

Antibacterial, Anticoagulant and Anti-inflammatory Activities of Marine *Bacillus cereus* S1

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A total of 11 bacteria were isolated from sea water and screened for their antibacterial activity against five bacterial pathogens. The most potent isolate (S1) was identified as *Bacillus cereus* S1 using 16S rRNA sequence analysis. *B. cereus* S1 exhibited antibacterial potentiality against, *Pseudomonas florescence*, *P. aeruginosa*, *Staphylococcus aureus*, *Vibrio damsela* and *Aeromonas hydrophila*. The inhibition zones diameter were 11, 12, 12, 12 and 16 mm respectively. 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 °C while they were inactivated up on exposing to ultraviolet radiation for 20 min. Moreover, the anticoagulant activity of *B. cereus* S1 was tested using activated Partial Thromboplastin Time (APTT) and Prothrombin Time (PT) 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 crude extract of *B. cereus* S1 was analyzed using gas chromatography–mass spectrometry (GC–MS). The main constituents were Cycloheptasiloxane, tetradecamethyl-, cyclooctasiloxane hexadecamethyl-, cyclononasiloxane octadecamethyl-, Heptasiloxane, hexadecamethyl- and 1,2-Benzenedicarboxylic acid, diisooctyl ester (phthalate).

Keywords: Antibacterial, Anticoagulant, Antiinflammatory, *Bacillus cerus* S1.

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, are some of the pathogens responsible for different diseases and economic losses^{3,4,5,6,7,8,9,10, 11, 12}.

Recently, side effects of new therapeutic agents which have entered the clinical area, have been reported¹³. 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 compounds¹⁶ with photo protective, anti-microtubule, anti-

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proliferative, anti-tumor, cytotoxic, and antifouling properties. Isolation of new microbes from marine environment has been reported^{17, 18}

Marine bacteria associated to invertebrate surfaces often produce secondary metabolites with amazing biological activities including antivirulence, anticancer and antibacterial¹⁹. Search of secondary metabolites produced by *Bacillus* species 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 aim of the present study was isolation of marine bacteria with antibacterial properties, maximization and characterization of the bioactive compounds in addition to studying its anticoagulation and anti-inflammation activities.

MATERIALS AND METHODS

Microorganism and culture conditions

Eleven bacterial isolates representing different colony morphologies were isolated from seawater samples collected from Alexandria sea shore, Egypt. Nutrient broth medium used for isolation consisted of (g/l); peptone, 5; yeast extract, 2, agar, 20, pH was adjusted to 7 and the incubation was for 24-48 hours²⁵.

Pathogenic indicators

Different Gram positive and Gram negative pathogens 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 pathogenic indicators were kindly provided from the Department of Poultry

and Fish Diseases, Faculty of Veterinary Medicine, Alexandria University

Antibacterial activity

The marine isolate (S1) was precultured in marine nutrient broth at 30 °C on a rotary shaker incubator until the absorbance of the culture at A₅₅₀=1. The culture broth was centrifuged at 10000 g for 15 minutes to remove bacterial cells²⁶. 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 the tested compound was added in each well. After incubation at 30°C for 24h, the radius of the clear zone around each well was linearly measured in mm²⁷.

Bacterial identification

DNA was isolated, purified using standard procedures²⁸ 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 sequences and 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. All the previous mentioned work of DNA extraction, PCR reaction and sequencing of PCR-DNA product was carried out at Macrogen (Seoul, Korea).

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). The main effect of each variable was determined with the following equation:

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

Where Ex_i is the variable main effect, and Mi+, Mi- are the inhibition zone (mm) in

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 (10 ⁵ CFU/ml) (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.

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 antibacterial 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, 0.5 ml of bacterial suspension were added to 250 ml sterile flasks containing five grams of porous support materials in 25 ml of optimized culture medium. Luffa pulp and sponge pieces were around 0.5 cm in diameter. The flasks were then shaken slowly at 120 rpm under the optimized conditions. The antibacterial activity was estimated using well cut diffusion technique and compared with the free cells. Entrapment was done in 3% sodium alginate. A twenty ml sodium alginate solution was prepared by dissolving 0.3 g in 16 ml distilled water and autoclaved at 121°C for 10 min. 0.5 ml of *B.cereus* S1 suspension was added to the sterilized alginate solution. 10 ml of alginate-bacterial combination was introduced into a cross linking solution (sterile 2% CaCl₂) using sterilized syringe to obtain spherical beads of calcium alginate beads entrapping the bacteria. The beads were left in the

solution for two hours, washed several times and transferred to 25 ml optimized medium³².

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 treatments (4, 30, 60, 100 °C) for 20 minutes³³. The antibacterial activity was estimated using well cut diffusion technique.

Effect of ultraviolet radiation

The effect of UV light on antibacterial 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 min³⁴. After each time interval, antimicrobial activity was analyzed by well-cut diffusion technique.

Assay of bacteriolytic activity

The culture filtrate of *B.cereus* S1 was used to test its bacteriolytic activity using a method previously described by Than *et al.*³⁵. A 24 hrs culture of *A. hydrophila* was centrifuged at 6000 g for 25 min to obtain the cell pellets. This was dispersed in 3 ml of sterile artificial sea water (ASW) and A mixture consisting of 7 ml of filtrate and 3 ml of *A.hydrophila* cell suspension in sterile seawater was adjusted to an initial absorbance of

0.5 (O.D 540 nm) using spectrophotometer then incubated at 30°C for 12 h investigating cell lytic activity. A scanning electron microscope (SEM) was used to observe the cells and a positive control is kept without addition of the filtrate.

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 solvent³⁶. Antibacterial activity was determined each time using well cut diffusion technique.

Antibiogram

Sensitivity of *A. hydrophila* to different standard antibiotics including Streptomycin (10 µg), Chloramphenicol (30 µg) and Ampicillin sulbactam (20 µg), Tetracyclin (30 µg) and Amoxicillin (10 µg) was tested. The antagonistic effect of these antibiotics were compared to that of the active metabolite using disk diffusion method³⁷

Scanning electron microscopy

Preparation of cells for microscopic examination was carried out according to Sakat *et al.*³⁸. Scanning-electron micrographs of *B. cereus* S1 and viewing the bacteriolytic effect of *B. cereus* S1 filtrate on *A. hydrophila* were produced using a JEOL JSM-5300 scanning electron microscope (JEOL Technics Ltd., Tokyo, 196-8558, Japan) at the Electron Microscope Unit, Faculty of Science, Alexandria University, Egypt.

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 standard³⁹.

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 seconds³⁹.

Anti-inflammatory activity

Inhibition of albumin denaturation

Anti-inflammatory activity of the supernatant was estimated using methods of Sakat *et al.* (2010)⁴⁰. The reaction mixture was consisting of test supernatant and 1% aqueous solution of bovine albumin fraction. The samples were incubated at 37°C for 20 min and then heated to 51°C for 20 min after cooling of the samples, the turbidity was measured spectrophotometrically at 660 nm. The experiment was performed in triplicate. Percent inhibition of protein denaturation was calculated as follows:

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

Spectral analysis

The active extract was analyzed using Gas-liquid chromatography mass spectra (GC-MS). The peaks were identified by comparing with WILEY MASS SPECTRAL DATA BASE LIBRARY⁴⁰.

Infrared analysis

Pellets for infrared analysis were prepared by grinding a mixture of 1mg sample with 200 mg dry potassium bromide, 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 bacterium S1 isolated from sea water was tested for its potentiality in production of antibacterial agents against some pathogens including *V. damsela*, *P. aeruginosa*, *P. fluorescence*, *S. faecalis*, *S. aureus* and *A. hydrophila*. Results in Figure 1 showed that the highest antibacterial activity was against *A. hydrophila* with inhibition zone diameter (16mm) followed by *P. aeruginosa*, *V. damsela* and *S. aureus* (12 mm), while the lowest antibacterial activity was against *P. fluorescence* (11

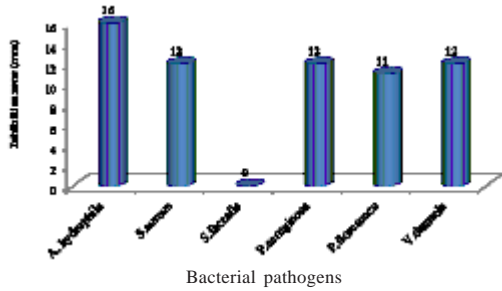


Fig. 1. Antibacterial activity of *B. cereus* S1 against some bacterial pathogens

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 pathogen. Results concluded that Gram negative bacteria was the most susceptible for the antagonistic compounds which is coincide with Telesmanich *et al.* (2000)⁴¹. The difference in the susceptibility may be due to physiological characteristic and metabolism of each strain, cell wall structure and degree of contact⁴¹.

Antimicrobial activity by *Bacillus* spp. was proven in previous studies^{43, 44, 45}. The results obtained in the present study indicated the potential production of bioactive compounds by marine *Bacillus* sp. Anand *et al* (2006)⁴⁶ reported the production of a highly active metabolites by

marine *Bacillus* sp. Mohan *et al* (2016)⁴⁷ 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 parahaemolyticus*, *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, 48}.

Molecular identification of the marine isolate (S1)

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

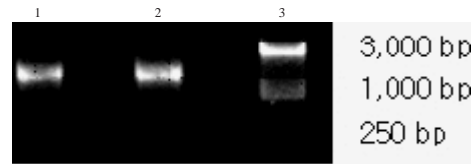


Fig. 2a. 1.6S agarose gel electrophoreses of the extracted and amplified DNA. Lanes 1, 2 are the purified PCR products. Lane 3 is molecular weight marker.

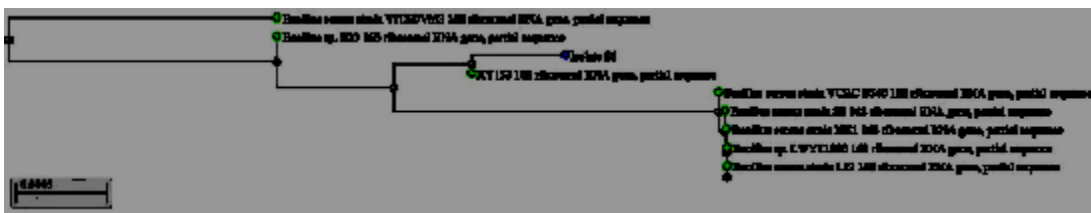


Fig. 2b. Phylogenetic relationships among the representative experimental strain and the most closely related *Bacillus* species. The dendrogram was generated using tree view program

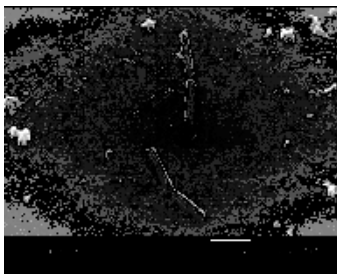


Fig. 3. Scanning electron micrograph showing *B. cereus* S1

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 accession number KX683220. The phylogenetic relationships of this experimental isolate and the closely related relatives were analyzed as shown in Figure 2b. Figure 3 shows the scanning electron micrograph of *B. cereus* S1

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. The main effect of each variable on the production of the bioactive compounds as well as t-values were estimated for

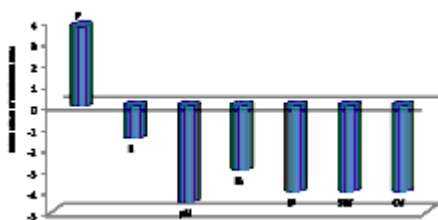


Fig. 4. Elucidation of fermentation conditions affecting the production of the antagonistic agents by *B.cereus* S1.

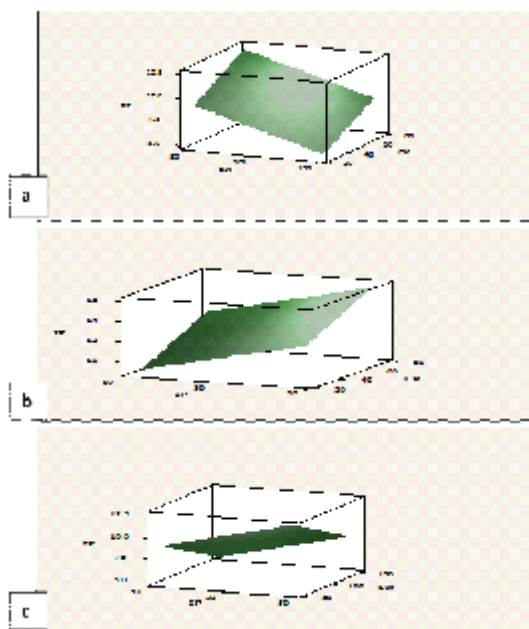


Fig. 5. Interaction effect between culture volume (CV) and sea water (%) (SW) (a); culture volume (CV) and incubation period (IP) (b) and seawater (%) (SW) and incubation period (IP) (c) levels, with respect to inhibition zone (IZ)(mm) based on Plackett-Burman results .

each independent variable as shown in Table 1 and graphically presented in Figure 4. It was observed that the production of the antibacterial 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 is in accordance with other previous studies⁴⁹ , 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)⁵⁰ 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. cereus* was in the acidic range as was also reported by Sana *et al* (2008)⁵¹. Regarding effect of NaCl, Balakrishnan *et al* (2014)⁴⁵ observed that effect of NaCl is dependent on the mechanism of expression of the bacterium to the particular salt concentration. 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 5.

Abd-Elnaby *et al* (2016)⁵² 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, 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

Table 2. A verification experiment: Antibacterial activity of *B. cereus* S1 grown on basal versus optimized medium

Response	Basal medium	Optimized medium
Inhibition zone diameter (mm)	16	21



Fig. 6. Verification experiment of the applied Plackett-Burman statistical design by comparing the antagonistic activity of *B. cereus* S1 growing on the resulting optimized medium, (a) the basal medium and (b) the anti-optimized medium.

that the inhibition zone diameter produced by *B. cereus* S1 raised to 21 mm and realized 1.3 fold increase when growing in optimized medium (Table 2 and Figure 6).

Effect of immobilization on production of the antibacterial agents

Immobilization on different support materials was investigated to enhance the production of the antibacterial agents. As shown in Table 3, the antibacterial activity of cells adsorbed on pumice was increased up to 1.6 fold than the cells grown in the basal medium, while adsorption on luffa pulp and clay 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 too⁵². Abd-Elnaby *et al.* (2016)⁵² also reported the potentiality of the immobilized cells in raising the antimicrobial activity of *Streptomyces parvus* compared to free cells.

Preliminary characterization of the bioactive compounds in cell free supernatant

Stability of antibacterial compounds produced by *B. cereus* S1 toward temperature and UV was examined. Screening the effect of different temperature on stability of the bioactive components (Table 4 and Figure 7) showed that the antibacterial compounds produced by *B. cereus* S1 were relatively stable even after boiling at 100 °C while they lost their antibacterial activity at 4 °C. They retained about 84% and 44% of the antibacterial activity after exposing to 60°C and 100 °C respectively for 20 min. Extreme temperatures, boiling for 10 min, had no effect on the antimicrobial agents produced by *B. subtilis* as was reported by Sabaté and Audisio (2013)⁵⁴. The same finding was observed by Risøen *et al.* (2004)⁵⁵ who stated that the antimicrobial compound retained activity over a wide range of temperatures even up to 100°C. in other study by

Table 3. Effect of immobilization on the antibacterial activity of *B. cereus* S1

Diameter of inhibition zones using different support materials and Ca -alginate beads						
	Free cells	Luffa pulp	Sponge	Pumice	Clay	Ca -alginate beads
Inhibition zone diameter (mm)	21	0	11	25	0	0

Table 4. Effect of temperature and UV on antibacterial activity of *B. cereus* S1

	Temperature (°C)				UV exposing time (min)			
	4	30	60	100	15	30	45	60
Inhibition zone diameter (mm)	0	25	21	11	0	0	0	0

Chalasanani *et al* (2015)⁵⁶, the antimicrobial compound was also stable at different temperatures 80% activity was retained at 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

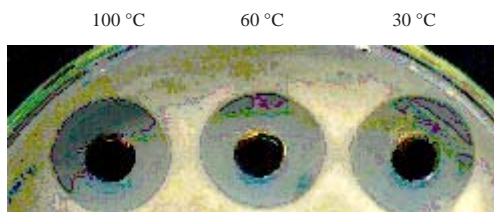


Fig. 7. Effect of temperature on inhibition zone diameter produced by *B.cereus* S1.

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 antimicrobial agents⁵⁷.

Assay of bacteriolytic activity

The bacteriolytic activity of a 0.45 µm culture filtrate from 12 hrs of *B. cereus* S1 culture against *A. hydrophila* was detected using SEM. Figure 8 represents the morphological changes of the nontreated and treated *A. hydrophila*. Figure 8a shows the SEM micrographs of bacterial cells without the supernatant treatment. The figure revealed the normal rod shape cell structure without any shrinkage or cavity formation as the surface was smooth and regular. Figure 8b 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. Figure 7c revealed that some cells showed more formation

of crumpled cells and cells lysis were formed up on treatment with supernatant for 12 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 bacteria⁵⁸. Only a few active compounds have been reported to lyse cells of Gram negative bacteria⁵⁹; 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.

Extraction of the bioactive compounds

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 diameter (25 mm) followed by chloroform (11 mm) while the diameter of inhibition zones were 10 mm up on using methanol and ethanol (Table 5). This may

Table 5. Efficiency of different solvents for extraction of the active compounds

Solvent	Inhibition zone diameter (mm)
Ethanol	10
Methanol	10
Chloroform	11
Hexane	25

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 solvents⁶⁰.

Antibiotic susceptibility test

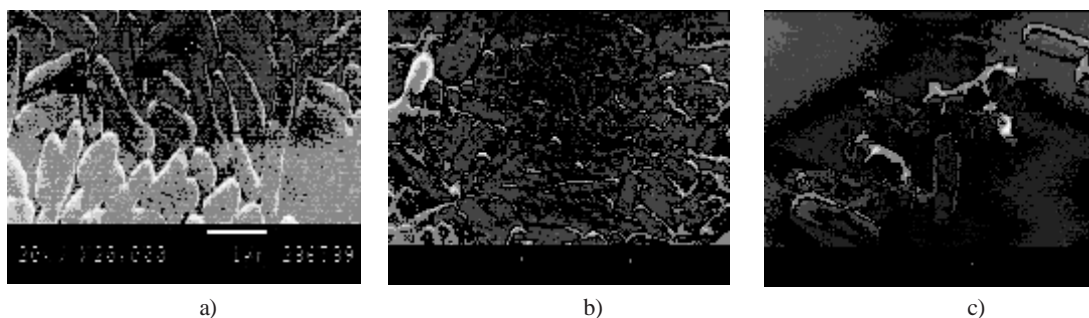


Fig. 8 . SEM photomicrographs showing effect of culture filtrate of *B.cereus* S1 on *A. hydrophila* **a)** control (no addition of the filtrate); **(b)** and **(c)** treatment with the filtrate.

Sensitivity of *A. hydrophila* to different standard antibiotics including Streptomycin (10 µg), Chloramphenicol (30 µg) and Ampicillin sulbactam (20 µg), Tetracyclin (30 µg) and Amoxicicillin (10 µg) 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 Amoxicicillin (10 µg) which showed no antibacterial activity against *A. hydrophila* (Figure

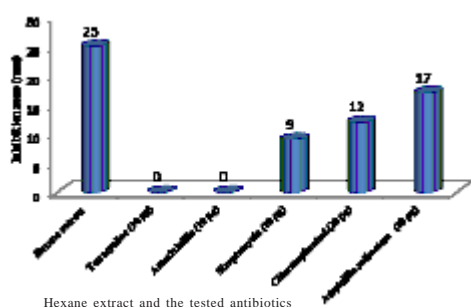


Fig. 9. Antibacterial activity of hexane extract compared with other different standard antibiotics

9) this was confirmed by study of Barakat and Beltagy (2015)⁶¹ who reported the sensitivity of *A. hydrophila* to the bioactive compounds produced by *Streptomyces ruber* EKH2 rather than that of some tested commercial antibiotics.

Anticoagulant activity

Pharmaceutical importance of the bioactive compounds depends up on the positive chemical interactions with microorganisms⁶¹. Bacterial supernatant was tested for blood coagulation effects in normal human plasma using heparin as standard. Results in Table 6 revealed that the supernatant exhibited greater 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)⁶³ reported that the clotting time of human blood serum in the presence 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)⁶⁴ stated that the fermented chickpeas from *B. amyloliquefaciens* showed anticoagulant activity, and the purified anticoagulant component showed higher anticoagulant activity than heparin sodium.

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 antiinflammatory activity (86%) compared to the control without addition of the supernatant (Table 6). Our finding is supported by study of Kurian *et al* (2015)⁶⁵ who proved the anti-inflammatory activity of *Bacillus* spp. BTCZ31

Spectral analysis

GC-MS analysis of hexane extract was carried out to identify the components in the extract. As shown in Figure 10 a, the main constituents in the hexane extract at retention time 22.61, 26.53, 29.92, 35.72 and 41.85 sec. and correspond to a molecular weight 519.0776, 593.231,667.38546, 533.14722 and 390.5561 and formula C₁₄H₄₂O₇Si₇, C₁₆H₄₈O₈Si₈, C₁₈H₅₄O₉Si₉, C₁₆H₄₈O₆Si₇ and C₂₄H₃₈O are identified as Cycloheptasiloxane, tetradecamethyl-, cyclononasiloxane hexadecamethyl-, cyclononasiloxane octadecamethyl-, Heptasiloxane, hexadecamethyl- and 1,2-Benzenedicarboxylic acid, diisooctyl ester

Table 6. Anticoagulant and anti-inflammatory activities of the produced compounds

	Anticoagulant activity		Anti-inflammatory activity
	Prothrombin time (PT) (sec.)	Partial thromboplastin tme (APTT) (sec.)	
Supernatant	40	253	86%
Heparin	87		

(phthalate). The major chemical component (Phthalate) is reported to have a known biomedical value in the pharmacological fields. Antibacterial activity of phthalate against *Staphylococcus aureus* (ATCC 6538), *Streptococcus faecalis* (ATCC8043), *Pseudomonas aeruginosa* (ATCC 8739), *Escherichia coli* (ATCC 8739), *Micrococcus luteus* (ATCC 10240) and *Candida albicans* was evidenced in previous studies^{66, 67}.

Barakat and Beltagy (2015)⁶¹ also stated that phthalate showed antibacterial activity with broad spectrum against the fish pathogens *A. hydrophila*, *Edwardsiella tarda*, *P. aeruginosa* and *V. ordalii*. The antibacterial properties of siloxanes have been earlier reported by Lessoy *et al.*⁶⁸ and also exhibit bioactivity as antibacterial and antioxidant agents as was documented by Avci and Dik⁶⁹. Anti-inflammatory activity of

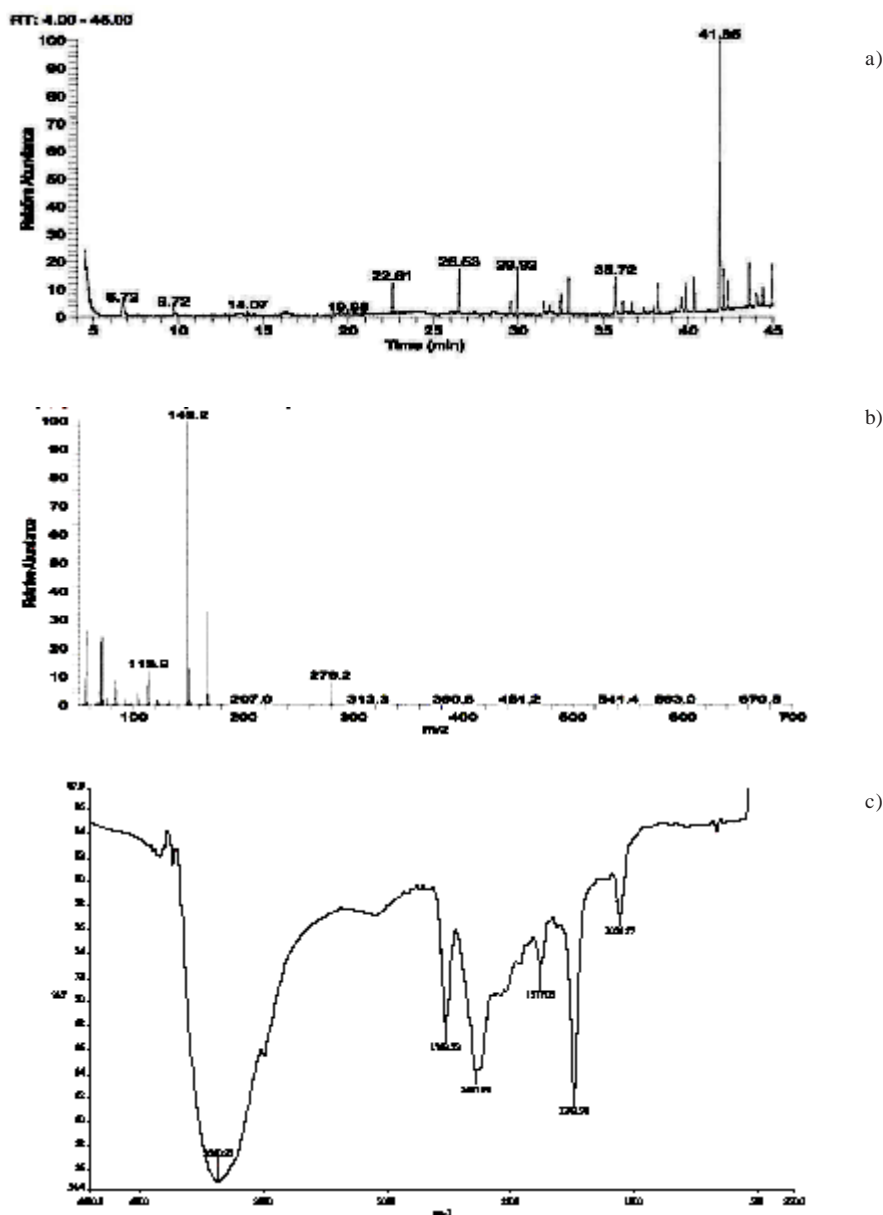


Fig. 10. Gas chromatogram spectral analysis showing (a) the main constituents of hexane extract, (b) mass spectral analysis of phthalic acid and (c) IR spectrum of the phthalic acid (c).

siloxane was also reported⁷⁰. The mass spectra of the major compound (Phthalate) is shown in Figure 10b. IR spectra of phthalate showed peaks at 2994 (CH₂), 1763 (C,O), 1637 (C,C), 1242 (C–O) and 1056 (C–H) cm⁻¹ (Figure 10c).

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 compounds.

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