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Original article

Turbinaria ornata and its associated epiphytic *Bacillus* sp. A promising molecule supplier to discover new natural product approaches

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ABSTRACT

Marine ecosystems are highly dependent on macroalgea in providing food and shelter for aquatic organisms, interacting with many bacteria and mostly producing secondary metabolites of potent therapeutic antibacterial property. Screening of marine microbial secondary metabolites of valuable biotechnological and therapeutical applications are now extensively studied. In this study, *Bacillus* spp. identified by DNA sequencing and found associated with *Turbinaria ornata*, was screened and characterized for its cell free supernatant (CFS) possible antimicrobial and antibiofilm applications. Among the 7 microbial isolates tested, CFS greatly affected *Bacillus subitilis* (12 mm) and inhibited equally the yeast isolates *Candida albicans*, *Candida tropicalis* and *Candida glabrata* (10 mm) and had no or negligible effect on *S.aureus, E.coli, P. aeruginosa.* As for the CFS antibiofilm activity, no difference was revealed from the positive control. Algal crude extracts (methanol, acetone and aqueous), on the other hand, were similarly tested for their antimicrobial activity against the seven microbial isolates, where highest activity was observed with the aqueous crude extract against *Staphylococcus aureus*(10 mm) and *Pseudomonas aeruginosa* (9 mm) compared to the negligible effects of methanol and acetone crude extracts. Chemical analysis was performed to reveal the major constituents of both crude algal extracts and *Bacillus* spp. CFS. FTIR spectrum of the bacterial CFS indicated the presence of bacteriocin as the major lipopeptide responsible for its biological activity. Whereas, methanol and water crude algal extract GC–MS spectra revealed different chemical groups of various combined therapeutical activity mainly Naphthalene, amino ethanesulfonic acid, pyrlene, Biotin and mercury chloromethyl correspondingly. Thus, the present study, demonstrated the moderate activity of both crude algal extract and the bacterial CFS, however, further investigations are needed for a better biological activity.

1. Introduction

Screening and identifying of Bacteria inhabiting marine macroalgae are now the main objective of research involving taxonomy and ecology. Proteobacteria and Firmicutes were the most abundant phyla encountered on macroalgae according to Hollants et al. (2013). Many combined techniques from bacterial culture, microscopic and molecular tools have been used to identify these epiphytic bacteria present on seaweeds and study their presence and occurrence in association with their metabolic functions (Steinberg et al., 2002; Goecke et al., 2010). However, further studying and better understanding are required to explain in details the algal interaction with associated microorganisms on algal surfaces (Steinberg et al., 1998; Steinberg and de Nys, 2002; Kubanek et al., 2003).

Brown algae (Phaeophyceae), example of which *Turbinaria ornata* (see Fig. 1), are among the most diverse marine algal groups which play important roles on coastal ecosystems (Andersen 2004; Cock et al. 2011). They provide a rich environment for the microorganisms' communities which provide certain natural compounds (Singh et al., 2011; Ali et al., 2012; Martin et al., 2014) defending the host algae from secondary biological fouling (Penesyan et al. 2010). Previous studies (Pedersén, 1968; Egan et al. 2013)

Abbreviations: CFS, cell free supernatant; FTIR, Fournier transformer infrared; LMW, low molecular weight; BCFS, *Bacillus* spp.CFS; BF, Biofilm; AMP, antimicrobial peptides.

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Fig. 1. *Turbinaria ornata* showing its morphological characteristics: color, leaves and pigmentation for its identification.

highlighted that seaweeds pharmaceutical and ecological roles cannot be understood without considering the association with the persisting microbiota (Bang et al., 2018).

Therefore, extensive studies focused on identifying the microbial communities associated with the algal species to better understand the dynamics of the bacterial algal interactions in relation to ecology and pharmacology (Hengst et al., 2010; Lachnit et al., 2011). Importance of microbial diversity, particularly on the bacterial genus level, associated with seaweeds, are highly host specific (Goecke et al., 2013; Mesquita et al., 2018). Thus, these bacterial species together with their active metabolites are now considered promising bioactive substances (Egan et al. 2008; Penesyan et al. 2009; Ismail et al. 2016), a prominent example of which comprises biosurfactants, a diverse group of amphiphilic lipids containing both hydrophilic and hydrophobic domains within one molecule. To date, soil-isolated microbes were mostly studied for biosurfactants technological access, predominately from *Bacillus*, *Lactobacillus* and *Pseudomonas* species. Recently, microorganisms associated with marine habitats are more considered as a prolific source that produce a variety of useful compounds of which biosurfactants (Jensen and Fenical, 1994; Romanenko et al., 2008; Satpute et al., 2010; Kennedy et al., 2011). The interest in these latter is due to their low toxicity and to their wide therapeutical effect as antibacterial agents and or to their biotechnological applications involved in soil and sand bioremediation (Banat et al., 2010; Lima et al., 2010) and their ability to reduce the surface tension property and hence can be effective anti-adhesives/antibiofilm agents (Das et al., 2009). Actinobacteria and Firmicutes including *Bacillus* are the dominant phyla for lipopeptide biosurfactant producers recently enlarged by marine isolates (Kubicki et al., 2019) nowadays highlighted as the main producers of novel biosurfactants with excellent bioactivity. Nevertheless, their morphological and bioactive characteristics are different between strains.

Biosurfactants are surface-active chemical compounds among which are lipopeptides. synthesized by many *Bacilli* and other species *Lactobacillus*, *Streptomyces*, *Pseudomonas* and *Serratia* often proved as good biofilm disruptive agents. Lipopeptides are derived from amino acids of low molecular weight and with prominent dual activities: bioactive surface tension reduction and antimicrobial effect. They can generate unfavorable environmental conditions for pathogens to grow (e.g., altering pH, competing for nutrients and surface (Baltz et al., 2005; Ines and Dhouha, 2015), depending on the amino acid chain length composition (Meena and Kanwar, 2015). For instance, bacteriocin, a lipopeptide, produced by different species of the genus *Bacillus* display a broad spectrum of activity at the same time a high degree of specificity against bacterial infection (Ramachandran et al., 2014; Wu et al., 2019) mainly against gram- positive bacteria (Wang et al., 2018), becoming as such a potent antibacterial alternative to the standard antibiotics, in addition to anti- biofilm ability.

Thus the aim of this study was (1) to isolate, identify new *Bacillus* strain associated with the brown alga *Trubinaria ornata* using molecular techniques, (2) to screen in in-vitro assay the antimicrobial activity of the algal crude organic and aqueous extracts and their chemical composition, and (3) to determine the cell free supernatant (CFS) both biological and chemical characteristics as an antimicrobial and biosurfactant agent.

2. Materials and methods

2.1. Collection and identification of algal sample

The algal sample was collected from the shallow surface of the Red Sea, Jeddah, Saudi Arabia. Samples were conserved in sterile plastic bag and were kept cold in coolers until processed to the laboratory, at the Botany and Microbiology department, King Saud University. Algae were first washed thoroughly with water to remove any debris, sedimentations and loosely attached microorganisms, and then washed with sterile distilled water. Algal samples were first identified as brown seaweed *Turbinaria ornata* based on their special morphological characteristics: fronds, branching, presence of air bladders, midribs, and reproductive structures (Pankow, 1971).

2.2. Bacterial isolation and identification

Bacterial strains associated with the brown alga *Turbinaria ornata* (bacterial symbiont) were isolated from the surface of the macroalga using two different methods: (1) a small piece of the algal sample was suspended into sterile saline water from the red sea according to Wiese et al. (2009) with slight modifications and then swabbed into two different culture media: Tryptone Soy agar (TSA) (Oxoid, USA) and Marine agar (MA) (Oxoid, USA) ; the second method is direct swabbing from the algal surface onto TSA and MA agar plates respectively using sterile cotton swabs. All plates were incubated aerobically at 37C for 18–24 hrs. Bacterial strains obtained from both techniques were selected and purified on the TSA and MA plates. Isolated single colonies were studied macroscopically and microscopically (Gram staining technique) for a preliminary identification. Susceptible strains were further analyzed at the genus level using molecular techniques: PCR and DNA sequencing to be conserved in 15% glycerol at −50C for future analysis.

2.3. Single colony PCR amplification reaction

Single colonies of the presumptive strains were dissolved in 50 μL sterile distilled water for single colony PCR reaction (Moubayed et al., 2019). Amplification of the 16srRNA gene was performed using the universal primer set 1429R (5′–AAGGAGGT GATCCAGCCGCA–3′) and 27F (5′–AGAGTTTGATCCTGGCTCAG– 3′). A total volume of 25 μL PCR reaction was carried in Genepro thermal cycler (Bioer, China); each reaction mixture contained 2 μL of each of the DNA samples, 12.5 μL of the GoTaq green Master Mix (Promega, USA), 0.125 μL of each of the primers forward and

reverse (Invitrogen, USA), 10.25 μL of RNAse DNAse free water. The cycling parameters were as follows: initial denaturation for 2 min at 94 °C followed by 35 cycles of 94 °C for 15 sec, 63 °C for 1 min as for the annealing of each primer, 72 °C for 2 min and a final elongation step at 72 °C for 5 min. DNA concentration and quality were determined using the genova nanodrop (Italy) and 1.5% agarose gel electrophoresis; samples were run alongside with the positive strain *Bacillus subtilis* ATCC6633 showing bands at about 1500 bp.

2.4. DNA sequencing

Following the amplification reactions, PCR products obtained were purified using Qiagen QIA 250 Qiagen purification kit (Germany) and sequenced using the Applied biosystem sequence analyzer (Spain). According to the manufacturer's instructions, ABI PRISM® BigDye™ Terminator Cycle Sequencing Kit (version3.1) was used with the same set of primers previously applied in the amplification reactions 1429R and 27F. The National Centre for Biotechnology Information (NCBI-BLAST) software was used to identify the DNA sequence identity and the evolutionary relationship between the 16S rRNA genetic marker of the standard strain used as positive control.

2.5. Phylogenetic tree

Phylogenetic analysis of the isolate in study was performed by the neighbor joining method using the Mega version 7.1 (Tamura et al., 2007). The 16sRNA gene sequence of the presumptive strain was compared to the National Center for Biotechnology Information (NCBI) GeneBank using BLAST (Basic Local Alignment Search Tool).

2.6. Crude algal extract preparation

Algal samples were washed thoroughly with sterile distilled water to remove any impurities present, then samples were air dried at room temperature for 3 days. Dried samples were then grinded into powder, of which 10 g were weighed and extracted with 100 mL of acetone, methanol and sterile distilled water. Samples were kept incubated in a rotary shaker (140 rpm) at room temperature for 3 days, following incubation the crude extract was filtered through Whatman filter papers and then evaporated using a rotavapor, and the dried extract was dissolved in 5 mL sterile distilled water, sterilized through syringe Millipore of 0.45 μm pore size (Moubayed et al., 2017). The crude extract, of 100μg/mL concentration, was kept in sterile microcentrifuge tube at −4℃ for further analysis.

2.7. GC–MS analysis for the crude algal extract

GC–MS was carried out following standard method (Gaddaguti et al., 2012) with the Agilent technologies (USA) equipped through silica capillary column (30 mx0.25 mm × 0.2 μm) composed of 5% diphenyl/95% dimethylpolysiloxane to reveal the major chemical constituent of both crude extracts: aqueous and methanol. Thus for GC–MS detection, 70 eV ionizing energy was used using an electron ionization system, Helium as the carrier gas (99.9%) was used at a constant flow rate of 1 mL/min, and 2 μL of the sample was inj*ected* at 250℃ with a split ratio of 10:1. The oven temperature ranged from 110℃ to 280℃ with a rise of 100℃/min to 200℃ and then 5℃ /min to reach the 280℃.

2.8. CFS preparation

Overnight grown *Bacillus* spp. isolates were inoculated and incubated in TSB medium in a rotary shaker at 140 rpm and 37℃, for 24 hrs. cells were then separated by centrifugation at 10,000 rpm and 4℃ for 10 min (Sigma Hereus XR) and CFSs were collected after filtration at 0.22 mm. CFS were halved adjusted at pH 6.5 NaOH 1 M (Toba et al., 1991), while the rest are kept intact just to compare the difference between their biological activities.

2.9. Dual antimicrobial activity determination of crude algal extracts and CFS

Crude algal organic (acetone and methanol) and aqueous extracts together with *Bacillus* CFS (BCFS) were tested for their antimicrobial activity against Gram- positive bacteria listing *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923 and against Gram- negative bacteria particularly against *Escherichia coli* ATCC 25966, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhymirium* (LT2) ATCC 27106 and against *Candida albicans* ATCC 60193, *Candida tropicalis* ATCC 66019 and *Candida glabrata* (clinical isolate) as yeast species. Wells were performed at the surface of Mueller Hinton agar (Oxoid, USA) with a sterile corck borer (6 mm) inoculated with the tested strains using sterile cotton swabs. 100 μL of each of the extracts and the BCFS were loaded into the wells. Plates were incubated aerobically at 37℃ for 18–24 h. The antimicrobial effect was determined by measuring the inhibition zones in comparison to standard antibiotic discs inhibition zones, as positive control. The experiments were performed in triplicates and data were tabulated as average inhibition zone in mm.

2.10. MIC determination for water crude extract and Bacillus spp. CFS

In the current study, minimum inhibitory concentration (MIC) of both crude water *T. ornata* extract (Tw) and the bacterial CFS was performed in triplicates against S. aureus and P. aeruginosa for the TW extract and against *B. subtilis*, *C. albicans*, *C. tropicalis* and *C. glabrata* for the CFS respectively by Micro-broth dilution assay. The tested concentrations were ranged from 0.1 to 100 μg/ mL. In brief, 2-fold serial dilutions were performed in 96 well plates where the first column was the positive control (Mueller Hinton broth and the microbial cells) and the last column as the negative control (broth and either the crude extract and or the CFS). 50 μL of each of the microbial suspensions was loaded, respectively, following incubation for 24 h at 37 °C the results were recorded at 600 nm using an ELISA reader (Biotech).

2.11. CFS characterization using FTIR

The purified collected CFS was subjected to FTIR analysis with KBr pellet method. About 0.1 mL of BCFS was mixed with KBr (0.1 g) and compacted in pellet form. Data were plotted on standard software provided with the instrument and the spectrum was recorded in the frequency range of 4000 to 400 cm − 1 using Agilent Technologies (USA).

2.12. Bacterial CFS antibiofilm activity

Tested organisms showing highest sensitivity to CFS were overnight cultured in Luria bertani broth (LB) and then tested for their biofilm activity. 100 μL of each tested organisms were loaded on 96-well plates with 100 μL of LB as positive control. Similarly, 100 μL of each of the tested organisms were added to 100 μL of CFS whereas the negative control was LB medium only. Plates were read at 600 nm for determining the CFS antibiofilm activity following staining and distaining with crystal violet.

3. Results

3.1. Bacterial identification

The present study showed no significant difference in the isolation method for the bacterial strains associated with the brown alga. Altogether, using the Gram staining technique, 12 bacterial isolates obtained from both methods used were similar in shape, color and microscopically. All strains were preliminary identified as gram positive, non-motile, endospore forming rods fermenting starch when streaked on 1% starch agar plates flooded with Gram's iodine, and with 98% similarity to *Bacilli* spp. upon the DNA sequencing supported by the NCBI 16srRNA gene cluster, and given the following GenBank accession number MW857094. The phylogenetic tree was then constructed to relate the similarity to other phylotypes where the newly identified bacterium was highly and equally similar to both strains *Bacillus pacificus* and *Bacillus cereus* (Fig. 2).

3.2. In vitro antimicrobial assay for the crude algal extract and B. Pacificus CFS

Data indicated that the aqueous extract showed the highest activity mainly against *S. aureus* (10 mm) and *P. aeruginosa* (9 mm). All other tested microbial strains showed negligible or no effect with algal crude extract as indicated in Table 1. CFS, on the other hand, in vitro inhibited *B. subtilis* (12 mm) and all the 3 tested candida species *C. albicans, C. tropicalis* and *C. glabrata* equally of 10 mm diameter inhibition zone. No significant difference was noted between the intact CFS and the pH6.5 CFS for their antimicrobial activity.

3.3. GC–MS chromatograms

The crude aqueous extract revealed the presence of different chemical constituents having variable therapeutical effects listing antimicrobial, antioxidant and anti-inflammatory effect, however; still the mechanism and the potent activity are not very clear, whether it is a synergistic effect of all the chemical constituents or it is a single active chemical compound having the dominant therapeutical activity. GC–MS chromatograms obtained, when compared with the NIST library v.2.3, revealed the presence of 10 chemical constituents showing largest peaks listing Naphthalene, aminoethano-sulfonic acid, Biotin, Benzeneselenol, Benzenamine, Tridecanedioic acid, Perylene, Dichlorophen, Dienestrol, Ergotaman. Each of these components has a different activity either, anti-inflammatory, antimicrobial and antioxidant activity (as polyphenols) to which the in-vitro moderate activity obtained in the present work is being attributed. In contrast, the aqueous extract revealed the presence of two major chemical constituents' mercury chloromethyl, and Perylene with its derivatives known with their antimicrobial activity (Fig. 5**a, b**).

3.4. Antibiofilm activity

As noted from the present data, the CFS anti-biofilm and anti-adherent effect was moderate, this is because the bacteriocin which is the main lipopeptide present in the CFS in study is known for its anti-adhesive and anti-biofilm activity. The highest anti-biofilm activity was observed in the following order *C. albicans* > *C. tropicalis* > *B. subtilis. C. glabrata*; however, showed a different pattern where the CFS anti-biofilm activity reading was higher than that for the positive BF control (Fig. 6).

3.5. FTIR analysis

Different bacterial strains together with their cellular components listing fatty acids, membrane proteins, intracellular proteins, polysaccharides and nucleic acids can be revealed by FTIR, due to FTIR spectra strain specificity. Each cellular component is distinguished in different spectral region, where fatty acids are found between 3000 and 2800 cm^{-1}, 1700–1500 cm^{-1} wavelength contains amide I and II bands of proteins and peptides; 1500– 1200 cm^{-1} is a mixed region of fatty acid, proteins, and phosphate, 1200–900 cm^{-1} reveals microbial cell wall carbohydrates, as for 900–700 cm^{-1} region is characterized by unique absorbencies for each bacteria. Since bacteriocin is a lipid or protein in nature, and *Bacilli* are among the main lipopeptide produc-

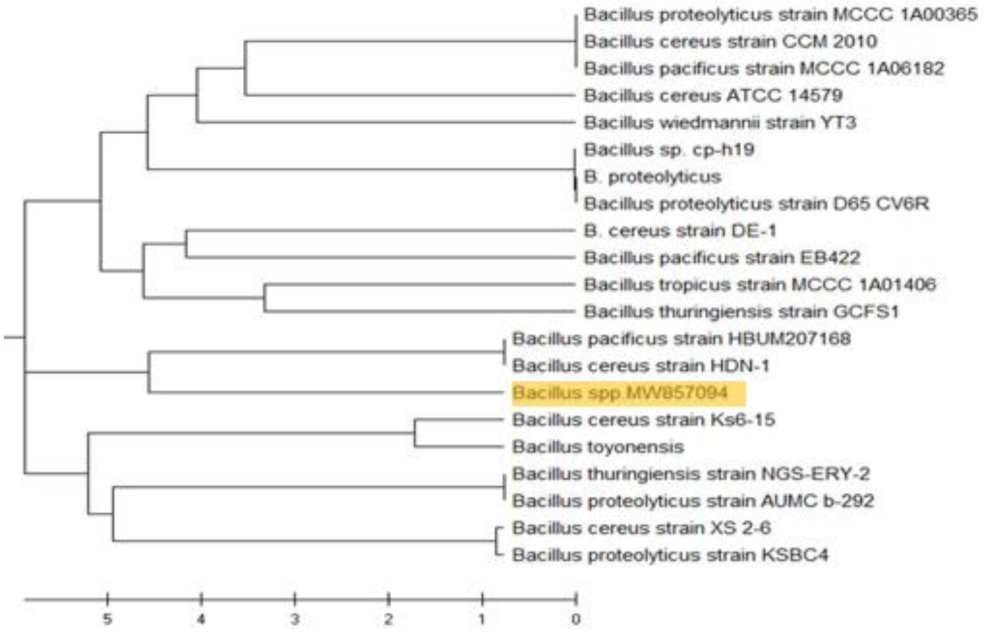

Fig. 2. Phylogenetic tree of the newly identified strain *Bacillus* spp. MW857094 showing highest and equal similarity to *Bacillus cereus* and *Bacillus pacificus.*

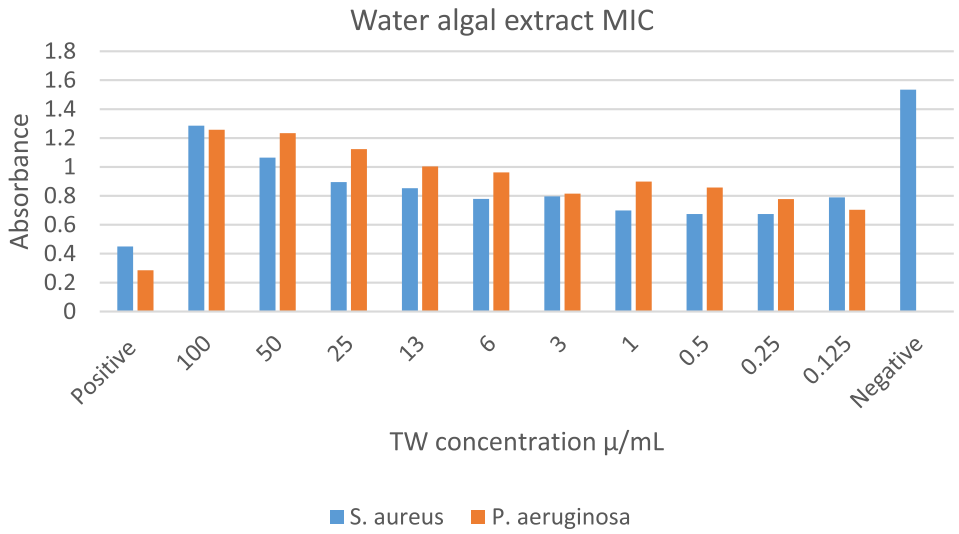

Fig. 3. Water algal extract MIC against *S. aureus* and *P. aeruginosa* where the minimal inhibitory concentration was observed for *S. aureus* at 0.5 µg/mL whereas for *P. aeruginosa* the MIC was noted at 0.125 µg/mL.

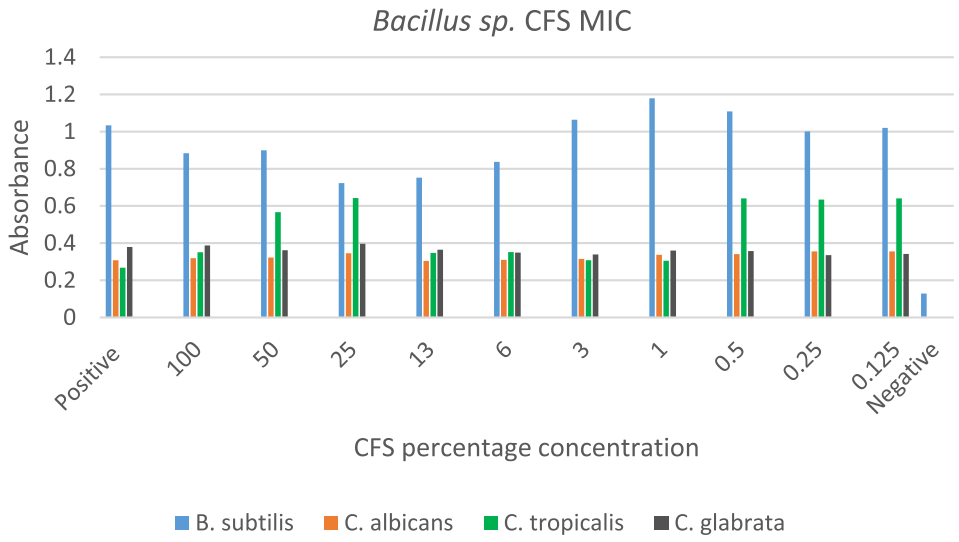

Fig. 4. *Bacillus* sp. CFS MIC revealing the highest antimicrobial activity of CFS against the Candida isolates in study compared to *B. subtilis*. The minimal inhibitory concentration was observed at 3 percentage concentration equally for *C. albicans*, *C. tropicalis* and *C. glabrata*. As for *B. subltilis* the MIC was determined at 25%.

Table 1
Antimicrobial activity of both crude algal extracts and BCFS.

Microorganisms	Average inhibitory effect of crude algal extract in mm						CFS (mm)		Standard disc
	Acetone	SD	Methanol	SD	Ethanol	SD		SD	Tetracycline (TE)(30 µg)
		Gram -positive							
S. aureus ATCC 25923	–	0	–	0	10	1	–	0	19
B. subtilis ATCC 6633	–	0	–	0	–	0	12	1	12
		Gram- negative							
E. coli ATCC 25966	–	0	–	0	–	0	–	0	15
P. aeruginosa ATCC 27853	–	0	–	0	9	0.5	–	0	15
		Yeast							Fluconazol 1 mg/mL
C. albicans ATCC 60193	–	0	–	0	–	0	10	1	7
C. tropicalis ATCC 66019	–	0	–	0	–	0	10	1	15
C. glabrata	–	0	–	0	–	0	10	0.5	15

Water algal and bacterial CFS MIC determination.

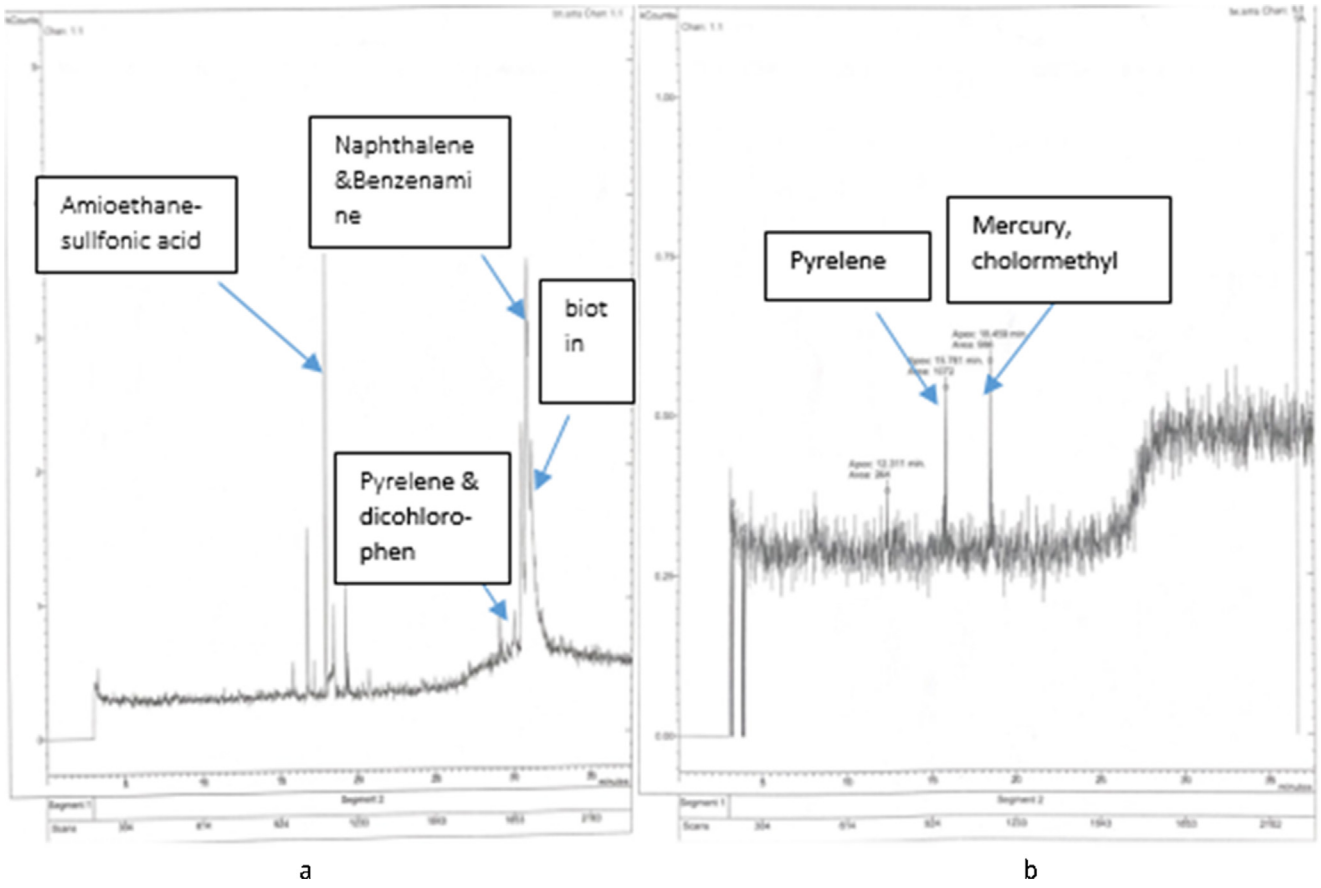

Fig. 5. GC–MS chromatograms of the algal crude extracts. a: methanol algal extract reveals the presence of 12 bioa*ctive chemical compounds, among which:* Naphthalene, aminoethano-sulfonic acid, Biotin, Benzenamine, Pery*lene, Dichlorophen are indicated,* each of *these compounds* has a different biological activity listing antimicrobial, antioxidant and anti-inflammatory, while b: aqueous extract chromatogram indicates the presence of 2 main chemical constituents: mercury chrolomethyl and perylene and its derivatives.

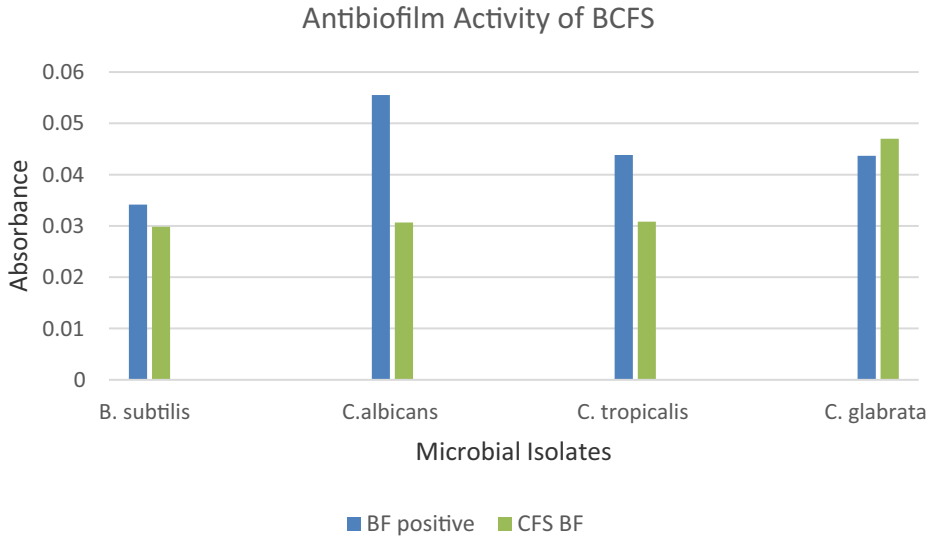

Fig. 6. Biofilm formation and inhibition in response to the BCFS. Moderate antibiofilm activity was observed with CFS on all microbial isolates with slight difference with *C. tropicalis* where BCFS showed higher absorbance than the positive BF (Control).

ers herein, we came to conclude that the present FTIR analysis of two spectral regions mainly for C = C stretch and amine group coupled with a C–N group (Fig. 7) of 3284.28 cm^{-1} represents the Amine stretch (N–H), in contrast, the 1635.32 cm^{-1} represents the Alkene (C = C) group indicating as such the presence of bacteriocin as the single main chemical constituent of the bacterial CFS identical to the FTIR spectrum obtained with Kadirvelu et al. (2015).

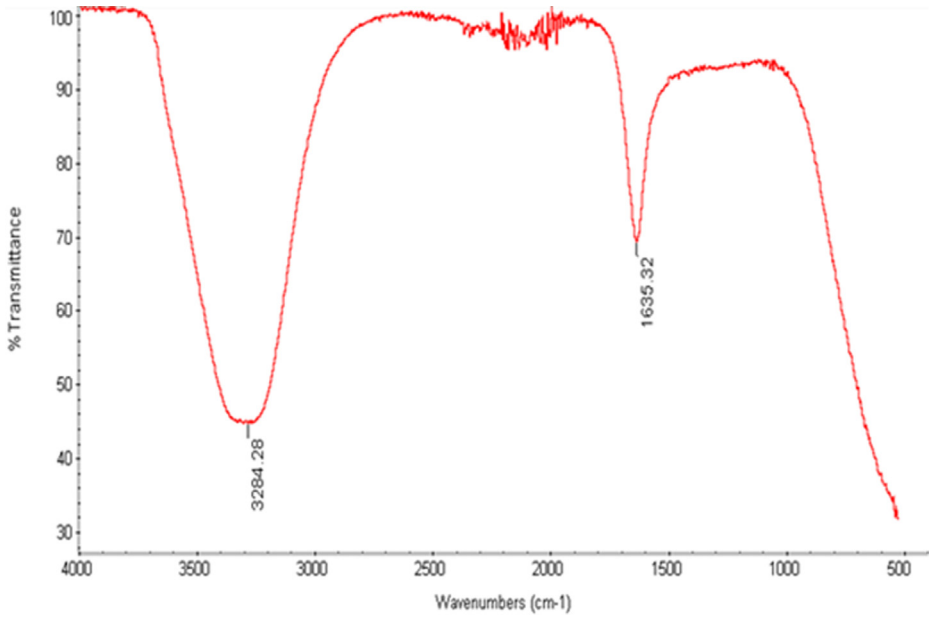

Fig. 7. BCFS FTIR spectrum revealing the presence of bacteriocin at 3284 cm^{-1} as the main peptide to which both antimicrobial and antibiofilm activity was attributed.

4. Discussion

Brown algae with an almost inexhaustible, and renewable resource for obtaining polyfunctional biological compounds now offer a high biotechnological potential. As is known from previous studies, the content and chemical composition of these secondary metabolites, naming polyphenols, depend mainly on the algal type, the geographical zone, the method of extraction and the collection time (Moubayed et al., 2017) and hence have wide range of potential biological activities either on the bacterial structure and or function for example with low toxic effect (Besednova et al., 2019). Brown algae, on the other hand, offer a safe habitat for bacteria to grow, reproduce and produce secondary metabolites with promising bio-application (Reen et al., 2015; Gupta et al., 2019; Paulsen et al., 2019). The relationship between brown algae and associated bacteria is mutualistic, where the host is providing the bacterial cells with nutrients while the bacteria provide the host with the defense mechanism against harmful colonization by other microorganisms. However, the rate of bacterial occurrence, distribution and presence associated with the brown algae in addition to their ecological contribution still are not determined.

The genus *Bacillus* is a predominant microbial flora in marine ecosystem that differed with terrestrial strains in production of diverse classes of AMPs (Tareq et al., 2014; Chen et al., 2017). Due to advancement in protein purification technology and characterization of AMPs, reports on bioactive from marine bacterial sources have also increased in the recent past. In this context, *Bacillus* spp. was identified in this study and was found taxonomically related to *Bacilli* species listing *B. cereus* and *B. pacificus* (Fig. 2) with its ability to adapt to marine environment and production of LMW lipopeptide bacteriocin with promising biological activity (antibiofilm and antimicrobial activities) as revealed with the FTIR spectrum of two spectral regions for amine 3284.28 $cm^{-1}$and carbohydrates 1635 cm^{-1}indicating the presence of bacterioncin as the unique constituent in agreement with Kadirvelu et al. (2015) who demonstrated exactly identical FTIR spectrum of purified bacteriocin from *Enterococcus casseliflavus* MI001 strain at 3284.28 cm^{-1} for the Amine stretch (N–H) and 1635.32 cm^{-1} for the Alkene (C = C) group. Even though, few studies are reported on this field, however; the available data gives us hope that seaweed secondary metabolites and or associated bacterial extracts may develop methods for controlling multicellular associations of bacteria.

Data from this study demonstrated that the bacterial peptide CFS in study inhibited the growth of only gram- positive bacteria mainly *Bacillus subtilis* (12 mm) (Table 1) in agreement with Oman et al. (2011). It was revealing the fact that bacteriocins generally exhibit antimicrobial activity mainly against phylogenetically related bacteriocin-producing bacteria (Hassan et al. 2012). Earlier reports showed that antimicrobial lipopeptides spectrum depended on their fatty acid moiety length (Strieker and Marahiel, 2009; Mandal et al., 2013; Meir et al., 2017), hence the presence of different fatty acid resulted in broad spectrum of antimicrobial activities. On the other hand, the observed antibiofilm activity of CFS bacteriocin in this study, in agreement with previous study (Bhattacharya et al., 2018), revealed that biofilm formation may be suppressed but the microbial growth is not stopped, bacteria and or *Candida* sp. in study reached a number sufficient to inactivate the peptide effect and resume the biofilm formation subsequently, bacterial derived bacteriocin may prevent but does not eradicate biofilm formation, this explains the non - significance difference between the control and the tested CFS biofilms (Fig. 5). CFS minimal inhibitory assay (MIC) revealed equally its antimicrobial activity against *B.subtilis*, *C. albicans, C. tropicalis* and *C. glabrata* at different concentrations 25 and 4 μg/mL correspondingly (Fig. 4).

On the other hand, the crude algal extract had a different antimicrobial effect against the strains in study. Data, from the present work, indicated that aqueous extract had the most potent antibacterial activity mainly against *S. aureus* (10 mm) and *P. aeruginosa* (9 mm), oppositely had no significant effect with the remaining isolates. Similar results were observed with Rahelivo et al. (2015) where *T. ornata* crude extract was mostly active against *S. aureus* and *P. aeruginosa* at different concentrations as revealed by the MIC (Figs. 3 and 4) (1 and 0.25 μg/ mL) correspondingly. This distinct antimicrobial activity could be due to the thickness and composition of target bacterial cell walls (Purnama et al., 2011) as well as due to the synergistic effect of the algal secondary metabolites produced which overcomes the difficulties of isolating a single active ingredient and or enhances the low doses of one

active constituent's efficacy (Williamson, 2000). Synergistic therapy can be used to expand the antimicrobial spectrum, to prevent the emergence of resistant mutants and to minimize toxicity, to the extraction method and to the geographical differences which can produce different biological compounds of hence different biological activities; however, more studies are required through a dedicated study to possibly clarify the mechanism of action. Moubayed et al. (2017) and Venkatesan et al. (2019) both reported that this variable sensitivity between gram-positive and gram-negative bacteria towards these compounds could be related to polyphenols binding to the bacterial proteins where aromatic ring and OH-groups of polyphenols bind with –NH– groups of bacterial proteins through hydrogen bonds and hydrophobic interactions) and cell membrane proteins and thus causes cell lysis. Moreover, as revealed by the GC–MS chromatograms (Fig. 5 **a, b**), different bioactive compounds among which polyphenols are present in the algal crude extract; however, as reported by Lopes et al. (2012) the main challenge in the extraction of these compounds arises from the strong complexes formed between these compounds and the main components of the cell wall polysaccharide including polyphenols. Perylene and its derivatives, for instance, the main chemical compound of the aqueous algal crude extract, was widely reported as a "killer" and a "marker", namely a membrane-intercalating broad-spectrum antimicrobial agent and an excellent fluorescence imaging agent (Niu et al., 2019); this explains the recorded antimicrobial activity of the aqueous algal extract in study against the experimented *Bacillus subtilis* and the three fungal isolates. Thus as noted from the present work, each component either the crude algal extracts or the bacterial CFS had a different biological activity, revealed by a moderate antimicrobial activity mainly against *Bacillus sbutilis* and a promising anti-biofilm activity, however; further investigations are required with suggested combination of both crude algal extract and the CFS that may exert better synergistic biological activity, and develop as such an innovative safe therapy for microbial and biofilm-related infections.

5. Conclusion

A combination of physical, geographical conditions is required for the optimal biological activity of seaweeds secondary metabolites and its associated bacteria. In this study, each of the crude extract and the newly identified *Bacillus* spp. CFS showed different effect against the tested microorganisms. Maximal antimicrobial activity was noted with the aqueous *T. ornata* extract against mainly *S. aureus* and *P. aeruginosa* whereas the bacterial CFS was found to be mostly active against *B. subtilis* and against all three *Candida* isolates, revealing as such therapeutical differences between the algal extracts and its associated bacteria and opening a possible promising outline for marine-microbial separate or combined biological activities.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

NM: conceived the idea, performed the experimental work and wrote the manuscript.

HH: provided the alga, collected the data.

Data availability

Data are included within the manuscript and available upon request from the corresponding author.

Sample Availability:

Samples of the algal and bacterial compounds are available from the corresponding author.

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