiation for different time intervals caused complete absence of antagonistic activity. It can be stated that bioactivity of the compounds is dependent on the mutation effect on the active gene which responsible for the production of antibiotics[56].

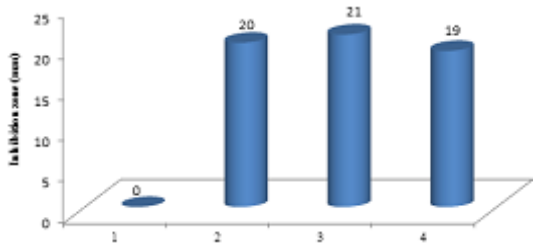


Fig. 6. Effect of temperature on stability of the inhibitory activity produced by *B.cereus* S1

Assay of bacteriolytic activity

The bacteriolytic activity of a 0.45 µm culture filtrate from 24 hrs of *B. cereus* S1culture against *A. hydrophila* was detected using SEM. Figure 8 represents the morphological changes of the nontreated and treated *A. hydrophila*. Fig. 8 shows the SEM micrographs of bacterial cells

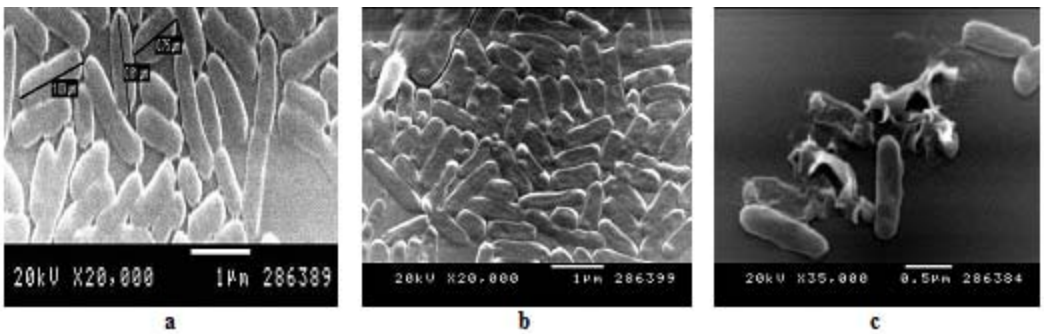


Fig. 7. SEM photomicrographs showing lysis of *A.hydrophila* cells by a 24 hrs culture filtrate (0.45 µm) of *B.cereus* S1a) control (no addition) (b) and (c) treatment with supernatant after 24 hrs incubation

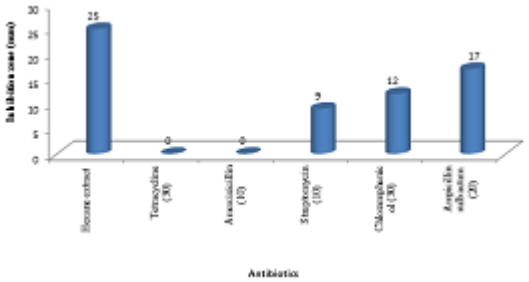


Fig. 8. Antibacterial activity of hexane extract compared with other different standard antibiotics

without the hexane extract treatment. The figure revealed the normal rod shape cell structure without any shrinkage or cavity formation as the surface was smooth and regular. Fig. 8 shows the morphology of the cell after 24 h of treatment with the supernatant. The bacterial cells started to show multiple defects with many of cells exhibited crumpled or shrunken cell surface. Fig. 8 revealed that more formation of crumpled cells and cells lysis were formed up on treatment with hexane extract for 24 h. It was reported that the cell wall of Gram negative bacteria (*A.hydrophila*) is surrounded by an outer membrane consisting of lipopolysaccharides, phospholipids and lipoproteins and thus they are less sensitive to bacteriolytic enzymes than Gram positive bacteria[57]. Only a few active compounds have been reported to lyse cells of Gram negative bacteria[58]; therefore, the present study is interesting that the isolate *B.cereus* S1 was active against *A.hydrophila* which can be applied as probiotic in aquaculture.

Anticoagulant activity

Pharmaceutical importance of the bioactive compounds depends up on the positive chemical interactions with microorganisms[59]. Bacterial supernatant was tested for blood coagulation effects in normal human plasma using heparin as standard. Results in Table 4 revealed that the supernatant exhibited grater activity with prolonged the clotting time 40 sec which represents about 3.3 fold compared to the control which suggests that the metabolites produced by *B. cereus* S1 is an effective antithrombotic agent. On the other hand the tested supernatant was capable of increasing the normal coagulation time up to 253 sec with 7.2 fold in relation to normal APTT time. Hassanein *et al* (2011)[60] reported that the clotting time of human blood serum in the presence

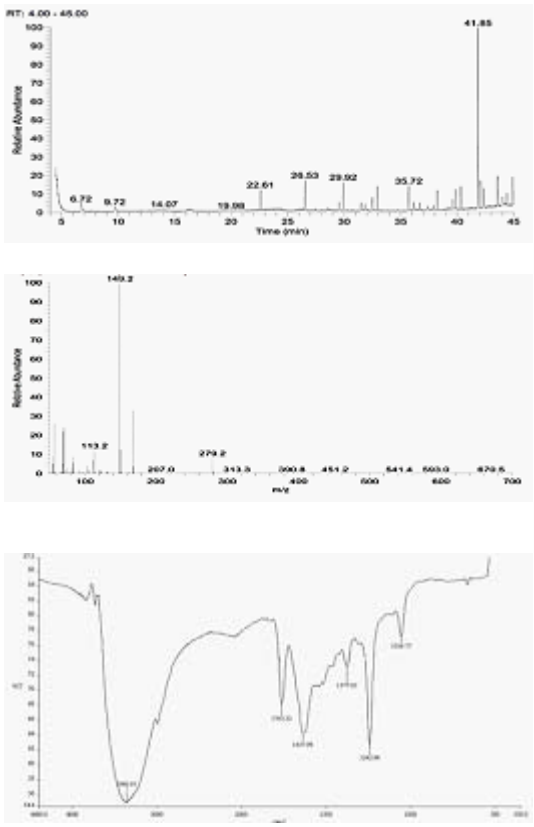


Fig. 9. Gas chromatogram spectral analysis showing the major peak of the active compound **(a),** mass spectral analysis of phthalic acid in the crude extract **(b)** and IR spectrum of the phthalic acid derivatives **(c)**

Table 3. Anticoagulant and anti-inflammatory activities of the bioactive metabolites

	Anticoagulant activity		Anti-inflammatory activity
	Prothrombin time (PT) (sec.)	Partial Thromboplastin Time (APTT) (sec.)	
Supernatant	35	253	85%
Heparin	87		

of active metabolites produced by *B. subtilis* K42 reached a relative PTT of 241.7% with a 3.4-fold increase, Similarly Wei *et al* (2011)[61] stated that the fermented chickpeas from *B. amyloliquefaciens* showed anticoagulant activity, and the purified anticoagulant component showed higher anticoagulant activity than heparin sodium.

In vitro anti-inflammatory activity

Inhibition of albumin denaturation

Denaturation of proteins is a well documented cause of inflammation. As part of the investigation on the mechanism of the invitro anti inflammation ability of the metabolites produced by *B.cereus* S1, protein denaturation was studied. It showed relatively good antiinflamatory activity (86%). Our finding is supported by study of Kurian *et al* (2015)[62] who prove the anti-inflammatory activity of *Bacillus* spp. BTCZ31

Extraction of the antimicrobial agents

Different solvents were screened for their efficiency in extracting the bioactive compounds. Hexane was the most efficient solvent exhibiting the highest value of inhibition zone (21 mm). The other solvents showed varied efficiencies against the indicator bacteria (Table 4). The antibacterial effect was not observed up on using methanol, ethanol and chloroform once the extraction procedures had attempted to isolate and concentrate the biologically active compound. This may have been a consequence of the organic solvents denaturing the compound during the procedure or that the compound is labile or that the compound is a type of molecule not extracted into these solvents[63] .

Antibiotic susceptibility test

Sensitivity of *A.hydrophila* to different standard antibiotics including was tested . The antagonistic effect of these antibiotics were compared to that of the active fraction. It was superior in its effect than Streptomycin (10 µg), Chloramphenicol (30 µg) and Ampicillin sulbactam (20 µg) with 2.7, 2.1 and 1.5 fold respectively and also than Tetracyclin (30 µg) and Amoxixicillin (10 µg) which showed no antibacterial activity against *A. hydrophila* (Figure 7) this was confirmed by study of Barakat and Beltagy, 2015[64] who reported the sensitivity of *A. hydrophila* to the bioactive compounds produced by *Streptomyces ruber* EKH2 rather than that of some tested commercial antibiotics.

Table 4. Efficiency of different solvents for extraction of the active compounds

Solvent	Inhibition zone diameter (mm)
Ethnol	0
Methanol	0
Chloroform	0
Hexane	21

Spectral analysis

GC-MS analysis of hexane extract (Figure 9a) was carried out to identify the components in the extract The major compound was at retention time 45sec. corresponds to a molecular formula of $C_{24}H_{38}O_4$, is identified as 1,2-Benzenedicarboxylic acid, diisooctyl ester (phthalate). The mass spectra of this compound is shown in Figure 9b. Antibacterial activity of phthalate against *Staphylococcus aureus* (A TCC6538), *Streptococcus faecalis* (ATCC8043), *Pseudomonas aeruginosa* (A TCC 8739), *Escherichia coli* (A TCC 8739), *Micrococcus luteus* (A TCC 10240) and *Candida albicans* was evidenced in previous studies[65, 66]. Barakat and Beltagy (2015)[64] stated that phthalate showed antibacterial activity with broad spectrum against *Aeromonas hydrophila, Edwardsiella tarda, Pseudomonas aeruginosa and Vibrio ordalii*. IR spectra showed peaks at 2994 (CH_2), 1763 (C,O), 1637 (C,C), 1242 (C–O) and 1056 (C– H) cm^{-1} (Figure 9c).

CONCLUSIONS

The present research indicates that the marine bacterium *B.cereus* S1 has the potentiality to target the growth of both Gram negative and Gram positive bacteria which supports the successful use of the strain as a biological control agent. Moreover, it was proved as anticoagulant and anti-inflammatory agent. One of the challenges in future will be the large scale production of these compounds to meet the demand for different applications which can be applied as probiotic in aquaculture system. Heat stability of the inhibitory substances produced by *B. cereus* S1 can effectively be used as bio-preservative in food. Further studies are needed for complete identification of the active compound.

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