A Comparative biochemical study on two marine endophytes, *Bacterium SRCnm* and *Bacillus sp. JS*, Isolated from red sea algae

Eman Fadl Ahmed[1]*, Hossam Mokhtar Hassan[2], Mostafa Ezzat Rateb[2,4], Noha Abdel-Wahab[3], Somayah Sameer[4], Rainer Ebel[4], Hanan Anwar Aly Taie[5], Mohammed Sayed Abdel Hameed[3] and Ola Hammouda[3]

[1]Department of Chemistry of Natural and Microbial Products, National Research Centre, Dokki, Cairo, Egypt
[2]Department of Pharmacognosy, College of Pharmacy, Beni-Suief University, Egypt
[3]Department of Botany, faculty of Science, Beni-Suief University, Egypt
[4]Marine Biodiscovery Centre, University of Aberdeen, AB24 3UE Scotland, UK
[5]Department of Plant Biochemistry-National Research Centre, Dokki, Cairo, Egypt

Abstract: Two marine endophytic bacteria were isolated from the Red Sea algae; a red alga; *Acanthophora dendroides* and the brown alga *Sargassum sabrepandum*. The isolates were identified based on their 16SrRNA sequences as *Bacterium SRCnm* and *Bacillus sp. JS*. The objective of this study was to investigate the potential anti-microbial and antioxidant activities of the extracts of the isolated bacteria grown in different nutrient conditions. Compared to amoxicillin (25µg/disk) and erythromycin (15µg/disk), the extracts of *Bacterium SRCnm* in media II, III, IV and V were potent inhibitors of the gram-positive bacterium *Sarcina maxima* even at low concentrations. Also, the multidrug resistant *Staphylococcus aureus* (MRSA) was more sensitive to the metabolites produced in medium (II) of the same endophyte than erythromycin (15µg/disk). A moderate activity of the *Bacillus* sp. *JS* extracts of media I and II was obtained against the same pathogen. The total compounds (500ug/ml) of both isolated endophytes showed moderate antioxidant activities (48.9% and 46.1%, respectively). LC/MS analysis of the bacterial extracts was carried out to investigate the likely natural products produced. Cyclo(D-*cis*-Hyp-L-Leu), dihydrosphingosine and 2-Amino-1,3-hexadecanediol were identified in the fermentation medium of *Bacterium SRCnm*, whereas cyclo (D-Pro-L-Tyr) and cyclo (L-Leu-L-Pro) were the suggested compounds of *Bacillus sp. JS*.

Keywords: Endophytes, antimicrobial activity, antioxidant, LC/MS analysis.

INTRODUCTION

Marine microorganisms have recently been isolated from a variety of marine habitats, such as seawater, sediments, algae and different animals, with the aim of discovering new natural products (Blunt *et al*., 2009; König*et al*., 2006). Microbial natural products are of considerable importance in the discovery of new therapeutic agents (Newman and Cragg, 2007), and they form a mainstay of the drug discovery industry due to their structural diversity and biological activity. More than 22,000 microbial secondary metabolites have been described up to 2009 (Demain and Sanchez, 2009). By 2012, more than 40,000 secondary metabolites have been isolated and identified from microorganisms and this number is increasing significantly (Laatsch *et al*., 2012). Apart from plants, bacteria and fungi are the most important producers of such compounds (Harvey, 2008).

Bacillus species have repeatedly been found to produce chemical compounds with anticancer activity. Although this type of bacteria can grow in almost any substrate, it was possible to suggest that these species seemed to have acquired the skill to synthesize compounds capable of inhibiting HCT-116 colorectal cancer cells (Villarreal-Gḿez *et al*., 2010).

**Corresponding author:* e-mail: emanfadl69@yahoo.com

Moreover, antibiotics are used to save billions of lives but pathogenic microorganisms constantly acquire resistance towards them by altering their metabolism and genetic structure (Raghunath 2008; Maragakis, 2008). Accordingly, there is an incessant need to find novel efficient drug molecules against multi drug resistant pathogenic microorganisms, like methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* (VREF) (Cassell and Mekalanos, 2001).

Production of anti-microbial peptides by *Bacillus* strains has been reported on several occasions, and many peptides produced by this group of bacteria were found to be suitable for various applications (Abriouel *et al*., 2011). The anti-microbial peptides produced by *Bacillus* spp. include various classes of bacteriocins (Klaenhammer, 1993), Mukherjee *et al*., 2006 and Rodrigues *et al*., 2006 also reported the isolation and identification of anti-microbial surface-active biosurfactants like lipopeptides, glycopeptides and non-ribosomally synthesized cyclic peptides. During the screening for antibiotic producing microorganisms, the supernatant of the *Bacillus* isolate FAS was found to have antibacterial and anti-fungal activity, with a maximum inhibition zone of 25 mm on *Aspergillus niger* pathogen (Moshafi *et al*., 2011).

Marine bacteria have been found to produce potential antibacterial agents, allowing the ecological stability of the multiple marine ecosystems, inhibiting the rival organisms and pathogenic microbes. The sharing or competition mechanisms that are known between these microorganisms are diverse, including antibiotic production, bacteriocines, siderophores, lysosomes, proteases and even the pH alteration through the production of organic acids (Avendaño-Herrera 2005). The aim of this study is to explore new bioactive compounds from natural sources, this was achieved by the isolation of marine bacteria and screening the antibiotic and antioxidant potential of the extracted metabolites, also the isolation and identification of the compounds in the active extract was one of our purposes.

MATERIALS AND METHODS

Materials

All solvents that used for cell mass and media extraction as well as LCMS preparation were purchased from Fischer Scientific, Newcastle, UK and used as it is without further purification. Glucose, potassium chloride, potassium dihydrogen phosphate, magnesium sulphate and disodium hydrogen phosphate were products from BDH chemical LTO, Pok, England.

Methods

Collection and identification of the samples

The red and brown algae were collected from the Red Sea at a depth of 2 m from the Institute of Marine Science (Hurghada, Egypt). The collected marine algae were washed with tap water to remove the attached soil, surface-sterilized by submerging in 70% ethanol for 3 min and rinsed 2 times with sterile distilled water. The algal samples were identified by the Coral Reef Ecology and Biology group, National Institute of Oceanography and Fisheries, Suez, Egypt.

Isolation of endophytic bacteria

Samples were cut out and macerated in droplets of distilled water and placed on the surface of nutrient agar medium (NAM) that contained the following components (g/l): glucose 10, peptone 6, yeast extract 3, meat extract 1.5 and agar 25. The media were incubated for 28 h at 30˚C. Colony morphology was recorded for the following characters: size, shape, color, and growth rate. The distinct colonies were sub-cultured and maintained on NAM.

DNA extraction and PCR amplification of 16SrDNA region

DNA was isolated according to Sambrook *et al*., 1989. The 16SrDNA was amplified by polymerase chain reaction (PCR) using primers designed to amplify a 1500 bp fragment of the 16SrDNA region. The forward primer was 5'AGAGTTTGATCMTGGCTCAG3' and the reverse primer was5'TACGGYTACCTTGTTACGA-CTT3'. The PCR mixture consisted of 30pmol of each primer, 10ng of chromosomal DNA, 200 µM dNTPs and 2.5 Units of Taq polymerase in 50µl of polymerase buffer. The PCR was carried out for 30 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 2 minutes. After completion, a fraction of the PCR mixture was examined using agarose gel electrophoresis (Qiagen) (Ausuble *et al*., 1999) (fig.1). DNA sequences were obtained using 3130 X DNA Sequencer (Genetic Analyzer, Applied Biosystems, Hitachi, Japan), Big Dye Terminator Cycle Sequencing (see details below). The PCR product was sequenced using the same PCR primers. Blast algorithm was used to assess the DNA similarities and multiple sequence alignment and molecular phylogeny were performed using Bio Edit software (Hall, 1999). The phylogenetic tree was displayed using the TREEVIEW program (Page, 1996).

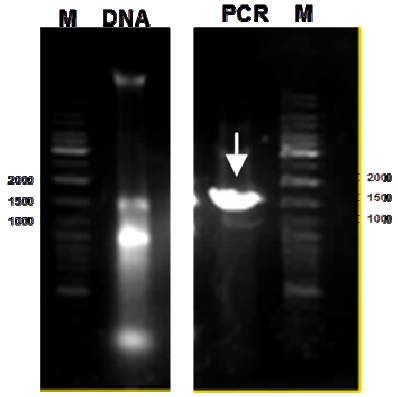

Fig. 1: M: DNA marker, Lane DNA and PCR products of 16srRNA for isolate (A).

DNA sequencing

Automated DNA sequencing based on enzymatic chain terminator technique, developed by the method of Sanger *et al*., 1977 was done using 3130 X DNA Sequencer (Genetic Analyzer, Applied Biosystems, Hitachi, Japan). The sequencing reaction was performed with four different fluorescent labels identifying the dNTPs, instead of the radioactive labels. These fluorophores were excited with two argon lasers at 488 and 514 nm, respectively when the respective bands passed the lasers during the electrophoresis. The specific emissions were detected and the data were collected for analysis (Prober *et al.,* 1987 and Freeman *et al*., 1990). The thermal cycling mixture was as follows: 8 µl of BigDye terminator mix, 6 µl of the sequencing primer (10 pmol) and 6 µl of the sample (PCR product or plasmid), then the reaction was run in the thermal cycler. The cyclic reaction composed of 1 min at 95°C, then 49 cycles of 30 s at 95°C, 10 s at 52°C and 4min at 60°C. The products were purified using a special column according to the instruction of the manufacturer. Dye formamide was added to the elute with 1:1volume ratio, run at 95ºC for 5 min for denaturation, shock on ice, then sequenced in 3130 X DNA sequencer and analyzed.

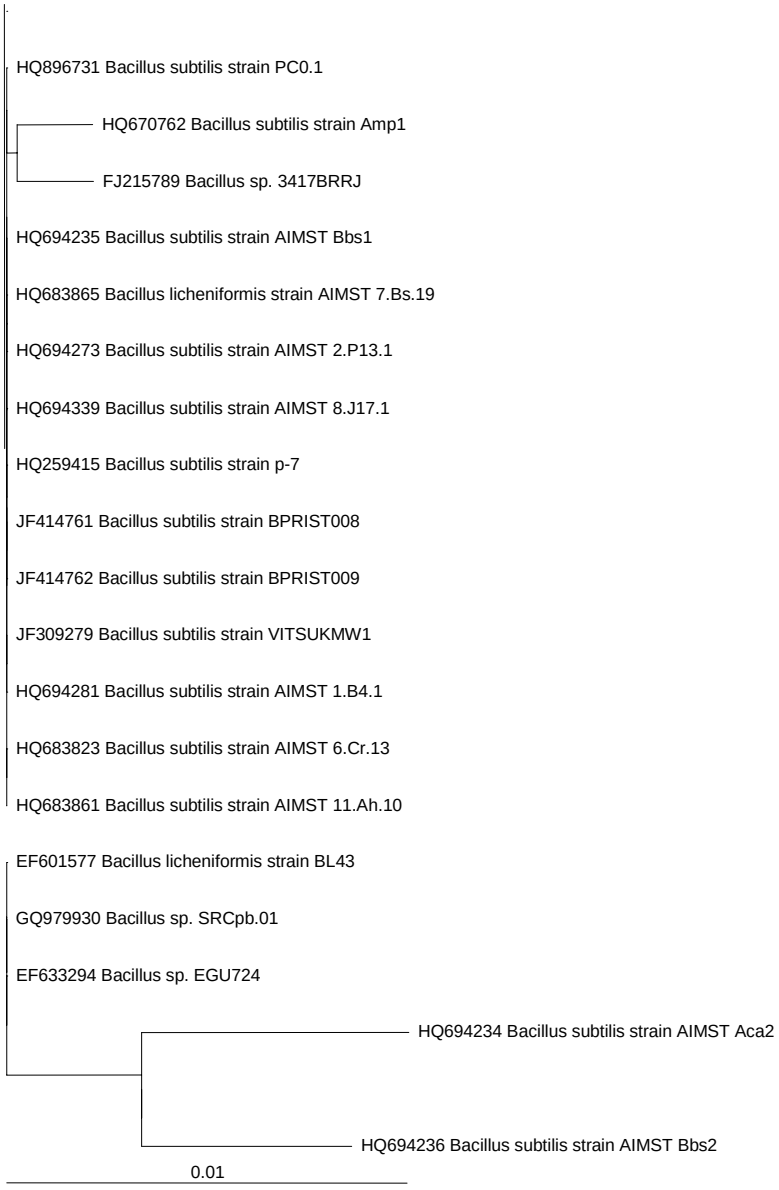

Fig. 2: The phylogenetic tree based on PCR product sequencing of DNA isolated from endophyte (A)

Extraction of metabolites

Spore suspensions of the isolated endophytes were cultured in 12 L nutrient broth media for 72h. The cells were separated by centrifugation and the filtrate was extracted using ethyl acetate. The resultant extract was dried using a rotary evaporator at reduced pressure and a water temperature of 40°C.

Antimicrobial assay

The anti-microbial activity test was carried out by the disk diffusion method (Lisboa *et al*., 2006). The crude extracts (1mg/ml) were dissolved in EtOAc and sterile paper disks were impregnated with different concentrations of the extracts and placed on the surface of NAM previously spread with pathogen suspensions. Petri dishes were incubated at 30°C and the diameter of the inhibition zones was measured after 24/48 h and expressed in mm.

Pathogenic test organisms

All test organisms were supplied by Biotechnological Research Center, Al-Azhar University, Cairo, Egypt.

Gram positive bacteria: Bacillus subtilis NCTC 1040, *Staphylococcus aureus,* NCTC 7447 and *Sarcina maxima* ATCC 33910,
Gram negative bacteria: Escherichia coli NCTC 10416, *Pseudomonas aeruginosa,* ATCC 10145.
Yeast: Candida albicans IMRU 3669.

The test organisms were maintained on NAM for the bacteria and potato dextrose agar (PDA) medium:(Peeled potato 200 g, dextrose 20 g, agar 18 g in 1000 ml distilled water) for the yeast.

Optimization of production of anti-microbial compounds

To evaluate the influence of different media on the production of anti-microbial metabolites, the isolated bacteria were cultivated in 100 ml of the following fermentation media:

Medium I (50% sea water)**,** contained (g/l): yeast extract 3, glucose 10, meat extract 1.5 and peptone 6.

Medium II (50% sea water)**,** contained (g/l): yeast extract 4, glucose 4, malt extract 10.

Medium III (100% sea water)**,** contained (g/l): yeast extract 4, glucose 4, malt extract 10.

Medium IV (50% sea water)**,** contained (g/l): Peptone 5 and yeast extract 1.

Media V (100% sea water)**,** contained (g/l): Peptone 5 and yeast extract 1.

Antioxidant activity (DPPH assay)

The free radical scavenging activity using 1,1- diphenyl-2- picryl-hydrazil (DPPH) reagent was determined according to the method described by Brand-Williams *et al*., 1995.

Antioxidant activity was calculated using the following equation:

Antioxidant activity% = [(control absorbance-sample absorbance /control absorbance × 100)]

Physiological studies on marine endophytes

Effect of temperature and pH on the growth of the bacteria

To determine the optimum growth temperature, the isolates were incubated on nutrient agar slants at different temperatures (40-70°C) for 28 h. Nutrient broth media (50 ml) were adjusted to cover the pH range 3-10 using phosphate buffer solutions. Spore suspension (1ml) was inoculated in the flasks and shaken at 250rpm for 24 h. Cell density was assessed at 600nm.

Influence of different salts

Salt solutions (0.1M) of the following salts; $FeCl_3$, Fe_2SO_4, $CuSO_4$, potassium phthalate, $ZnSO_4$, disodium EDTA, Na_2HPO_4, KH_2PO_4, $MgSO_4$. $7H_2O$, NaCl, $CaCl_2$, KCl, KI, $CH_3COONa.3H_2O$, Na_2HPO_4, $NaHCO_3$, Na_2CO_3 $MnCl_2$, were prepared separately in 50 ml distilled water that contained the following nutrients (g/l): (peptone 0.3, yeast extract, 0.15 and glucose, 0.5). 1ml spore suspension was used to inoculate the media, incubated at 30°C and the Cell density was measured at 600 nm after 48h.

Effect of different carbon sources

Galactose, glucose, raffinose, lactose, mannose, cellobiose, manitol, glycerol, sucrose and maltose were used in weights equivalent to the carbon amounts of 2g glucose and added separately in 50ml media that contained the following nutrients (g/l): yeast extract 0.5, KH_2PO_4 0.1 and $MgSO_4.7H_2O$ 0.5. The media were inoculated with 1 ml spore suspension at30°C, shaken for 28 h. Cells turbidity was measured at 600 nm.

LC/MS analyses and dereplication

High resolution mass spectrometric data were obtained using a Thermo Instruments MS system (LTQ XL/LTQ Orbitrap Discovery) coupled to a Thermo Instruments HPLC system (Accela PDA detector, Accela PDA auto sampler, and Accela pump). The following conditions were applied: capillary voltage 45 V, capillary temperature 260°C, auxiliary gas flow rate 10-20 arbitrary units, sheath gas flow rate 40-50 arbitrary units, spray voltage 4.5 kV, mass range 100-2000 amu (maximum resolution 30 000). The exact mass obtained for eluted peaks was used to deduce the possible molecular formulae for such mass, and these formulae were searched in Dictionary of Natural Products, CRC press, online version, for matching chemical structures.

RESULTS

By definition, endophytes are microorganisms (mostly fungi and bacteria) which inhabit healthy living plant tissues for all or part of their life cycle without causing apparent harmful symptoms to the host (Sturz *et al*., 2000, Wellington and Marcela, 2004).

In this study we aimed to isolate marine bacteria from the red alga *Acanthophora dendroides* (isolate A) and the brown alga *Sarggassum sabrepandum* (isolate B) and to screen the antagonistic effects of their crude extracts against some pathogenic bacteria. Molecular characterization of the selected endophytic bacteria was carried out by PCR amplification of 16S rDNA genes. A fraction of the PCR mixture was examined using agarose gel electrophoresis (fig. 1). According to sequencing similarities and multiple alignment, the isolates were found to be in a close relation to *Bacterium SRCnm* (ac: GQ979939.) and *Bacillus sp. JS*, (ac: CP003492) with 100% identity. The phylogenetic tree of *Bacterium SRCnm* was displayed using the TREEVIEW program (fig. 2).

Investigation of the most favorable medium to *Bacillus subtilis* for the production of antimicrobial metabolites was one of our purposes. The results revealed that the extracted compounds in Media I and IV showed a reasonable inhibition to the pathogen growth (fig. 3, A and B). *Sarcina maxima* exhibited the highest sensitivity to the metabolites produced in media II, III, IV and V by endophyte A, and to the total extract in media I and II where isolate B was grown (fig 4). The most active extracts against *Staphylococcus aureus* growth were

obtained after incubation of *Bacterium SRCnm* in media II and IV (fig. 5). Similar inhibitory activities of isolate A against *Escherichia coli* and *Pseudomonas aeurginosa* were shown in all extracted media (figs. 6 and 7).

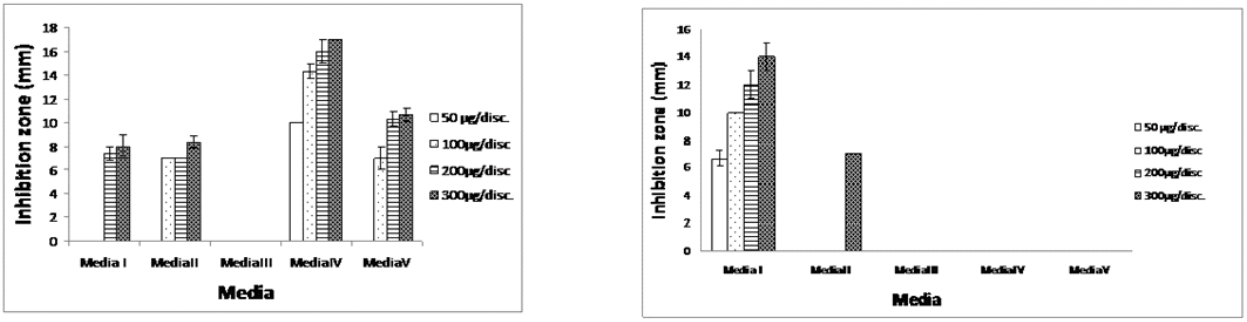

Fig. 3: Anti-microbial activity of *Bacterium SRCnm* (A) and *Bacillus sp. JS* (B) against *Bacillus subtilis*

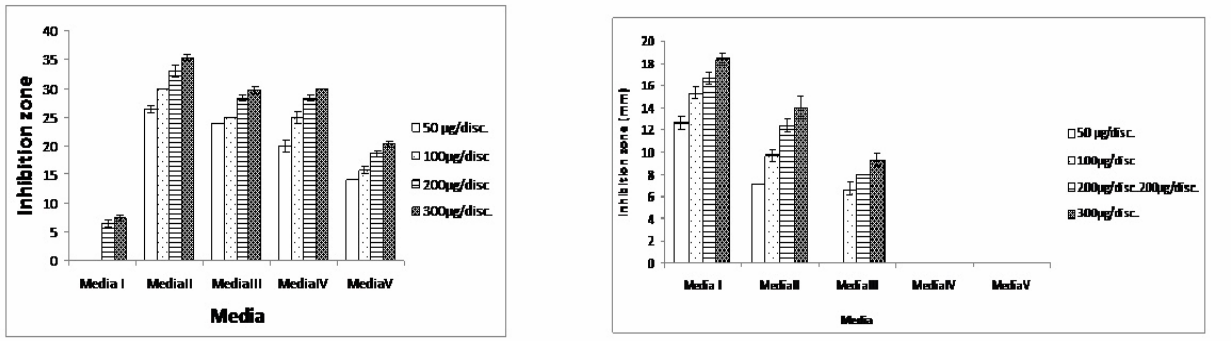

Fig. 4: Bioactivity of *Bacterium SRCnm* (A) and *Bacillus sp. JS* (B) against *Sarcina maxima*

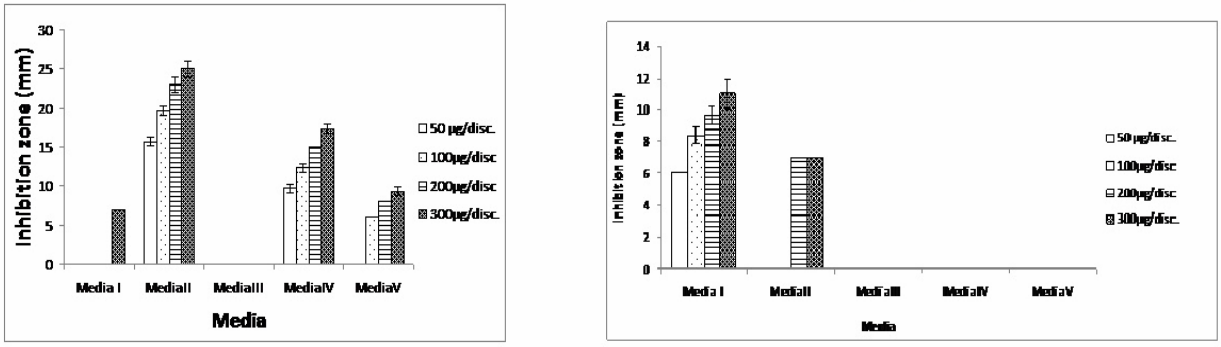

Fig. 5: Antagonistic potential of *Bacterium SRCnm* (A) and *Bacillus sp. JS* (B) against *Staphylococcus aureus*

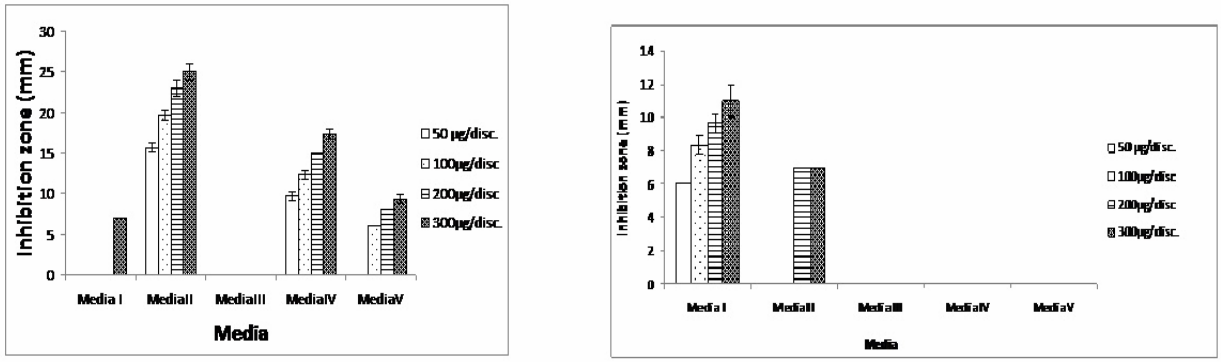

Fig. 6:Growth inhibition of *Escherichia coli* by extracted compounds of *Bacterium SRCnm* (A) and *Bacillus sp. JS* (B)

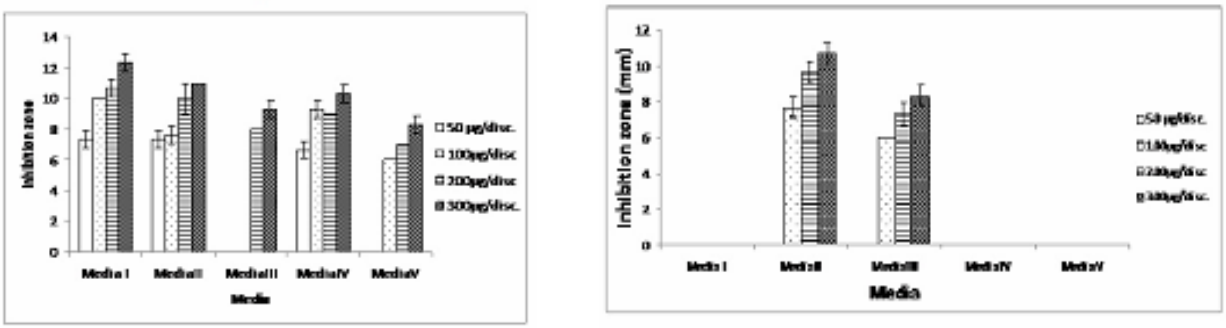

Fig.7: Sensitivity of *Pseudomonas aeurginosa*to the extracted metabolites of *Bacterium SRC nm* (A) and *Bacillus sp. JS* (B).

The second goal in this investigation focused on improving the growth yield of the isolated bacteria through changing some growth parameters, such as pH, temperature and carbon sources. The results in table1 revealed that the isolates should be considered neutrophils, because the optimum growth was noticed at pH 8.On the other hand, the most favorable temperature for the growth of the tested bacteria was found to be 30°C (table 2). Different Bacillus species have similar growth patterns and enzyme profiles, but their optimized conditions vary, depending upon the strain (Vijayalakshmi *et al*., 2012).

Investigation of the role of different carbon sources on bacterial growth revealed that maltose was the best nutrient for the isolated endophytes. Also the addition of sucrose (a disaccharide composed of the monosaccharides glucose and fructose) to the culture medium of *Bacterium SRCnm* caused an increase in its growth (table 3).

The influence of adding different salts to the fermentation medium of the bacterial isolates presented variable results. These results grouped in table 4 showed that $CaCl_2$ and Na_2HPO_4 increased the growth capacity of *Bacterium SRCnm* and *Bacillus sp. JS* to a maximum value.

Table 1: The growth capacities of *Bacterium SRCnm* (A) and *Bacillus sp. JS* (B) in relation to pH level

Phosphate buffer solution pH	Growth %	
	(A)	(B)
3.0	1.76	0.36
4.0	2.31	0.4
5.0	28.0	1.12
6.0	38.0	21.9
7.0	87.16	27.7
8.0	100	100
9.0	56.58	3.6
10.0	8.5	1.4

Table 2: Effect of temperatures on the growth of *Bacterium SRCnm* (A) and *Bacillus sp. JS* (B)

Temperature ˚C	A	B
10	_	_
20	++	+
30	++++	++++
40	++	+++
50	+	+
60	_	_

Table 3: The role of different carbon sources on the growth% of *Bacterium SRCnm* (A) and *Bacillus sp. JS* (B)

Carbon sources	(A)	(B)
Non	33.3	13.9
Galactose	79.4	24.5
Glucose	36.1	43.0
Raffinose	85.9	21.4
Lactose	66.1	22.9
Mannose	48.4	30.5
Cellobiose	73.09	0.66
Manitol	87.30	25.9
Glycerol	22.7	13.1
Sucrose	90.8	34.8
Maltose	100	100

The antioxidant activity for the metabolites of, *Bacterium SRCnm & Bacillus sp. JS* was also investigated using DPPH assay. The metabolites that were potential in scavenging of DPPH radicals could be related to their hydrogen donating ability (Spandana *et al*., 2012).

Table 6 and figs. 8-20 showed that 7 compounds of *Bacterium SRCnm* and 10 compounds of *Bacillus sp. JS* had the ability to be ionized. HRESIMS of these compounds were determined and compared to previously isolated ones using different libraries data bases (Dictionary of Natural Products; version 22.1 and Anti-Base 2012). Cyclo (D-*cis*-Hyp-L-Leu),

dihydrosphingosine, 2-Amino-1,3-hexadecanediol, and other hits not of bacterial origin were identified in *Bacterium SRCnm* fraction , on the other hand cyclo (D-Pro-L-Tyr) and Cyclo (L-Leu-L-pro) were the suggested compounds for *Bacillus sp. JS* extracts, the same identified compounds of *Bacterium SRCnm*. The compound eluting at 21.73 min showed the molecular formula $C_{53}H_{94}O_{13}N_7$, which warrants further investigation as is most likely a cyclic peptide.

Table 4: Influence of salts on the growth% of *Bacterium SRCnm* (A) and *Bacillus sp. JS* (B)

Salt	(A)	(B)
Non	48.4	40.3
Fe_2SO_4	-	52.6
$CuSO_4$	1.35	20.4
k.phthalate	1.14	0.6
$ZnSO_4$	1.3	6.9
EDTA.2Na	0.5	0.39
NaH_2PO_4	47.43	0.5
KH_2PO_4	12.05	7.2
$MgSO_4.7H_2O$	47.0	82.9
Nacl	38.0	37.1
$CaCl_2$	100	28.9
KCl	40.3	42.7
KI	34.36	0.42
$CH_3COONa.3H_2O$	7.2	37.1
Na_2HPO_4	50.5	100
$NaHCO_3$	1.08	3.6
Na_2CO_3	8.3	7.0
$MnCl_2$	-	1.6

Table 5: Antioxidant activity of *Bacterium SRCnm* (A) and *Bacillus sp. JS* (B)

Concentration (µg/ml)	Antioxidant Activity %	
	(A)	(B)
500	48.89	46.13
250	37.12	31.05
125	22.36	19.69
62.5	9.74	8.11

DISCUSSION

Endophytic bacteria have received considerable attention due to their diverse bioactivities, including biological control of plant diseases, plant growth stimulation, nitrogen-fixing, etc (Harris *et al.,* 1994; Chen *et al*., 1995; Graner *et al*., 2003; Cui *et al*., 2003; Qiao *et al*., 2006). A general call exists for new antibiotics, chemotherapeutic agents, and agrochemicals that are highly effective, possess low toxicity, and have a minor environmental impact where endophytes could be good sources of such chemical entities. This is because natural products from endophytic microbes have been observed to inhibit or kill a wide variety of harmful disease-causing agents including, but not limited to, phytopathogens and bacteria, fungi, viruses and protozoans that affect humans and animals (Strobel and Daisy, 2003).

It was suspected that such Specific invertebrate-microbial associations play a major ecological role in maintaining marine macroorganism's life by protecting the host from invasion of potentially pathogenic microbes through competing for nutritional sources or excretion of biologically-active metabolites as defense mechanism (Klaus *et al*. 2007; Salyers and Whitt 1994).

Antibacterial activity among marine bacterial derived natural products is a well-known phenomenon and has been demonstrated in a number of studies (Isnansetyo and Kamei, 2003; Uzair *et al*., 2006).

It is well known that the marine bacteria bacilli have long been associated with the production of antibacterial compounds, which could be used in the biotechnology industry to manufacture antibiotics, biochemicals and consumer products (Erickson, 1976; Eveleigh, 1981; and Austin, 2001).

Romanenko *et al*., 2001 isolated *Bacillus* strains from internal tissues of the ascidian *Halocynthia aurantium* which was capable to inhibiting the growth of *S. aureus* and *B. subtilis*. Anti-MRSA and VRE compounds were isolated from *B. licheniformis* strain EI-34-6, which was associated with a marine alga (Yan *et al*., 2003).

Jamal and Mudarris (2010) isolated *Bacillus subtilis* from *Sargassum sp*., which exhibited anti-microbial activity against resistant pathogenic bacteria. Such results were also in agreement with our observation.

Previously, Davey and Daughtry 1995, reported in validation of a model for predicting the combined effect of three environmental factors on both exponential and lag phases of bacterial growth: temperature, salt concentration and pH. In this work, the effect of each factor was tested separately. Changes in pH levels had the most noticeable effects on bacterial enzymes and growth, where extreme changes in the pH balance of the local environment for bacteria tended to kill them.

The optimum temperature for growth of *Bacillus subtilis* 115 & *Bacillus subtilis* was 50˚C (Jansova *et al.,* 1993) whereas, *Bacillus subtilis* KC3 has optimum growth temperature around 33°C, (Demirkan *et al*., 2005). The maximum growth of *Bacillus subtilis KO* was achieved at 45°C and pH was in range 6.5-7.0 but it exhibited no growth above 50°C (Magdi *et al*., 2010). Bacillus sp. AQ1 could grow and produce enzyme at the pH (7-9) and temperature (30-50°C) observed (Budiasih *et al*., 2009).

It is well known that maltose could be broken down into two glucose molecules by hydrolysis. In living organisms, the enzyme maltase can achieve this very rapidly. Our finding was in accordance with that previously obtained by, Vijayalakshmi *et al*. (2012), who demonstrated that induction of α-amylase requires substrates having α-1, 4 glycosidic bond, including starch and maltose, but glucose represses its production. The biosynthesis of α-amylase in most species of the genus *Bacillus* is repressed by readily metabolizable substrates especially glucose, by a mechanism of catabolite repression (Haseltine *et al*., 1996 &Kato *et al.,* 1996). The results revealed the importance of $CaCl_2$ and Na_2HPO_4 because these salts are naturally found in the sea water which required for growth of such marine bacteria (Robert and Onofrey, 1956). Deshpande and Cheryan (1984) referred to the significant effect of Ca^{2+} on the metabolism and physiology of bacteria as it was found to be effective on enzyme activity. Potassium, calcium and phosphorus elements were found to serve a structural or physiological role in the bacterial cell. Potassium is a main cellular inorganic cation and cofactor for certain enzymes, calcium is Inorganic cellular cation, cofactor for certain enzymes and phosphorus is a component of endospores and Constituent of nucleic acids, nucleotides, and phospholipids. In the same manner, Robert and Onofrey (1956) reported that none of the marine bacteria could grow, unless Na^+, K^+&$P0_4^-$added to their cultivated media. Thus, growth temperature, pH level and nutrients in the culture media of the endophytes have significant effects on the mass production of the microorganisms and thus the yield of the compounds produced by the growing cells. Tomohiro *et al*.1994 isolated 112 bacterial strains from different marine organisms which exhibited antioxidant activity as a kind of adaptation to the aerobic conditions, this activity was correlated to the presence of uric acid, indole, 3,4-dimethoxyphenol and 3-hydroxyindolin-2-one in their fermentation broth.

LC/MS analyses results indicated that the similar compounds that were identified are of bacterial and sometimes fungal secondary metabolites. Many of the constituents still not known and may be correlated to the genetic contents of the investigated bacteria. Previous results were of great interesting as many of the other compounds did not show any hits to be isolated from nature before. More investigation of these fractions on large-scale fermentation, fractionation, isolation and structure elucidation are needed.

CONCLUSION

In the present work, we have isolated two algae-associated bacterial strains, identified them using 16S rDNA and fermented them on five different media and screened their extracts for their anti-microbial and antioxidant potential. Using LCMS and natural product databases, we managed to identify some ionized compounds and some were potentially new based on finding no hits for their formulae in these databases, which required further investigation on larger scale. Finally, the biological potential of the compounds produced by *Bacterium SRCnm* and *Bacillus sp. JS* was tested to figure out the possibility of using these metabolites against resistant pathogens. Different parameters; temperatures, pH, salt concentrations, and carbons were studied to optimize the growth conditions of the isolated endophytes.

REFERENCES

Abriouel H, Franz CM, Ben Omar N and Galvez A (2011). Diversity and applications of *Bacillus bacteriocins*. *FEMS. Microbiol. Rev*., **35**: 201-232.

Austin B (2001). Novel pharmaceutical compounds from marine bacteria. *In*: Recent Advances in Marine Biotechnology, Bio-organic Compounds: Chemistry and Biomedical Applications, R. Nagabhushanam, M. Fingerman, (ed.) (New Delhi: Oxford and IBH), **6**: 1-19.

Ausubel FM, Brent R, Kingston RE, More DD, Seidam JG, Smith JA and Struhl K (eds). (1999). *Short protocols in Molecular Biology.* John Willey and Sons, Inc. NY, pp.53-64.

Avendaño-Herrera R, Lody M and Riquelme CE (2005). Production of inhibitory substances among bacterial biofilms on marine substrates. Producción de substancias inhibitorias entre bacterias de biopelículas en substratos marinos. *Revista. de. Biología. Marinay Oceanografía*., **40**: 117-125.

Blunt JW, Copp BR, Hu WP, Munro MH, Northcote PT and Prinsep MR (2009). Marine natural products. *Nat. Prod. Rep*., **26**: 170-244.

Brand-Williams W, Cuvelier ME and Berset C (1995). Use of free radical method to evaluate anti-oxidant activity. *Lebensmittel Wissenschaft und Technologie*, **28**: 25-30.

Budiasih W, Nisa RM and Siswa S (2009). Effect of pH, Temperature and medium composition on xylanase production by bacillus sp. AQ-1 and partial characterization of the crude Enzyme. *Microbioogy*., **3**: 17-22.

Cassell GH and Mekalanos J (2001). Development of anti-microbial agents in the era of new and reemerging infectious diseases and increasing antibiotic resistance. *J. Am. Med. Assoc*., **285**: 601-605.

Chen C, Bauske EM, Musson G, Rodriguezkabana R and Kloepper JW (1995). Biological control of Fusarium Wilt on cotton by use of endophytic *bacteria. Biol. Control*, **5**: 83-91.

Cui L, Sun Z, Sun F, Yuan J, Tian H, Wang L and Xu H (2003). Isolation of endophytic bacteria from potato and selection of antagonistic bacteria to potato ring rot disease. *Acta Phytopathol. Sinica*., **33**: 353-358.

Davey KR and Daughtry BJ (1995). Validation of a model for predicting the combined effect of three environmental factors on both exponential and lag phases of bacterial growth: Temperature, salt concentration and pH Original Research Article. *Food Res. Int.* **28**: 233-237.

Demain AL and Sanchez SJ (2009). Microbial drug discovery: 80 years of progress. *Antibiot*., **62**: 5-16.

Demirkan E S, Mikami B, Adachi M, Higa T and Utsumi S (2005). α-amylases from B. amyloliquefaciens: Purification, characterization, raw starch degradation and expression in E. coli. *Process Biochem*., **40**: 2629-2636.

Deshpande SS and Cheryan M (1984). Effects of phytic acid, divalentcation and their interactions on α-amylase activity. *J. Food. Sci*., **49**: 516-519.

Erickson RJ (1976). Industrial applications of the bacilli: A review and prospectus. *In*: D. Schlesinger, (ed.), Microbiology. Washington, D.C: American Society for Microbiology, pp.406-419.

Eveleigh DE (1981). The microbial production of industrial chemicals. *Sci. Am*., **245**: 155-178.

Freeman M, Baehler C and Spotts S (1990). Automated Lazer fluorescence sequencing. *Biotechnol*., **8**: 147-148.

Graner G, Persson P, Meijer J and Alstrom S (2003). A study on microbial diversity in different cultivars of *Brassica napus*in relation to its wilt pathogen. Verticillium longisporum. *FEMS. Microbiol. Lett*., **224**: 269-276.

Hall TA (1999). Bio Edit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acid. Symp. Ser*., **41**: 95-98.

Harris GH, Hesterman OB, Paul EA, Peters SE and Jahnke RR (1994). Fate of legume and fertilizer nitrogen-15 in a long-term cropping systems experiment. *Agron. J*., **86**: 910-91.

Harvey AL (2008). Natural products in drug discovery. *Drug Discov. Today*, **13**: 894-901.

Haseltine C. Rolfsmeier M and Blum P (1996). The glucose effect and regulation of α-amylase synthesis in the hyperthermophilic archaeon sulfolobus solfataricus. *J. Bacteriol*., **178**: 945-950.

Isnansetyo A and Kamei Y (2003). *Pseudoalteromonas phenolica* sp. nov., a novel marine bacterium that produces phenolic anti-methicillin resistant *Staphylococcus aureus* substances. *Int. J. Syst. Evol. Microbiol*., **53**: 583-588.

Jamal MT and Mudarris MSA (2010). Separation of YbdN bioactive protein from bacillus subtilis isolated from the red sea algae sargassum sp. with bioactivity against antibiotic resistant bacterial pathogens. *JKAU: Mar. Sci*., **21**: 53-64.

Jansova E, Schwarzova Z and Chalupka J (1993). Sporulation and synthesis of extra cellular proteinases in Bacillus subtilis are more temperature-sensitive than growth. *Folia Micro. Biologica*., **38**: 22-24.

Kato M, Sekine K and Tsukagoshi N (1996). Sequence-specific bindingsites in the Taka-amylase A G2 promoter for the CreA repressor mediating carbon catabolite repression. *Biosci. Biotechnol. Biochem*., **60**: 1776-1779.

Klaenhammer T (1993). Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiol. Rev*., **12**: 39-86.

Klaus JS, Janse I, Heikoop JM, Sanford RA and Fouke BW (2007). Coral microbial communities, zooxanthellae and mucus along gradients of seawater depth and coastal pollution. *Environ Microbiol*., **9**: 1291-305.

König GM, Kehraus S, Seibert SF, Abdel-Lateff A and Müller D (2006). Natural products from marine organisms and their associated microbes. *Chem. Bio. Chem*., **7**: 229-238.

Laatsch H (2012). Anti-Base: The natural compound Identifier, Wiley VCH, Weinheim, Germany, **84**: 4277-4283.

Lisboa MP, Bonatto D, Bizani D, Henriques JA and Brandelli A (2006). Characterization of a bacteriocin-like substance produced by *Bacillus amyloliquefaciens* isolated from the Brazilian Atlantic forest. *Int. Microbiol.* **9**: 111-118.

Magdi AM, Younis, Francis F, Hezayen, Moustafa A, Nour-Eldein and Mohamed SA (2010). Optimization of cultivation medium and growth conditions for *Bacillus subtilis* KO strain isolated from sugar cane molasses. *American- Eurasian J. Agric. & Environ. Sci.,* **7**: 31-37.

Maragakis LL, Perencevich EN and Cosgrove SE (2008). Clinical and economic burden of anti-microbial resistance. *Expert Rev. Anti-Infect Ther*., **6**: 751-763.

Moshafi MH, Forootanfar H, Ameri A, Ameri A, Shakibaie M, Dehghan-Noudeh G and Razavi M (2011). Anti-microbial activity of bacillus sp. strain FAS1 isolated from soil. *Pak. J. Pharm. Sci*., **24**: 269-75.

Mukherjee S, Das P and Sen R (2006). Towards commercial production of microbial surfactants. *TRENDS in Biotechnology*, **24**: 509-515.

Newman DJ and Cragg GM (2007). Natural products as sources of new drugs over the last 25 years. *J. Nat. Prod*., **70**: 461-477.

Page RDM (1996). Tree view: An application to display phylogenetic trees on personal computers. *Comp. Appl. in Biosci.* **12**: 357-358.

Prober JM, Trainor GL, Dam RJ, Hobbs FW, Robertson CW, Zagursky RJ, Cocuzza AJ, Jensen MA and Baumeister K (1987). A system for rapid DNA sequencing with fluorescent chain-terminating dideoxy nucleotides. *Science,* **238**: 336-341.

Qiao HP, Huang LL and Kang ZS (2006). Endophytic bacteria isolated from wheat and their anti-fungal activities to soil-borne disease pathogens. *Chin. Appl. Ecol*., **17**: 690-694.

Raghunath D (2008). Emerging antibiotic resistance in bacteria with special referenceto India. *J. Biosci*., **33**: 593-603.

Robert AM and Onofrey E (1956). Nutrition and metabolism of marine bacteria II. Observations on the relation of sea water to the growth of marine bacteria, *J. Bacteriol*., **71**: 661-671.

Rodrigues L, Banat IM, Teixeira J and Oliveira R (2006). Biosurfactants: potential applications in medicine. *J. Antimicrob. Chemother*., **57**: 609-961.

Romanenko LA, Kalinovskaya LI, Mikhailov VV (2001). Taxonomic composition and biological activity of the microorganisms associated with marine ascidian Halocynthia aurantium. Biologiya Morya Russ. *J. Marine Bio.*, **27**: 291-295.

Salyers AA and Whitt DD (1994). *In*: Bacterial pathogenesis: A molecular approach. American society for microbiology Press, Washington, DC, USA, pp.-97-110.

Sambrook J, Fritsch EF and Maniatis T (1989). Molecular cloning A. Laboratory Manual., 2_{nd} ed. Cold Spring Harbor Laboratory Press NY, New York, pp. 754-760.

Sanger F, Nicklen S and Coulson AR (1977). DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci*., **74**: 5463-5467.

Spandana M, Ratna MT, Aruna kD and Susmitha K (2012). Investigation on anti-oxidant and anti-microbial activities of marine actinomycetes. *IJPWR*., **3**: 1-20.

Strobel G and Daisy B (2003). Bioprospecting for microbial endophytes and their natural products. *Microbiol. Mol. Biol. Rev*., **67**: 491-502.

Sturzet AV, Christie BR and Nowak J (2000). Bacterial endophytes: *Potential Plant. Sci*., **19**: 1-30.

Tomohoro T, Futoshi K, Naoharu W. Akihito Y and Kanzo S (1994). A simple screening method for anti-oxidants and isolation of several anti-oxidants produced by marine bacteria from fish and shellfish. *Biosci. Biotech. Biochem*., **58**: 1780-1783.

Uzair B, Ahmed N, Ahmed V and Kousar F (2006). A new antibacterial compound produced by indigenous marine bacteria; fermentation, isolation and biological activity. *Nat. Proc. Res*., **20**: 1326-1331.

Vijayalakshmi K, Sushma S, Abha and Chander P (2012). Isolation and characterization of bacillus subtilis KC3 for amylolytic activity. *Int. J. Biosci., Biochem. and Bioinfor.* **2**: 336-341

Villarreal-Gmez LJ, Soria-Mercado IE, Guerra-Rivas G and Ayala-Sánchez NE (2010). Anti-bacterial and anticancer activity of seaweeds and bacteria associated with their surface. *Revista. de Biología. Marina Oceanografía*., **45**: 267-275.

Wellington B and Marcela TB (2004). Delivery methods for introducing endophytic bacteria into maize. *Bio. Control*., **49**: 315-322.

Yan L, Boyd KG, Adams DR and Burgess JG (2003). Biofilmcross-specific induction of anti-microbial compounds in bacilli. *Appl. Environ. Microbiol*., **69**: 3719-3727.