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Novel Egyptian bacterial strains exhibiting antimicrobial and antiaflatoxigenic activity

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ABSTRACT

The aim of this study was to isolate and characterize new Egyptian marine bacterial strains and to evaluate the antimicrobial, antioxidant and total phenolic content of their bioactive secondary metabolites. Eight bacterial strains were isolated from marine sediment samples collected from different areas in Red sea governorate, Egypt. Antifungal activity was recorded as zone inhibition of growth between the fungus and the organism tested and two bacterial isolates were selected (designated 21 and 30). These isolates significantly reduced fungal growth and AFB_1 production especially the isolate 30. These promising isolates were selected and identified using molecular identification technique and identified as *Brevundimonas* and *Bacillus*. The crude extracts from the two bacteria were extracted and the biological activity of these crude extracts was studied. The crude extracts exhibited potent antimicrobial activities against a set of microbial pathogens as well as antioxidant activity and total phenolic content.

INTRODUCTION

Many fungal strains belonging to the genera *Fusarium*, *Aspergillus* and *Penicillium* may produce several mycotoxins; these mycotoxins are highly toxic secondary metabolic products (Hathout and Aly, 2014). These mycotoxins were assessed and they found that at least 300 of these mycotoxins were potentially toxic to animals and humans (Heidler and Schatzmayr, 2003). The impact of these mycotoxins on human and animal health has also been studied extensively (CAST, 2003). Because of the detrimental effects of mycotoxins, some strategies have been developed to prevent the growth of mycotoxigenic fungi and also to detoxify mycotoxins (Kabak *et al*., 2006). Recently, investigators have reported that many microorganisms including bacteria have the ability to remove or degrade mycotoxins. These microorganisms produce antifungal agents that could be used as biocontrol agents (Chitarra *et al*., 2003). The use of *Bacillus* sp., has been investigated due to their properties to control diseases caused by phytopathogenic fungi (Schisler *et al*., 2004), and to produce antifungal metabolites that keep the plants free from fungal infection (Morita *et al*., 2005; Siddiqui *et al*., 2005; Nourozian *et al*., 2006). Recently, Malleswari (2014) reported that *Bacillus* sp. have become the bacterium of the choice for their flexibility and capability to contain a large number of plant pathogens in diverse target environments. *Bacillus* sp. are considered safe biological agents (Kim *et al*., 2003), and their ability to produce and secrete extracellular enzymes has placed them among the most significant industrial enzyme producers (Schallmey *et al*., 2004). *Bacillus* species with important roles which date back in time are considered main industrial microorganisms (Horikoshi, 1999).

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Brevundimonas species are aerobic straight and slender gram-negative bacilli and were formerly classified with the genus *Pseudomonas.* Segers *et al.* (1994) proposed the genus *Brevundimonas* by their classification of two *Pseudomonas* species and currently comprise 12 species. With the transfer of several species of the genus *Caulobacter* to *Brevundimonas*, the description of the genus was corrected considerably (Abraham *et al*., 1999). *Brevundimonas* can be beneficial to the plant because of its suppressive effect on pathogenic fungi and its nematicidal capability (De Boer *et al*., 2007; Zheng *et al*., 2008), and it has also been recovered as an endophyte from various plant tissues such as cotton roots, banana shoots, carrots, and tomato leaves (Hallmann *et al*., 1999; Surette *et al*., 2003; Enya *et al*., 2007; Thomas *et al*., 2008).

Bacillus sp. and *Brevundimonas* sp. are considered alkaliphiles as they grow optimally at pH values above 9, and cannot grow or grow slowly at near neutral pH values (Horikoshi, 1999). *Bacillus* sp. have the characteristics of, having high thermal tolerance, and showing rapid growth in liquid culture, and they have been isolated mostly from soil samples and other environments like saline lakes, sea water as well as by-products of food processing industries (Ntougias and Rusell, 2000).

Alkaliphilic bacteria are reported to be a rich source of alkaline active enzymes e.g. amylase, protease, cellulase, xylanase, which have several applications in industrial processes (Horikoshi, 1991).

These bacteria due to their great potential for biotechnological applications and research of ecological diversity, physiologic, biochemical and molecular specifics have attracted increased consideration during last decades (Rossi *et al*., 2003). Therefore, the aim of the current study was to isolate and characterize new Egyptian marine bacterial strains. The isolates showing high antifungal and antiaflatoxigenic activities were further screened for bioactive potential secondary metabolites to evaluate their antimicrobial and antioxidant activity.

MATERIALS AND METHODS

Microorganisms

Gram +ve *Staphylococcus aureus*; *Pseudomonas aeruginosa*, as well as *Candida albicans*, *Aspergillus niger*, *Aspergillus parasiticus* and *Aspergillus ochraceus* were obtained from the culture collection of the Microbial Resources Centre (MlRCEN), Faculty of Agriculture, Ain Shams University, Cairo, Egypt. *Fusarium solani* and *Fusarium oxysporum* were isolated from maize samples in Egypt in a previous study (Hathout *et al*., 2015)

Sampling

Marine samples (marine algae, soft corals, sea sediments and sea water) were collected from Red Sea governorate, Egypt and used for the isolation of alkaliphilic bacteria. Red Sea Governorate is located between the Nile and the Red Sea in the southeast of the country, its southern border forms part of Egypt's border with Sudan. Its capital is the city of Hurghada. Its Latitude is 25° N and Longitude is 33° E.

Isolation of alkaliphilic bacteria

The isolation medium used for the isolation of alkaliphilic bacteria was alkaline agar medium and contained the following ingredients: glucose (1.0%), peptone (0.5%), yeast extract (0.5%), KH_2PO_4 (1.0%), $MgSO_4$. $7H_2O$ (0.02%), $NaNO_3$ (1.0%) and agar (2.0%), pH 10.5 (Horikoshi, 1990). Serial dilution was done for each sample and each dilution was used to inoculate the isolation medium described by Aftab *et al*. (2006). Agar plates (15-cm diameter) containing 50-mL solidified medium were inoculated with 20 microliters of each dilution and incubated at 37°C for 24-48h. The plates that showed considerable single colonies were selected for this purpose. Inoculums from these grown colonies were transferred into replicates of slants containing the same specific media. Purified isolates were maintained on agar slants of the same medium at 4°C and were sub-cultured at monthly intervals.

Antifungal activity of the bacterial isolates

The antifungal activity of the isolated alkaliphilic bacteria was tested against fungal cultures. Alkaliphilic bacteria were grown on Nutrient Agar (NA, Sigma-Aldrich Corp. St. Louis, MO USA) and incubated at 37°C for 24 h. The isolates were removed from their agar using a sterile cork borer (5 mm in diameter) and placed onto Potato Dextrose Agar plate (PDA, BD Difco™, MD 21152, USA), lawn with the pathogenic fungi. The contact biocidal property was determined by measuring the diameter of the zone of inhibition around the bacteria.

Effect of bacterial isolates on AFB_1 production

The two bacterial isolates showing high antifungal activity (Isolate 21 and 30) were studied for their ability to detoxify AFB_1. AFB_1 standard was obtained from Sigma-Aldrich Corp., St Louis, MO, USA. All chemicals used in this study were HPLC grade (Merck KGaA, 64271 Darmstadt, Germany). Mycotoxin-producing fungi (*Aspergillus flavus*) preserved in the Department of Food Toxicology and Food Contaminants, National Research Centre, was maintained on slants of PDA and incubated at 28°C for 7 days. Fungal spore suspensions (10^6spores/mL) were prepared in an aqueous solution of 0.1% Tween 80. The bacterial cultures (isolates 21 and 30) were grown in Nutrient Broth (NB, Sigma-Aldrich Corp. St. Louis, MO USA) at 37°C for 12 h. One mL of each of the bacterial cultures for each of the strains was transferred into 250 mL conical flasks containing 100 mL yeast extract (yeast extract 2%-Sucrose 20%) broth and inoculated with 1 mL fungal spore suspension. The cultures were incubated for 7 days at 28°C. AFB_1 was extracted from culture filtrates using chloroform according to the Association of Official Analytical Chemists (Tosch *et al*., 1984). The culture filtrates were extracted three times with chloroform and the chloroform extracts were evaporated under nitrogen gas, the residue was dissolved in methanol, and then completely passed through immunoaffinity

column (AflaTest, VICAM, Ma 01757 USA) at a rate of about 1-2 drops/second. After passing the samples, the immunoaffinity column was washed twice with 10 mL purified water at a rate of about 2 drops/second. Elution was performed with 1.0 mL methanol and then analysed by HPLC.

The HPLC system used for AFB_1 analyses was an Agilent 1200 series system (Agilent, Berks, UK) with a fluorescence detector (FLD G1321A), an autosampler ALS G1329A, FC/ALS therm G1330B, Degasser G1379B, Bin Bump G1312A and a C18 (Phenomonex, Luna 5 micron, 150 × 4.6 mm) column joined to a pre-column (security guard, 4 × 3-mm cartridge, Phenomenex Luna). The mobile phase was water: acetonitrile: methanol (3:1:1, v/v/v) using an isocratic flow rate of 1 ml/min at 360 nm excitation and 420 nm emission wavelengths. The mycelium mats were collected by filtration through Whatman filter paper No. 4, washed twice with water and dried in an oven at 95°C until constant weight and weighed.

Effect of bacterial isolates on AFB_1 degradation

The bacterial cultures were grown in NB at 37°C for 12 h, and 2.4 mL of the bacterial culture broth was added to a 77.6 mL NB containing AFB_1 at a concentration of 20 ppb. The flasks were incubated at 37°C in a rotary shaker incubator at 200 rpm for 0, 24, 48 and 72 h. Flasks were taken at each interval and centrifuged at 10000 rpm for 5 min. The remaining AFB_1 in the supernatant was extracted using chloroform.

Molecular identification of the bacterial isolates

Bacterial isolates showing antifungal and antiaflatoxigenic activity were identified. Bacterial colonies were picked up with a sterilized toothpick, and suspended in 0.5 mL of sterilized saline in a 1.5 mL centrifuge tube, and centrifuged at 10,000 rpm for 10 min. After removal of the supernatant, the pellets were suspended in 0.5 mL of InstaGene Matrix (Bio-Rad, USA). Incubated at 56°C for 30 min and then heated 100°C for 10 min.1 µl of template DNA was added in 20 µl of PCR reaction solution. Sequencing of 16S rRNA gene from bacterial strains was carried out by using the forward primer of 16S rRNA gene sequence 27F 5'-AGA GTT TGA TCM TGG CTC AG-3', and the reverse primer of 1492R 5'-TAC GGY TAC CTT GTT ACG ACT T-3' (Martin and Collen, 1998). Amplification of DNA was carried out under the following conditions: denaturation at 94° C for 5 min followed by 30 cycles of 94° C for 45 s, 55° C for 60 s, 72° C for 60s and a final extension at 72° C for 10 min. DNA fragments were amplified at about 1,400 bp. A positive control (*Candida sp.* genomic DNA) and a negative control were used. Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA).

Data analysis

PCR product was sequenced subsequently and analysed with the GenBank database using BLASTN program (NCBI). The phylogenetic tree was constructed using ClustalW program editor to determine the phylogenetic relatedness of the different species. They were aligned using the UPGMA algorithm, which considers the rate of evolution to be constant between species, to develop a phylogenetic tree based on sequence homology. The resulting alignment was opened into a program called Tree View which allowed the phylogenetic tree to be viewed (Swofford, 1993).

Fermentation, extraction, and isolation of bioactive compounds

Erlenmeyer flasks (1L-volume) each containing 250 mL of NB medium were inoculated with a cell suspension from 24h old nutrient agar slants inoculated with 21 and 30 strains (1 slant for 2 1L-Erlenmeyer flasks). The flasks were kept at 37°C using rotary shaker (150 rpm) and harvested after 24 h. Another set of flasks were incubated static at 37°C. The bacterial cells were separated from the supernatant by centrifugation at 5000 rpm at 4°C. The obtained yellowish culture broth was extracted with ethyl acetate and the biomass was extracted with acetone. The acetone extract was concentrated, and the remaining water residue was re-extracted with ethyl acetate.The methods of extraction of the crude extracts and the isolates used are mentioned in Table (1).

Table 1: Crude extracts of bacterial bioactive secondary metabolites.

Extract number	Bacterial name	Incubation condition		Extraction from	
		Rotary shaker (150 rpm)	Static	Bacterial Supernatant	Bacterial Cell
I	*Brevundimonas* sp.	✓		✓	
II		✓			✓
III			✓	✓	
IV			✓		✓
V	*Bacillus* sp.	✓		✓	
VI		✓			✓
VII			✓	✓	

Determination of biological activities of the crude extracts

Antimicrobial activity

The antimicrobial activities of crude extracts of the bioactive secondary metabolites were tested against four different microbial strains. The obtained extracts were dissolved in methanol and filter paper discs (5 mm diameter, Whatman No.1) were saturated with 10 µL from each extract (Bauer *et al*., 1966; Kokosha *et al*., 2002), and dried for 1 h at room temperature under sterilized conditions (final extract conc. was 100 µg per disc). The paper discs were placed on inoculated agar plates with the tested microbes and incubated at the appropriate temperature and time for each of the tested microorganism. Both bacterial and yeast strains were grown on NA medium while the fungal strain was grown on Czapek-Dox medium (DSMZ GmbH, Germany). After the incubation period, the diameter of the growth inhibition zones was measured averaged and the mean values were recorded.

Determination of total phenolic content

The total phenolic content was determined using Folin-Ciocalteu's reagent and by using, gallic acid as standard. Crude extracts of the bioactive secondary metabolites (50 µl) at a concentration of 200 µg/mL, Folin-Ciocalteu's reagent (250 µl), and 20% of sodium carbonate (0.75 mL) were prepared. The mixture was shaken and made up to 10 mL using distilled water. The mixture was allowed to stand for 2 hrs. Then the absorbance was measured at 765 nm.

The total phenolic content was expressed as (mg gallic acid equivalent (GAE) per g dry extract) (Kumar *et al*., 2008; El-Sayed *et al*., 2009; Shoeb *et al*., 2014).

Determination of total antioxidant capacity

The antioxidant activity was determined according to phospho-molybdenum method, using ascorbic acid as standard. In this method, crude extracts of the bioactive secondary metabolites (0.5 mL) at a concentration of 200 µg /mL were dissolved in methanol in dried vials with 5 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate).

The vials containing the reaction mixture were capped and incubated in a thermal block at 95°C for 90 min. After the samples were cooled to room temperature, the absorbance was measured at 695 nm against a blank. The blank consisted of all reagents and solvents without the extract and it was incubated under the same conditions. The antioxidant activity of the extracts was expressed as the number of equivalents of ascorbic acid (AAE) per g dry extract (Prieto *et al*., 1999; El-Sayed *et al*., 2009; Ghareeb *et al*., 2014).

Determination of Reducing Power Antioxidant Activity (RPAA)

The modified spectrophotometric method described by Ferreira *et al*. (2007) was used. For this method, crude extracts of the bioactive secondary metabolites (2.5 mL) were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and 1% potassium ferricyanide (2.5 mL, 10 mg/mL). The mixture was incubated at 50ºC for 20 min, then rapidly cooled, mixed with 10% trichloroacetic acid (2.5 mL) and centrifuged at 6500 rpm for 10 min.

An aliquot (2.5 mL) of the supernatant was diluted with distilled water (2.5 mL) and then ferric chloride (0.5 mL, 0.1%) was added and allowed to stand for 10 min. The absorbance was read spectrophotometrically at 700 nm, ascorbic acid used as a standard (Ferreira *et al*., 2007; Ghareeb *et al*., 2014; Shoeb *et al*., 2014).

RESULTS AND DISCUSSION

Isolation of bacteria

Marine samples were collected from Red Sea governorate, Egypt. Screening of alkaliphilic bacteria was carried out by spread plate method having alkaline pH. A total of eight bacterial strains were isolated and given laboratory codes.

Antifungal activity of the isolated bacteria

All the bacterial isolates were tested for their ability to inhibit four fungal pathogens namely *Aspergillus parasiticus*, *Aspergillus ochraceus*, *Fusarium solani*, and *Fusarium oxysporum*. These fungi were selected for this study for their importance because *Fusarium* species are considered field fungi that infect the crops during the vegetation stage of plants, and gradually decrease during storage and are replaced by *Aspergillus* genera which are considered storage fungi that infect grains after harvesting and during storage (Piotrowska *et al*., 2013).

The bacterial isolates showed antifungal activity in variable degrees. Out of these 8 isolates, only 2 isolates showed significant antifungal activity (designated isolates 21 and 30) against the four pathogenic fungi. The mean values of zone inhibition diameter of F. oxysporum recorded 38.3 and 20.5 mm for isolate 21 and 30 respectively (Table 2). Similar results were recorded for the mean value of zone inhibition diameter of the other fungal pathogens. The isolates 1 and 9 showed relatively low antifungal activity, whereas both isolates showed antifungal activity against *A. parasiticus* only. The same was noticed for the isolate 17, which showed antifungal activity against *F. oxysporum* only.

Table 2: Antifungal activity of bacterial isolates against various fungal pathogens.

Bacterial isolates	Zone inhibition (mm)			
	A. parasiticus	*A. ochraceus*	*F.solani*	*F.oxysporum*
1	12.5±2.1	ND	ND	ND
9	22.5±10.6	ND	ND	ND
17	ND	ND	ND	40.0±7.0
19	18.0±0.0	ND	ND	34.0±8.4
21	20.0±0.0	14.0±4.2	24.0±5.6	38.3±3.5
30	20.0±0.0	23.5±2.1	30.0±0.0	20.5±6.3
31	ND	32.5±3.5	ND	35.0±7.0
MB	13.6±2.3	32.5±3.5	ND	30.5±27.5

Results are mean ± SD (n=3).
ND: Not detected.

Our results are similar to those reported by De Boer *et al*. (2007) who reported that non-antagonistic soil bacteria suppressed some plant pathogenic fungi. The antifungal effect was due to the sensitivity of fungi to the bacterial secondary metabolites, as well as to the competitive interactions between bacterial strains. Kim *et al*. (2003) reported that members of the genus *Bacillus* are effective in controlling a wide range of fungal species.

In the same trend, Sivanantham *et al*. (2013) demonstrated the fungicidal effect of the bacterial species *Bacillus circulans* and revealed the possibility of these bacterial species to be used as biocontrol agents against fungal species. Cook and Baker (1983) demonstrated that *Bacillus* species have shown potential antifungal activity for the biological control of fungal plant pathogen. Similar observations were reported by Ben Maachia *et al*. (2011) who revealed that two *Bacillus* species showed antifungal activity against filamentous fungi *Mucorramannianus* and *Botrytis cinerea*. In a recent study, Melentiev *et al*. (2014) isolated *Bacillus* sp. IB-OR17 from soda lake sediments and demonstrated that this strain showed slight

antifungal activity against some plant pathogen fungi. Similar results were reported by Malleswari (2014). On the other hand, De Boer *et al*. (1998) reported that antifungal properties of bacterial isolates might be due to the production of chitinase that could enable bacteria to live on hyphae. It could also be due to the production of extracellular antifungal secondary metabolites such as iron chelators that could inhibit or avoid the growth of pathogenic organisms (Osman, 2004).

Antiaflatoxigenic activity of the isolated bacteria

The effect of the bacterial isolates showing high antifungal activity on AFB_1 producing *A. flavus* was studied (Table 3). Results revealed that the bacterial isolate 30 significantly reduced fungal growth (mycelium dry weight) and AFB_1 production. On the other hand, the bacterial isolate 21 slightly stimulated fungal growths and reduced significantly the production of AFB_1. Detoxification of AFB_1 in liquid culture by the bacterial isolates 21 and 30 was also investigated. We observed that AFB_1 decreased during the first 24 h (Figure 1).

Table 3: Effect of bacterial isolates on AFB_1 production and mycelium dry weight.

Treatments	*A. flavus* mycelium dry weight g/100 mL medium [a]	AFB_1 production (ppb)[b]	% of inhibition
Control	2.312±0.292	45.067±18.354	-
Isolate 21	2.529±0.059	2.821±0.834	93.74
Isolate 30	1.175±0.210	0.125±0.044	99.72

Results are mean ± SD (n=3).
[a]Fungal cultures were grown in YES medium for 7 days at 28°C, values of dry. mycelium weight were obtained by weighing mycelium mats.
[b]Mycotoxin production was determined using HPLC.

The percentage of AFB_1 removal by the bacterial isolates 21 and 30 recorded 95.55% and 96.41%, respectively. It was also noticed that the percentage of AFB_1 removal increased by increasing the incubation period and reached 98.38 and 100% by the bacterial isolates 21 and 30 respectively after 72 h. Our results are similar to those reported by Petchkongkaew *et al*. (2008) who isolated *Bacillus licheniformis* from soybean and found that it removed 92.5 % of ochratoxin A and 74% of AFB_1 after 48 h at 37°C. Moreover, Tinyiro *et al*. (2011) found that *Bacillus subtilis* 168 and *Bacillus natto* were more efficient in the removal of zearalenone from the liquid medium. On the other hand, Cho *et al*. (2010) reported that *Bacillus subtilis* strain degraded 99 % of zearalenone in a liquid medium. The removal of AFB_1 could be due to degradation or enzymatic transformation of mycotoxins to less toxic compounds. Biological transformation reactions include acetylation, glucosylation, ring cleavage, hydrolysis, deamination, and decarboxylation (Hathout and Aly, 2014).

Molecular identification of the bacterial isolates

The molecular identification of the two bacterial isolates that showed the highest antifungal and antiaflatoxigenic activities was compared with that of NCBI databases using BLAST network and the partial sequences of 16S rRNA obtained were aligned with the available 16S rRNA sequences in GenBank database. The phylogenetic tree was also constructed using the neighbour-joining (N-J) method based on the 16S rRNA sequences (Figure 2 and 3). The16S rRNA gene sequence analyses showed that isolate 21 was most closely affiliated with members of the genus *Brevundimonas*. In the phylogenetic tree based on the neighbour joining algorithm, isolate 21 fell within the cluster comprising *Brevundimonas* and *Mycoplana* species (Figure 2).

The results revealed that the first bacterial isolate 21 of the sequenced 16S rRNA gene (~700 bp) was identified as *Brevundimonas* sp. The second bacterial isolate 30 of the sequenced 16S rRNA gene (~1046 bp) was identified as *Bacillus* sp. (Figure 3). It was noticed that the 16S rRNA gene sequence analysis of bacterial isolates showed a high similarity (99%) between the bacterial strains and their closest phylogenetic relative, thus indicating that 16S rRNA gene sequence data are helpful for identification of alkaliphiles bacteria. The gene sequence was deposited in GenBank database as; *Bacillus* sp. MERNA97 (Accession No. KM222498.1) and *Brevundimonas* sp. MERNA4 (Accession No. KM235741.1).

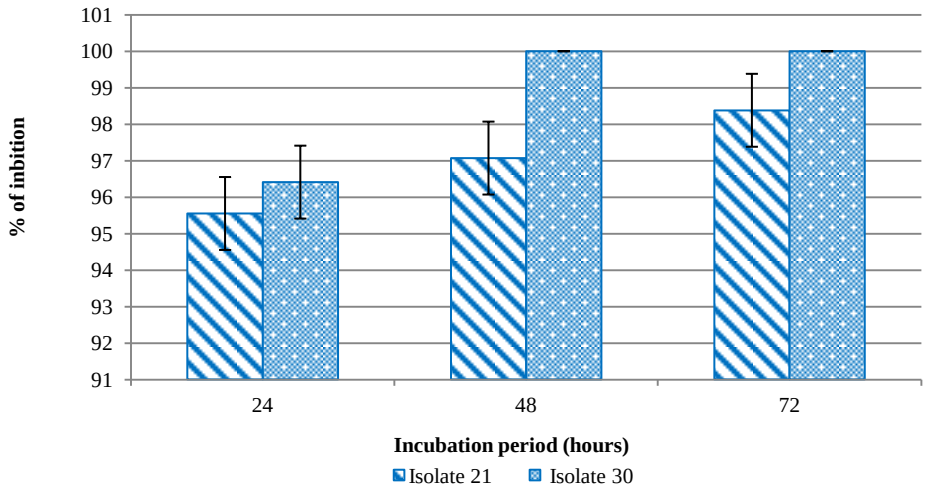

Fig. 1: Effect of bacterial isolates on AFB_1 degradation. Results are mean ± SD (n=3). Bars represent the standard errors.

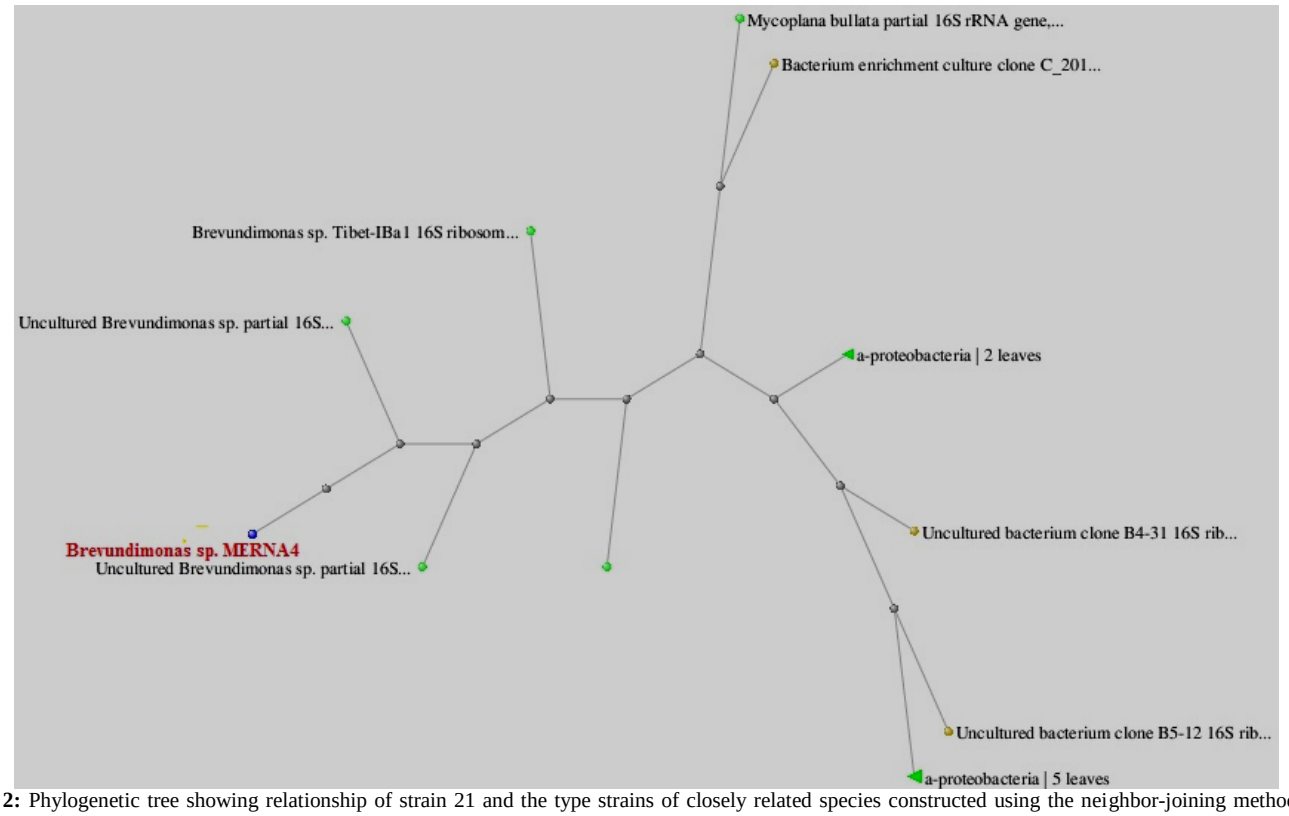

Fig. 2: Phylogenetic tree showing relationship of strain 21 and the type strains of closely related species constructed using the neighbor-joining method and based on 16S rRNA gene sequences.

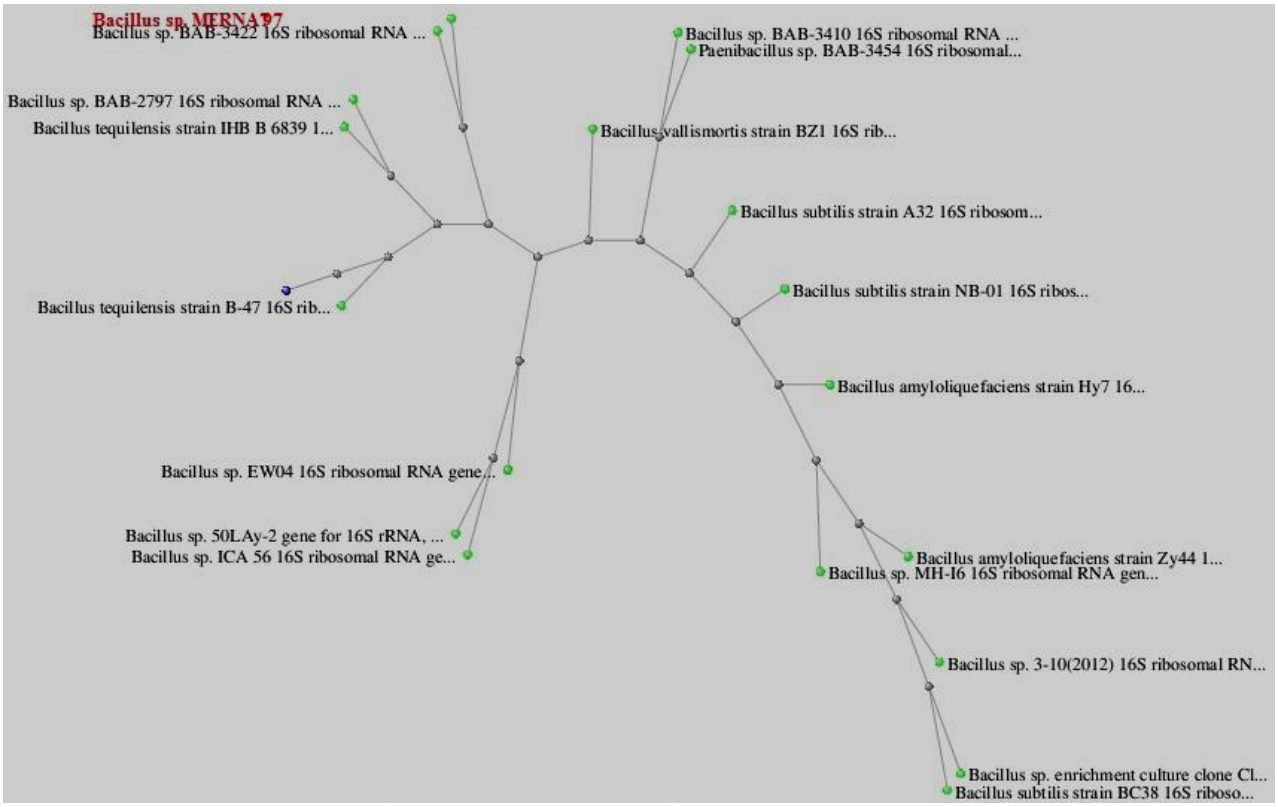

Fig. 3: Phylogenetic tree showing relationship of strain 30 and the type strains of closely related species constructed using the neighbor-joining method and based on 16S rRNA gene sequences.

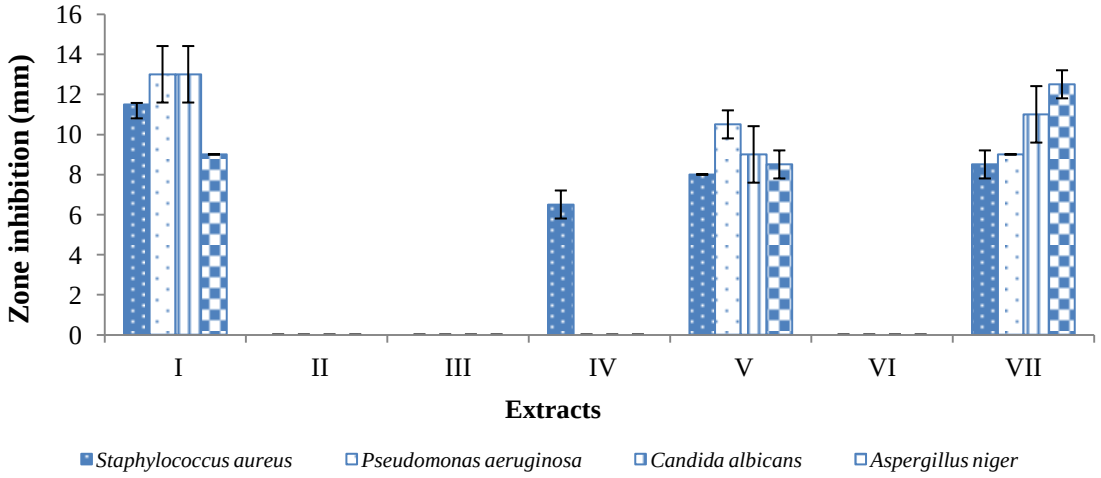

Fig. 4: Antimicrobial activity of bacterial crude extracts. Results are mean ± SD (n=2). Bars represent the standard errors.

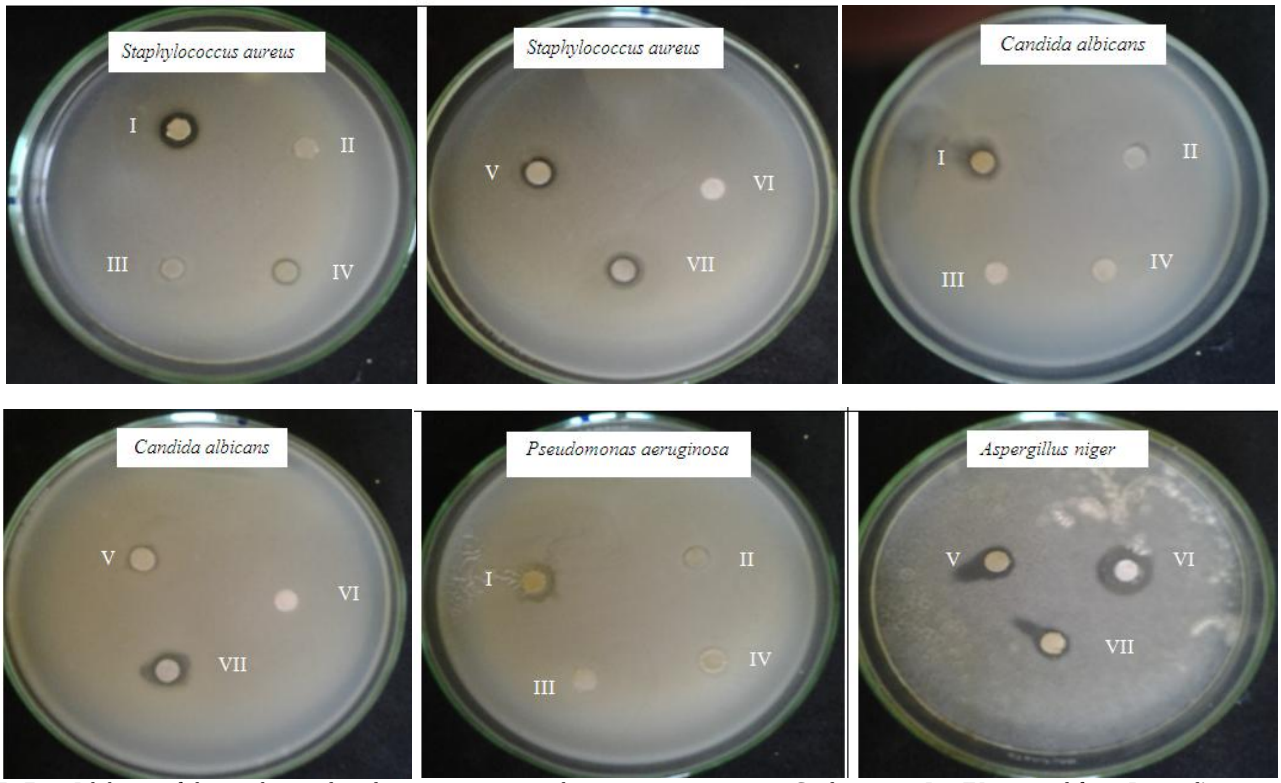

Fig. 5: Zone Inhibition of the two bacterial crude extracts against pathogenic microorganisms. Crude extracts I to IV extracted from *Brevundimonas* sp. and crude extracts VI to VII extracted from *Bacillus* sp.

Determination of biological activities of the crude extracts

Antimicrobial activity of Brevundimonas and Bacillus species crude extracts

The crude extract of the two isolates of bacteria was used to investigate the antimicrobial activity against pathogenic microorganisms. Results indicated that the extracts I, V extracted from *Brevundimonas* sp.and VII extracted from *Bacillus* sp. inhibited the growth of all the test microorganisms (*Staphylococcus aureus Pseudomonas aeruginosa Candida albicans Aspergillus niger*), whereas the extract IV only inhibited the growth of one pathogen (i.e. *Staphylococcus aureus*) (Figure 4). Figure (5) revealed the zone inhibition of the crude extracts against pathogenic bacteria (*Staphylococcus aureus, Pseudomonas aeruginosa*), yeast (*Candida albicans*) and fungi (*Aspergillus niger*). The antimicrobial activity of the *Bacillus* species could be due to its ability to produce a wide range of extracellular enzymes, one of which is amylase, which might be of industrial significance (Swain *et al*., 2006). It has been reported that *Bacillus* species have been associated with antifungal activities due to their ability to produce volatile and non-volatile

compounds (Ryder *et al*., 1999; Park *et al*., 2001; Bhaskar *et al*., 2005). In a similar study by Petatán-Sagahón *et al*. (2011), the authors found that extracellular filtrates of *B. subtilis* inhibited considerably the growth of the fungi *Stenocarpella maydis* and *Stenocarpella macrospora*.

Total phenolic content and total antioxidant activity of Brevundimonas and Bacillus species crude extracts

The phenolic content of each crude extract of the bioactive secondary metabolites was estimated as 174.00, 123.56, 130.10, 106.34, 119.30, 141.65, and 156.00 mg GAE/g for extracts I, II, III, IV, V, VI, and VII respectively (Table 4). On the other hand, the total antioxidant activity of the crude extracts recorded 451.50, 240.25, 253.46, 221.20, 231.63, 325.81, and 351.30 mg AAE /g for extracts I, II, III, IV, V, VI, and VII respectively (Table 4).The highest antioxidant capacity of crude extracts could be attributed to the presence of high total polyphenol contents (Katalinic *et al*., 2006). The presence of the phenolic groups in the crude extracts is considered to be a key element for the antioxidant efficiency (Marković and Manojlovi, 2010). Data in Figure (6) revealed a positive correlation between phenolic content and antioxidant activity.

These results were confirmed by several authors who indicated that there was a positive correlation between antioxidant activity and total phenolic content (Cai *et al*., 2004; Li *et al*., 2008).

Reducing power antioxidant activity of Brevundimonas and Bacillus species crude extracts

The reducing power of the crude extracts was studied by the reduction of Fe^{3+} to Fe^{2+}. The reducing power of the crude extracts was studied in the presence of ascorbic acid. The measured values of absorbance varied from 0.200 to 0.371. Amongst the tested crude extracts, extract VII gave the highest reducing power, whereas the reducing activity was lower than ascorbic acid standard (Figure 7). The reducing capacity of a compound might be due to its potential antioxidant activity (Shobha *et al*., 2013).

Table 4: Total phenolic content and total antioxidant capacity of crude extracts of bacterial bioactive metabolites

Extracts	Total phenolic content (mg GAE/ g dry extract)[a]	Total antioxidant capacity (mg AAE /g dry extract)[b]
I	174.0 ± 1.20	451. 50 ± 1.00
II	123.56 ± 1.25	240.25 ± 1.50
III	130.10 ± 1. 00	253. 46 ± 1.20
IV	106.34 ± 1.10	221.20 ± 1.32
V	119.30 ± 1.0	231.63 ± 1.52
VI	141.65 ± 0.94	325.81 ± 1.25
VII	156.0 ± 1.32	351.30 ± 1.13

Results are mean ± SD (n = 3).
[a] Total phenolic content values are expressed as mg gallic acid equivalent/g extract (mg GAE/g dry ext.).
[b] Total antioxidant capacity values are expressed as mg ascorbic acid equivalent/g extract (mg AAE/g dry extract).

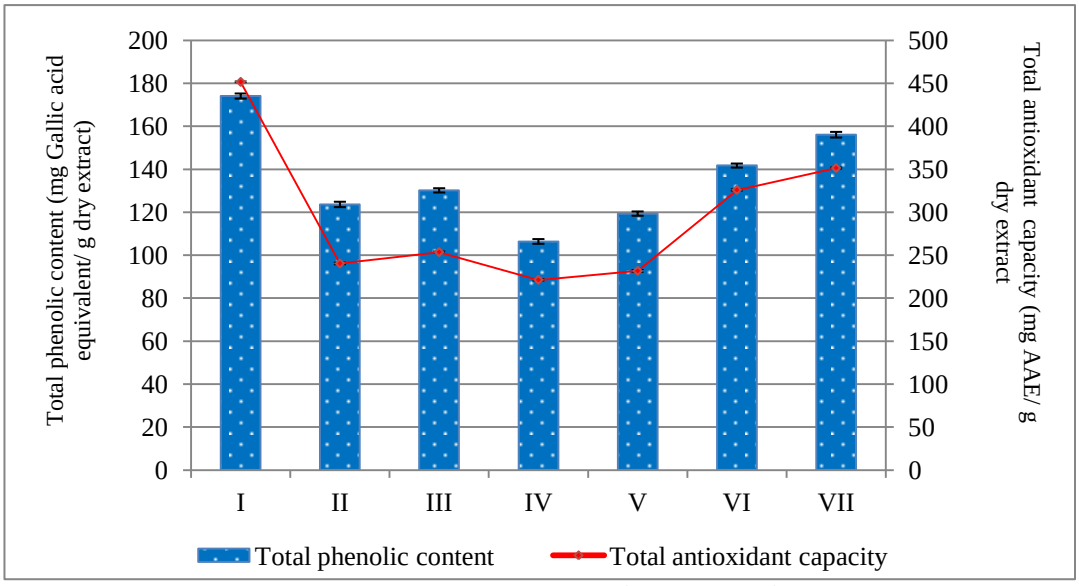

Fig. 6: Correlation between total phenolic content and total antioxidant capacity of crude extracts of bacterial bioactive secondary metabolites. Results are mean ± SD (n = 3). Bars represent the standard errors.

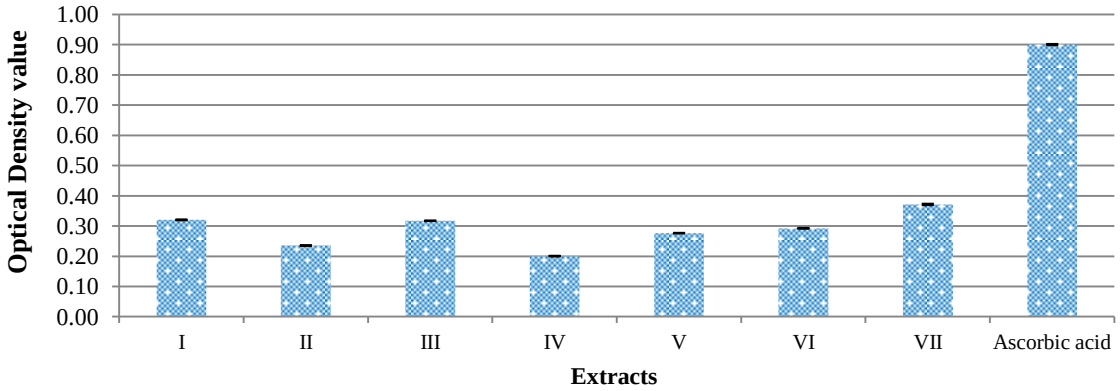

Fig. 7: Reducing power antioxidant activity (RPAA) of crude extracts of bacterial bioactive secondary metabolites against ascorbic acid. Results are mean ± SD (n = 3).

CONCLUSION

Isolation of alkaliphilic bacteria from marine samples led to the finding of eight bacterial isolates, two of which showed high antifungal and antiaflatoxigenic activity. Molecular characterization confirmed that the two isolates (21 and 30) as *Brevundimonas* and *Bacillus* species respectively. The bacterial secondary metabolites crude extracts I and VII were found to have excellent biological activities. Up till now, there is no data concerning the antimicrobial activity of *Brevundimonas* species, and thus, this manuscript is considered the first report for the antifungal and antiaflatoxigenic activity of these species. The results obtained were promising and thus further studies concerning purification and identification of the active crude extracts are necessary.

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