



# Pharmacological properties of marine macroalgae-associated heterotrophic bacteria

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## Abstract

The rich diversity of marine macroalgae and their associated bacterial flora represent a potential reservoir of bioactive compounds with valuable biotechnological and pharmaceutical use. Heterotrophic bacteria associated with the intertidal macroalgae were isolated and evaluated for their pharmacological properties using various in vitro models. Among 148 cultivable isolates, more than 50% were dominated by *γ-Proteobacteria* and *Firmicutes*, wherein 53 of them showed consistent antibacterial activity against a broad spectrum of clinically significant pathogens. The bacteria were characterized by extensive microbiological, molecular and chemical identification tools. The heterotrophs *Bacillus amyloliquefaciens* MTCC 12716 and *Shewanella algae* MTCC 12715 isolated from a red marine macroalga *Hypnea valentiae* exhibited potential anti-infective properties against multidrug-resistant pathogens, such as methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecalis* (minimum inhibitory concentration of 6.25–12.5 µg/mL). The organic extract of *B. amyloliquefaciens* displayed significantly greater antioxidative properties ( $IC_{90} < 1$  mg/mL) and the activities showed considerable positive correlation ( $r^2 > 0.8$ ,  $P < 0.05$ ) with the inhibitory activities against angiotensin converting enzyme-I, pro-inflammatory cyclooxygenases and 5-lipoxygenase, dipeptidyl peptidase-4 and hydroxymethylglutaryl coenzyme A reductase, which were associated with hypertension, inflammation, diabetes, and hypercholesterolemia, respectively. The applications of nuclear magnetic resonance-based fingerprinting to analyze the characteristic signals in the solvent extracts and to correlate them with the pharmaceutical properties were underlined. The heterotrophic bacterium *B. amyloliquefaciens* MTCC 12716 might, therefore, serve as a potential therapeutic candidate to develop products with wide pharmaceutical applications.

**Keyword** Intertidal marine macroalgae · Heterotrophic bacteria · *Firmicutes* · *Bacillus amyloliquefaciens* MTCC 12716 · Antibacterial · Pharmacological properties · Nuclear magnetic resonance-based fingerprinting

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## Introduction

Marine microbial heterotrophic symbionts were recognized for their capacity in imparting biochemical defences to the host eukaryotes to fight with competitive predators and pathogenic colonizers (Paul et al. 2011). These symbionts were reported to be endowed with potential bioactivities against pathogenic bacteria, inflammation, and cancer, to mention a few, and therefore, are valuable resources for drug discovery (Molinski et al. 2009). Recent research efforts established that, most often, the microbial symbionts are the sources of bioactivities associated with the host-eukaryotic symbiotic association, and apparently not the eukaryotic hosts (Li and Vederas 2009).

The fraction of bioactive bacteria in association with invertebrates (20%) and marine macroalgae (11%) was

described to be greater than those isolated from the surface seawater (more than 6%) and sediments (~5%) (Li and Vederas 2009) although lesser than 5% of them were cultivable under laboratory conditions. This directed to the conclusion that marine microbes have been uncultivable, and therefore, received meager attention to the scientists. However, recent research efforts proved that selected marine microorganisms could be cultivated successfully (Davidson 1995). Bacterial and fungal association were reported from the internal tissues of invertebrates and marine plant surfaces, whereas the symbionts harbored promising pharmaceutical properties (Webster and Taylor 2012). Bacterial groups, such as *Firmicutes*, *Bacteroidetes* and  $\gamma$ -*Proteobacteria* were predominantly present in association with marine macroalga *Laminaria saccharina* (Wiese et al. 2009). Among these,  $\gamma$ -*Proteobacteria* was found to be the predominant cultivable class in the *Haliclona simulans* sponge-associated heterotrophs (Kennedy et al. 2009) and marine macroalga *Jania rubens* associated (greater than 70%) microbes (Ali et al. 2012). As a sessile primary producer, marine macroalgae were reported to host wide varieties of microbes on its surface compared to other marine eukaryotes (Hollants et al. 2012). The comparative simplicity of culturing these bacteria, equated to other microbes that release bioactive metabolites, suggested that the macroalgae-associated bacteria might be useful in biotechnological applications, especially as the potential source of antimicrobial agents (Armstrong et al. 2001).

Microorganisms were recognized as valuable biological resources of chemically as well as biologically diverse compounds, whereas their potential capabilities to biosynthesize complex chemical entities from common nutrients in fermentation media have made them promising for the extensive use in the production of pharmaceutical preparations. More than 120 formulations of microbial origin are currently in clinical use for the treatment of cancer and infectious diseases, and as immune suppressors to aid organ transplantation (National Research Council 1999). They have also been demonstrated to possess varied properties of potential therapeutic applications, such as antioxidant (Tabbene et al. 2012), anti-diabetic adjuvant (Okamura et al. 1992), anti-inflammatory formulations (Tang et al. 2010), etc. Among different phyla of marine bacteria, *Firmicutes* and  $\gamma$ -*Proteobacteria* were recognized as important sources of bioactivities with potential pharmacological significance (Lachnit et al. 2011; Thilakan et al. 2016).

Earlier reports on macroalgae-associated bacteria contemplated their potential as antibacterial pharmacophores for use against human pathogenic microorganisms (Chakraborty et al. 2017a, b). As an ongoing programme to isolate and characterize marine macroalgae-associated symbionts, the present work has been directed to a culture-dependent method to isolate heterotrophic *Firmicutes* and  $\gamma$ -*Proteobacteria* associated with the intertidal marine

macroalgae, and evaluate for their pharmacological properties using various in vitro models. The bacteria were characterized by extensive microbiological, biotechnological and chemical identification tools. Based on the anti-infective potential against clinically significant pathogens, the heterotrophic *B. amyloliquefaciens* MTCC 12716 and *S. algae* MTCC 12715 were used to prepare organic extracts, and that were assayed for their antioxidant potential along with their abilities to inhibit various pharmacological targets, such as angiotensin converting inhibitory enzyme-1 (ACE-1), pro-inflammatory cyclooxygenases and lipoxygenase (COX-1, 2 and 5-LOX), dipeptidyl peptidase-4 (DPP-4) and hydroxymethyl glutaryl coenzyme A reductase (hMGCR) associated with hypertension, inflammation, diabetes and hypercholesterolemia, respectively. The  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR-based chemical fingerprint analyses of the prominent functionalities in the organic extracts of the heterotrophs were determined to underline the significance of electron-rich unsaturated centers towards the bioactive properties.

## Materials and methods

### Isolation of marine macroalgae-associated heterotrophs

The marine macroalgae referred to the classes of *Rhodophyceae*, *Phaeophyceae* and *Chlorophyceae*, were collected from the intertidal zone by hand, at low tide. The samples were collected from the coastal area of Mandapam situated at 9°17'0" North, 79°7'0" East, Gulf of Mannar region and Vizhinjam harbor located at 8.3932° North, 77.0046° East, in the Arabian Sea, at the Southern Coast of Peninsular India. The macroalgal samples were placed in a sterile polythene bag filled with seawater and were kept in the dark at 4 °C until further processing in the laboratory. Symbiotic heterotrophic bacteria associated with the marine macroalga *Hypnea valentiae* were isolated and purified by following previous reports of literature (Quevrain et al. 2014; Thilakan et al. 2016).

Briefly, the specimen samples (10 g) were thoroughly washed in sterile distilled water to remove loosely attached microbes and filth. Further, they were suspended in sterile seawater (10 mL) and homogenized using a pestle and mortar in a laminar airflow hood under an aseptic condition. The suspension was serially diluted in sterile seawater (9 mL), and different dilutions were plated on the isolation medium of Zobell Marine Agar, which was supplemented with sodium chloride (NaCl, 1% w/v). The incubation was performed in the dark at 30 °C for 7 days. The pure colonies were obtained by subsequent isolation and purification steps on Zobell Marine Agar medium supplemented with NaCl (1% w/v) (Wiese et al. 2009; Chakraborty et al. 2014). Streak

plate method was used to isolate an axenic culture. The bacteria isolated were then classified based on morphology and identified with biochemical tests, 16S rRNA fingerprinting followed by MALDI-TOF biotyping as discussed in the following section. The growth and bioactivity of the strains used in this study were optimized under various nutritional parameters, whereas an optimized media (modified basal salt agar) was used for the biomass production based on the study. The isolates were subjected to primary screening for their antibacterial potentials. The producer strains were maintained in the same media at 20 °C and found to be consistently active. Further, the most active strains were selected and their soluble secondary metabolites were extracted and bioactivities were analyzed against different disease models.

### Primary antibacterial screening for active strains

Antibacterial properties of the heterotrophic isolates was examined by spot on the lawn assay (Chakraborty et al. 2014). In brief, a lawn of the pathogenic bacteria was made to grow on Mueller–Hinton agar plates, over which the isolates at its late log phase were spotted in an aseptic condition. The test pathogenic organisms, such as *Escherichia coli* (MTCC 443), *Streptococcus pyogenes* (MTCC 1924), and *Edwardsiella tarda* (MTCC 2400) were obtained from the National Centre for Aquatic Animal Health-(NCAAH) of Cochin University of Science and Technology (CUSAT), Cochin. Pathogenic *Vibrio parahaemolyticus* (MTCC 451) was obtained from the Microbial Type Culture Collection and Gene Bank (MTCC) of Institute of Microbial Technology (Chandigarh, India), an affiliate member of the World Federation for Culture Collections (WFCC) and recognized as an International Depository Authority (IDA). These cultures were maintained in our laboratory, and multidrug-resistant pathogens including methicillin-resistant *Staphylococcus aureus* (ATCC 33592) and vancomycin-resistant *Enterococcus faecalis* (ATCC 51299) were procured from the American Type Culture Collection (Manassas, VA, USA). The pathogens were procured during the period of 2016, and their pathogenicities were assessed by performing PCR amplification with specific virulent factors for each pathogenic bacterium, immediately after obtaining the cultures. Their identities were confirmed by specific phenotypic and genotypic characterization, such as microbiological and biochemical testing, followed by 16S rRNA sequence similarity check (Armstrong et al. 2001). The pathogenic strains were maintained in Brain heart infusion agar and Nutrient agar supplemented with NaCl as per manufacturers' protocol.

The antibacterial activities were recorded according to the inhibition zone developed on the plates after a period of 48-h incubation at 30 °C. Further, the crude extracts of selected strains were assessed by Minimum Inhibitory Concentration (MIC) by broth microdilution assay

according to the National Committee for Clinical Laboratory Standards (NCCLS, 2004) with minor modifications. The MIC was determined by means of serial dilutions of the extracts (Balouiri et al. 2016) and positive control (chloramphenicol). The test samples of 100 µL were serially diluted in multi-well assay plates, whereas the pathogenic bacterial broth (100 µL,  $2 \times 10^6$  cell/mL) was added separately. The plate was then incubated for 24 h at 37 °C, and the minimum concentration of the sample, which inhibit the growth of the pathogen, where the OD<sub>600</sub> of the well was near to or equal to zero, has been considered as the MIC. Further, the bactericidal property of the samples was affirmed by observing the plates after the addition of tetrazolium salt of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) to the wells.

### Identification of the active isolates

The antagonistic bacteria were identified phenotypically, supported by the biochemical methods (Krieg and Holt 1984), Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS), followed by 16S rRNA sequencing (Armstrong et al. 2001) and analysis of bacterial membrane fatty acid (Zhu et al. 2005). Mass spectroscopic experiments were performed on a MALDI-TOF MS spectrometer (Microflex, BrukerDaltonics, Germany) operating in a linear positive mode. Mass spectra were evaluated between the *m/z* range of 2000 to 20,000, and analyzed by the MALDI Biotyper (version 3.1) for strain identification. Briefly, the bacterial proteins were extracted with ethanol/formic acid extraction method, whereas 1 µL of the extract was spotted on the target plate, and allowed to dry before being covered with 1 µL of  $\alpha$ -cyano-4-hydroxycinnamic acid. The cut-off score for similarity search was  $\geq 1.7$  for species-level, and any score lesser than this was considered as an unreliable identification. The isolates showing significant inhibitory activity were selected and phenol–chloroform extraction method was adopted for isolating DNA from the active strains. Further, the qualities of DNA samples were detected by agarose gel electrophoresis and their concentrations were evaluated using a Biphotometer (Applied Biosystems, USA). Universal primers AGAGTTTGATCC TGGCTCAG (forward) and ACGGCTACCTTGTTACGA CTT (reverse) (Weisburg et al. 1991) were employed to amplify the 16S rRNA gene. Further, the PCR products were sequenced and deposited in the NCBI GenBank.

### Assessment of pathogenicity

Pathogenicity of the selected microorganisms was studied by hemolytic assay (Gao et al. 2000) on sheep blood agar

plates. Briefly, blood agar with 5% defibrinated sheep blood was dispensed into a sterile Petri-plate aseptically. Overnight grown cultures of the bacteria were spot inoculated at the center of the plates and incubated at 30 °C for 18–24 h. *Streptococcus pyogenes* MTCC 1924 was used as the control organism.

### Preparation of crude extracts and in vitro bioassays

The extracellular metabolites of the potentially antagonistic bacteria *B. amyloliquefaciens* (MTCC 12716) and *S. algae* (MTCC 12715) were extracted with a suitable organic solvent. In brief, the bacteria were surface cultured over nutrient agar in the dark at 30 °C for 72 h in the case of *S. algae*, and 48 h for *B. amyloliquefaciens*. After the incubation period, the culture on the surface was removed, and the agar with the extracellular metabolites was exhaustively extracted by refluxing with solvent ethyl acetate on a temperature-controlled water bath before being concentrated *in vacuo* on a rotary vacuum evaporator (Heidolph, Schwabach, Germany). The ethyl acetate extract (470 g) of the antagonistic bacteria were assayed in vitro for their antioxidant potential along with their abilities to inhibit various pharmacological disease targets, such as angiotensin converting enzyme-1 (ACE-1), pro-inflammatory cyclooxygenases and lipoxygenase (COX-1, 2 and 5-LOX), dipeptidyl peptidase-4 (DPP-4), and hydroxymethyl glutaryl coenzyme A reductase (hMGCR) associated with hypertension, inflammation, diabetes and hypercholesterolemia, respectively.

### Antioxidant activity

Antioxidant activities of the crude extracts were evaluated by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and 2, 2-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) free radical scavenging abilities. The measurement of DPPH radical scavenging activity was performed by a previously described method (Brand-Williams et al. 1995). The changes in color were read at an absorbance of 517 nm after 20 min of reaction by a UV–VIS spectrophotometer (Varian Cary, USA). ABTS<sup>+</sup> assay was carried out by an established method of Re et al. (1999). The reaction mix was kept for 6 min, and the absorbance was recorded at 734 nm. DPPH/ABTS scavenging activities (in percentage) were expressed as  $(A_C - A_S)/A_C \times 100$ , where  $A_C$  represented the absorbance of control and  $A_S$  that of the sample. The scavenging activities on DPPH/ABTS<sup>+</sup> radical were documented, and the IC<sub>90</sub> values (concentration of the bacterial crude extracts that inhibit 90% of the radical/enzyme, mg/mL) calculated using the non-linear regression plot. Ascorbic acid was used as the standard during the assessment of free radical scavenging activity.

### Anti-hypertensive activity

Anti-hypertensive activities of the extracts were assessed based on the inhibition rate of ACE-1 (Holmquist et al. 1979). In brief, ACE-1 (20 μL, 1 U/mL, from rabbit lung) was mixed with the crude extracts (100 μg), and the mixture was added with *N*-[3-(2-furyl) acryloyl]-L-phenylalanyl-glycylglycine (FAPGG, 0.5 mM) dissolved Tris–HCl buffer (pH 7.5) supplemented with common salt. The reduction in absorbance at 345 nm was monitored with a multi-plate reader (Multiskan GO type 1510, Thermo Scientific, USA) within a 1.5 min interval at room temperature, and was stated as  $\Delta_{Ab}^{sample}/min$ . Blank was set with distilled water and expressed as  $\Delta_{Ab}^{blank}/min$ . Captopril was served as the positive control for ACE inhibition. The plots of ACE-1 inhibition by the extracts were recorded, and the IC<sub>90</sub> values (mg/mL) were calculated.

### Anti-diabetic activity

The crude extracts were assayed for their anti-diabetic activities by inhibition of  $\alpha$ -glucosidase,  $\alpha$ -amylase, and DPP-4 (dipeptidyl peptidase-4) enzymes. The  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibitory activities of the studied extracts were measured by the standard method (Ademiluyi and Oboh 2013) with suitable alteration. The absorbance of the reaction mixture was measured at 540 and 405 nm, respectively, whereas acarbose was used as a standard. For the inhibition of DPP-4, the extracts were made in different strengths in Tris–HCl buffer (0.05 M, pH 7.5). The substrate (gly-pro-*p*-nitroanilide) was added to the reaction mixture before an incubation period of 30 min at 37 °C. Glacial acetic acid (25%) was added to quench the reaction, and the absorbance was recorded at 450 nm, whereas diprotein-A (Ile-Pro-Ile) was used as a reference.

### Anti-inflammatory and anti-hypercholesterolemic activities

Dual blocking of both cyclooxygenases (COX-1 and COX-2) and 5-lipoxygenase (5-LOX) is a commonly used method for the treatment of inflammatory diseases without causing severe side effects (Charlier and Michaux 2003). The in vitro COX (Larsen et al. 1996) and 5-LOX inhibition assays (Baylac and Racine 2003) were used to determine the anti-inflammatory activities of the bacterial extracts. The 3-hydroxy-3-methyl-glutaryl-coenzyme A (hMG-CoA) reductase enzyme inhibition by crude *B. amyloliquefaciens* and *S. algae* extracts was evaluated with hMG-CoA reductase (hMGCR) assay kit from Sigma-Aldrich (St. Louis, MO, USA) to assess their anti-hypercholesterolemic activities. In brief, the crude organic extracts were added with the reaction mixture containing nicotinamide adenine dinucleotide



phosphate (0.4 mM) and hMG-CoA (0.4 mM) in a final volume of 0.2 mL of potassium phosphate buffer (0.1 M, pH 7.4) containing KCl (0.12 M), ethylenediaminetetraacetic acid (1 mM), and dithiothreitol (5 mM), before being added with HMG-CoA reductase (human recombinant, 2  $\mu$ L) and incubated at 37 °C in the multi-plate reader spectroscopic instrument (Multiskan GO, Thermo Scientific, USA) fitted out with thermostatically organized cell holder. The rates of NADPH consumption were monitored every 20 s for up to 15 min by spectral scanning at 340 nm. Percentage inhibition of COX-1, COX-2, 5-LOX and hMGCR were calculated, and the results were expressed in IC<sub>90</sub>, the concentration at which it inhibits 90% of the enzyme activity.

### Spectroscopic fingerprint analyses

Spectroscopic fingerprint characteristics of the organic extracts were comprehensively studied by Fourier transform infrared spectrometer (Perkin-Elmer Series 400 FTIR, USA) scanned between wave-numbers 4000–400 cm<sup>-1</sup> and nuclear magnetic resonance (<sup>13</sup>C NMR and <sup>1</sup>H NMR) spectral analysis on a Bruker AVANCE III 600 MHz spectrometer (Bruker, Germany). Chemical shift ( $\delta_H$  or  $\delta_C$ ) values were recorded as ppm ( $\delta$ , parts per million), and the protons at the definite regions of the <sup>1</sup>H NMR spectra were integrated (proton integral,  $\Sigma H$ ) to determine the aggregate number of protons in characteristic regions of the spectra (Fig. 2). The <sup>1</sup>H-NMR spectra were allocated into five discrete regions, such as alkane hydrocarbon (primary through tertiary,  $\delta_H$  0.1–2.0), allylic (CH<sub>2</sub>=CH–Me)/acetyl {–C(=O)Me}/derivatized hydride of alkanates {–CH<sub>2</sub>C(=O)O–,  $\delta_H$  2.1–2.5}, methoxyl (–OMe)/functionalized alkanol (–CH<sub>2</sub>OH,  $\delta_H$  2.6–3.5), anomeric (due to polysaccharides,  $\delta_H$  3.6–4.5), olefinic (–CH=CH–)/protons of the hydride group of alkanates {–CH<sub>2</sub>C(=O)OMe,  $\delta_H$  4.6–6.0}, and aryl protons (C<sub>6</sub>H<sub>5</sub>–H,  $\delta_H$  6.6–8.6) (Table 1). The <sup>1</sup>H NMR trace at  $\delta_H$  9–10 characterized the aldehydic protons. The <sup>13</sup>C NMR spectral data were used to recognize the carbons attributed to alkane

hydrocarbon ( $\delta_C$  up to 40), alkoxy (–CH<sub>2</sub>O–;  $\delta_C$  45–70), carbons of the hydride group of alkanates {–CH<sub>2</sub>C(=O)OMe,  $\delta_C$  75–80}, functionalized olefine ( $\delta_C$  110–140), aromatic ( $\delta_C$  140–160), ester alkanates {–C(=O)O–;  $\delta_C$  160–180} and carbonyl (aldehydic/ketonic) moieties ( $\delta_C$  180–210).

### Statistical analysis

Statistical analysis was carried out using the SPSS software (SPSS Inc, USA, ver. 10.0). The studies were carried out in triplicate, and the means were determined for significance at 5 percent level ( $P < 0.05$ ) by means of analysis of variance (ANOVA). The mean variances in the data set were identified with principal component analysis (PCA).

## Results

### Isolation of marine macroalgae-associated heterotrophs and antibacterial screening

Seven different intertidal macroalgae representing *Rhodophyceae*, *Phaeophyceae* and *Chlorophyceae* were collected from the intertidal zones, and that include brown algae, *Sargassum myricocystum*, *Padina gymnospora*, *Turbinaria ornata*, red algae *Hypnea valentiae*, *Kappaphycus alvarezii* and green algae *Ulva reticulata*, *Caulerpa racemosa*. A total number of 148 heterotrophic bacterial strains were isolated from the macroalgal species. The bacteria isolated were classified based on morphology and further with microbiological and biochemical tests, 16S rRNA fingerprinting followed by MALDI-TOF biotyping.

The bacterial classes of *Gamma proteobacteria* and *Firmicutes* represented the predominant bacterial communities comprising more than 50% of the total isolates screened. The isolated bacteria were further screened for their antibacterial activity by spot over lawn assay, and 53 of them showed consistent antibacterial activity against at least

**Table 1** Nature and integral values of protons obtained from the <sup>1</sup>H-NMR of the organic extracts of *B. amyloliquefaciens* MTCC 12716 and *S. algae* MTCC 12715

Integral values of protons			
Type of protons <sup>a</sup>	Chemical shift ( $\delta$ )	<i>S. algae</i> MTCC 12715	<i>B. amyloliquefaciens</i> MTCC 12716
Saturated hydrocarbons	0.5–2.0	329.7	152.9
–CH <sub>2</sub> =CH–CH <sub>3</sub> /RC(=O)CH <sub>3</sub> / RCH <sub>2</sub> C(=O)OR <sub>1</sub> /Ar-CH <sub>3</sub>	2.1–2.5	61.81	40.73
–OCH <sub>3</sub> /RCH <sub>2</sub> –X/RCH <sub>2</sub> OH	2.6–3.5	23.94	22.96
Anomeric (due to polysaccharides)	3.6–4.5	48.02	64.73
RCH=CHR <sub>1</sub> /RCH <sub>2</sub> C(=O)OCH <sub>3</sub>	4.6–6.0	18.82	12.42
Ar-H	6.6–8.6	25.80	28.77

<sup>a</sup>The protons at the defined regions of the <sup>1</sup>H NMR spectra were integrated to get the number of protons in specific regions

one test pathogen. Bactericidal activities were furthermore ascertained by MTT reaction, whereas the blue cells were indicated as live. The antibacterial activities of these heterotrophs against the pathogens were summarized in Table 2. Two of the most active cultures *B. amyloliquefaciens* and *S. algae* (Fig. 1), which were isolated from *Hypnea valentiae* were selected to study their pharmacological properties in greater details based on their superior bioactivity among other strains. The growth and bioactivities of the strains used in this study were optimized under various nutritional parameters, whereas an optimized media (modified basal salt agar) was used for the biomass production based on the study. The producer strains were maintained in the same media at 20 °C, and found to be consistently active under laboratory culture condition.

These isolates were characterized by biochemical, membrane analysis of fatty acids, 16S rRNA gene sequencing, followed by MALDI-TOF MS with a confidence score of 1.953 and 2.152 as *B. amyloliquefaciens* and *S. algae*, respectively. Among the selected bacterial strains, *B. amyloliquefaciens* belonged to Gram-positive, and *S. algae* was classified as Gram-negative, as determined by Gram staining and potassium hydroxide (KOH) screening experiments. Biochemical experiments deduced *S. algae* as rod-shaped, aerobic, and brown pigmented bacterium, which was able to ferment sugars to produce hydrogen sulfide on triple iron sugar (TSI) slants, whereas *B. amyloliquefaciens*, with the morphology of being white wavy, lobed margins, possessed casein hydrolysis and nitrate reduction properties. Blackish-brown pigmentation by *S. algae* was recorded at the late exponential phase due to the production of pyomelanin (Turick et al. 2008). Optimal growth of *S. algae* occurred

with pH 8.0 in the presence of 1–4% of NaCl at temperature 30 °C, and *B. amyloliquefaciens* at 37 °C with pH 7–9. The predominance of terminally methyl-branched *iso* and *anteiso* fatty acids with C–12 to 17 characterized *S. algae*, whereas C<sub>15</sub> and C<sub>17</sub> *isolanteiso* fatty acids were predominant in *B. amyloliquefaciens*. Among the *n*-9 fatty acids, *cis*-octadec-9-enoate (C18:1*n*-9c) was found to be present in *S. algae* other than *cis*-octadec-11-enoate (C18:1*n*-7c) and *cis*-heptadec-11-enoate (C17:1*n*-6c). Further, the similarity of 16S rRNA sequences was tested with known sequences of *B. amyloliquefaciens* and *S. algae* using BLAST search tool, and submitted in GenBank with accession numbers of KX272634 and KX272635, respectively. These bioactive isolates were deposited in Microbial Type Culture Collection (MTCC), an International microbial depository authority in South-East Asia under the Budapest Treaty, with accession numbers of *B. amyloliquefaciens* MTCC 12716 and *S. algae* MTCC 12715, respectively.

The crude organic (ethyl acetate) extracts of *B. amyloliquefaciens* MTCC 12716 and *S. algae* MTCC 12715 exhibited strong antagonism against a broad spectrum of pathogenic bacteria, *Escherichia coli* (gastrointestinal infection), *Streptococcus pyogenes* (skin infection), *Edwardsiella tarda* (gastrointestinal infection and myonecrosis), *Vibrio parahaemolyticus* (gastrointestinal infection), MRSA (methicillin-resistant *Staphylococcus aureus*) (skin infection) and VRE (vancomycin-resistant *Enterococcus faecalis*) (sepsis and meningitis) (Fig. 1). Inhibitory activities of the bacterial isolates were observed on MTT-sprayed Mueller Hinton agar plates. The microdilution method also displayed a significant MIC of 6.25–25 µg/mL against the test pathogenic bacteria (Table 2). Additionally, there was no zone

**Table 2** Antibacterial activities of marine macroalgae-associated heterotrophic *B. amyloliquefaciens* MTCC 12716 and *S. algae* MTCC 12715 against pathogenic bacteria and minimum inhibitory concentrations (MICs) of their ethyl acetate extracts

Test pathogens	<i>B. amyloliquefaciens</i> MTCC 12716		<i>S. algae</i> MTCC 12715	
	Inhibition zone <sup>a</sup> (mm)	Crude <sup>b</sup> MIC (µg/mL)	Inhibition zone <sup>a</sup> (mm)	Crude <sup>b</sup> MIC (µg/mL)
<i>Vibrio parahaemolyticus</i> (MTCC 451)	32 <sup>a</sup> ± 0.60	6.25	27 <sup>a</sup> ± 0.07	12.5
Methicillin resistant <i>Staphylococcus aureus</i> (ATCC 33592)	22 <sup>a</sup> ± 0.60	12.5	16 <sup>b</sup> ± 0.04	12.5
Vancomycin resistant <i>Enterococcus faecalis</i> (ATCC 51299)	20 <sup>b</sup> ± 0.37	6.25	14 <sup>b</sup> ± 0.03	12.5
<i>Escherichia coli</i> (MTCC 443)	10 <sup>c</sup> ± 0.25	12.5	11 <sup>c</sup> ± 0.05	25.0
<i>Edwardsiella tarda</i> (MTCC 2400)	21 <sup>a</sup> ± 0.30	6.25	17 <sup>b</sup> ± 0.02	6.25
<i>Streptococcus pyogenes</i> (MTCC 1924)	10 <sup>c</sup> ± 0.44	6.25	11 <sup>b</sup> ± 0.09	12.5
Chloramphenicol <sup>c</sup>	27 <sup>a</sup> ± 0.30	6.25	27 <sup>a</sup> ± 0.30	6.25

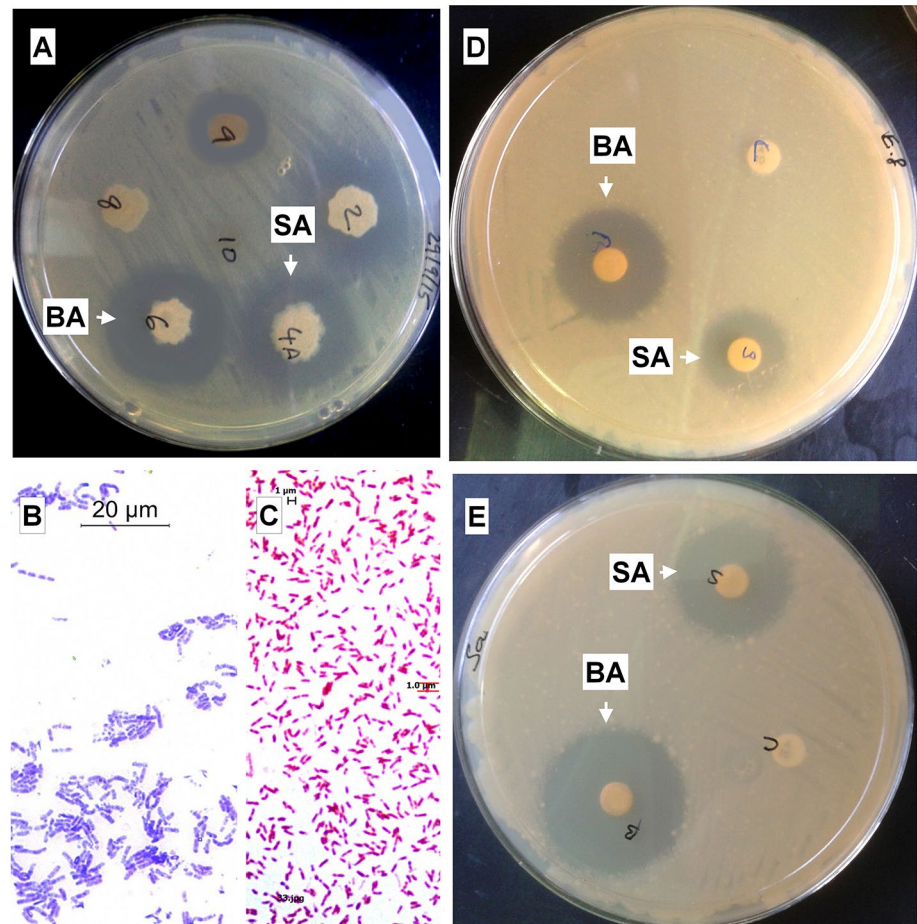
Data were expressed as mean ± standard deviation of three replicates. Means followed by the different superscripts (a–c) within the same row indicate significant difference ( $P < 0.05$ )

<sup>a</sup>Inhibition zone diameter (in mm)

<sup>b</sup>Minimum Inhibitory concentration (µg/mL) of ethyl acetate extract of the bacteria

<sup>c</sup>Standard antibiotic (positive control)

**Fig. 1** **a** Spot over lawn assay of the cultures *B. amyloliquefaciens* MTCC 12716 (BA) and *S. algae* MTCC 12715 (SA) against methicillin-resistant *Staphylococcus aureus* (ATCC 33592). The clear zones displayed by the isolates indicated the antibacterial activity. Antagonistic activities of the isolates were recorded as the inhibition zone diameter determined as a space of  $\geq 1$  mm between the circular area (=lawn of the isolate) and the end of the clear zone bounded by the lawn of the test strain. **b** View of the Gram stained culture of *B. amyloliquefaciens* MTCC 12716. **c** Gram stained image of *S. algae* MTCC 12715. **d** Disc diffusion assay with the organic extracts of *B. amyloliquefaciens* MTCC 12716 (BA) and *S. algae* MTCC 12715 (SA) against VRE (vancomycin-resistant *Enterococcus faecalis* ATCC 51299), and **e** MRSA (methicillin-resistant *Staphylococcus aureus* ATCC 33592). The zones of antibacterial activities were denoted by the arrows



of clearance indicating hemolytic activity developed on the blood agar plates with the test organisms *B. amyloliquefaciens* MTCC 12716 and *S. algae* MTCC 12715, whereas *S. pyogenes* exhibited complete hemolysis ( $\beta$ -hemolysis) around the colonies.

### Pharmacological activities of the extracts of *B. amyloliquefaciens* MTCC 12716 and *S. algae* MTCC 12715

The solvent extracts of *B. amyloliquefaciens* MTCC 12716 and *S. algae* MTCC 12715 were assayed in vitro for their antioxidant potential along with their abilities to inhibit various pharmacological targets, such as ACE-1, pro-inflammatory enzymes (COX-1, 2, 5-LOX), DPP-4 and hMGCR, which were associated with hypertension, inflammation, diabetes and hypercholesterolemia, respectively. The organic extract of *B. amyloliquefaciens* MTCC 12716 exhibited significantly greater antioxidative potential as determined by the scavenging activities of DPPH (IC<sub>90</sub> 278  $\mu$ g/mL) and ABTS<sup>+</sup> (IC<sub>90</sub> 136  $\mu$ g/mL) radicals compared to that obtained from *S. algae* MTCC 12715 (IC<sub>90</sub> 602 and 483  $\mu$ g/mL, respectively). The ethyl acetate extract derived from *B. amyloliquefaciens*

MTCC 12716 exhibited significantly greater inhibition towards ACE-1 (IC<sub>90</sub> 448  $\mu$ g/mL) than that of *S. algae* MTCC 12715 (IC<sub>90</sub> 638  $\mu$ g/mL) ( $P < 0.05$ ). Likewise, the organic extracts of *B. amyloliquefaciens* MTCC 12716 and *S. algae* MTCC 12715 were found to inhibit  $\alpha$ -amylase and DPP-4 with IC<sub>90</sub> 639–837 and 354–769  $\mu$ g/mL, respectively. Notably, the  $\alpha$ -glucosidase inhibitory potential of the organic extract from *B. amyloliquefaciens* MTCC 12716 (IC<sub>90</sub> 84  $\mu$ g/mL) and that of *S. algae* MTCC 12715 (IC<sub>90</sub> 530  $\mu$ g/mL) were significantly greater than the standard Acarbose (IC<sub>90</sub> 645  $\mu$ g/mL). The extracts exhibited considerable inhibition against the pro-inflammatory enzymes, which appeared to play significant functional roles in the metabolic pathway of inflammation. The organic extract from *B. amyloliquefaciens* MTCC 12716 and *S. algae* MTCC 12715 were found to be selective COX-2 and 5-LOX inhibitors (anti-COX-2 IC<sub>90</sub> 29–44  $\mu$ g/mL; anti-LOX-5 IC<sub>90</sub> 6.06–7.74  $\mu$ g/mL) than commercially available NSAID (aspirin anti-COX-2 IC<sub>90</sub> 83–86  $\mu$ g/mL; anti-LOX-5 IC<sub>90</sub> 79  $\mu$ g/mL). A dose-response in vitro analysis with regard to the inhibition of hMGCR displayed significant activity with the ethyl acetate extract of *B. amyloliquefaciens* MTCC 12716 (IC<sub>90</sub> 17.3  $\mu$ g/mL) and *S. algae* MTCC 12715 (IC<sub>90</sub> 74.6  $\mu$ g/mL) (Table 3).



## Spectroscopic fingerprinting of the functional groups

The labelling of carbons and protons allied with their characteristic magnetic fields of the functional groups in the ethyl acetate extracts of *B. amyloliquefaciens* MTCC 12716 and *S. algae* MTCC 12715 were recorded by the combined  $^1\text{H}$  NMR (Fig. 2, Fig. S1-S2) and  $^{13}\text{C}$  NMR experiments (Fig. 3, Fig. S3-S4). Aromatic proton signals ( $\delta_{\text{H}}$  6.6–8.6) were recorded in the  $^1\text{H}$  NMR spectral data of the bacterial extracts, whereas that extracted from *B. amyloliquefaciens* MTCC 12716 displayed the greater proton integral ( $\Sigma\text{H}$ ) of about 28.77 than that recorded with *S. algae* MTCC 12715 extract ( $\Sigma\text{H}$  25.80). Higher proton integrals ( $\Sigma\text{H}$  23.94) at  $\delta_{\text{H}}$  2.5–3.5 (attributed to the presence of  $-\text{OCH}_3/\text{RCH}_2\text{-X}/\text{RCH}_2\text{OH}$  groups) were documented in the ethyl acetate extract of *S. algae* MTCC 12715. The number of protons at  $\delta_{\text{H}}$  2–2.5 were attributed to the acetyl or allylic functionalities, and were recorded to be higher in *S. algae* MTCC 12715 ( $\Sigma\text{H}$  61.81), whereas comparatively weaker proton integrals at this position were noted for *B. amyloliquefaciens* MTCC 12716 extract ( $\Sigma\text{H}$  40.73). Notably, the proton integrals at the olefinic region ( $\delta_{\text{H}}$  4.5–6) of organic extract derived from *S. algae* MTCC 12715 was marginally higher ( $\Sigma\text{H}$  18.82) than that recorded with *B. amyloliquefaciens* MTCC 12716 extract ( $\Sigma\text{H}$  12.42). The ethyl acetate extract

of *S. algae* MTCC 12715 displayed greater proton integral owing to saturated hydrocarbons, whereas that of *B. amyloliquefaciens* MTCC 12716 exhibited greater signals in the downfield (with a predominance of electronegative functional groups) region of the NMR spectrum. It is interesting to note the greater proton integrals at  $\delta_{\text{H}}$  3.6–4.5 ( $\Sigma\text{H}$  64.73, due to anomeric protons associated with polysaccharides) of organic extract due to *B. amyloliquefaciens* MTCC 12716, whereas comparatively weaker proton integrals at this position were noted for *S. algae* MTCC 12715 extract ( $\Sigma\text{H}$  48.02).

The attributions of different types of carbons (primary through quaternary) in the EtOAc extracts of the heterotrophic bacteria were furthermore confirmed by extensive  $^{13}\text{C}$  NMR experiments. The EtOAc extract of *S. algae* MTCC 12715 exhibited a greater number of carbon atoms assigned to the saturated hydrocarbons ( $\delta_{\text{C}}$  10–40) (Fig. 3), and the results were in accordance with the greater proton integrals of the same ( $\Sigma\text{H}$  329.7). Likewise, a greater number of aromatic signals ( $\delta_{\text{C}}$  140–160) was recognized in the EtOAc extract of *B. amyloliquefaciens* MTCC 12716 than that displayed by *S. algae* MTCC 12715. The distinctive peaks attributed to the carbonyl groups were recorded at  $\delta_{\text{C}}$  180–200, which were abundant in the organic extract of *B. amyloliquefaciens* MTCC 12716, whereas meager traces of these highly

**Table 3** Pharmacological properties of the organic extracts of *B. amyloliquefaciens* MTCC 12716 and *S. algae* MTCC 12715 by various in vitro models

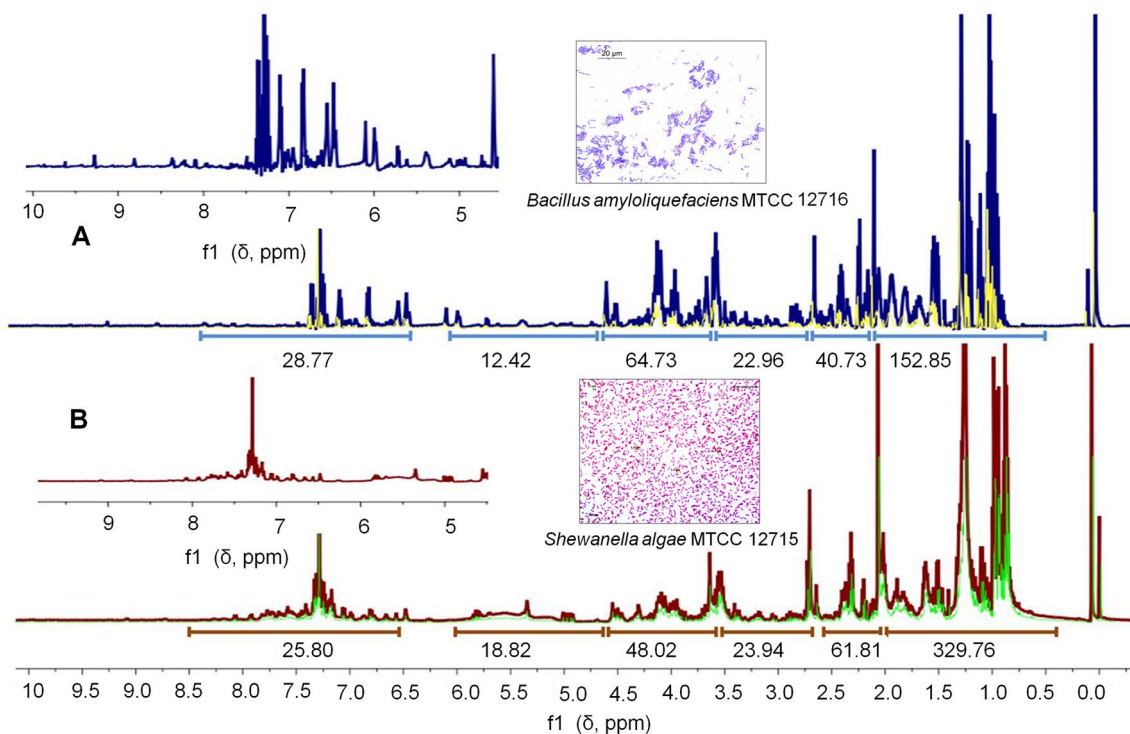
Pharmacological properties	Activities IC <sub>90</sub> (μg/mL)		
	Standard <sup>a</sup>	<i>B. amyloliquefaciens</i> MTCC 12716	<i>S. algae</i> MTCC 12715
Antioxidant activities			
DPPH radical scavenging	76.01 <sup>a</sup> ± 0.03	278.19 <sup>b</sup> ± 0.04	602.83 <sup>c</sup> ± 0.06
ABTS <sup>a</sup> radical scavenging	30.76 <sup>a</sup> ± 0.01	136.78 <sup>b</sup> ± 0.01	483.02 <sup>c</sup> ± 0.09
Anti-inflammatory activities			
COX-1 inhibitory activity	91.30 <sup>a</sup> ± 0.09	675.36 <sup>b</sup> ± 0.04	557.48 <sup>b</sup> ± 0.04
COX-2 inhibitory activity	86.93 <sup>a</sup> ± 0.07	29.10 <sup>b</sup> ± 0.07	44.00 <sup>c</sup> ± 0.04
5-LOX inhibitory activity	79.68 <sup>a</sup> ± 0.02	6.06 <sup>b</sup> ± 0.02	7.73 <sup>c</sup> ± 0.08
Anti-diabetic activities			
α-amylase inhibitory activity	312.44 <sup>a</sup> ± 0.01	639.54 <sup>b</sup> ± 0.05	837.69 <sup>c</sup> ± 0.03
α-glucosidase inhibitory activity	645.08 <sup>a</sup> ± 0.05	84.00 <sup>b</sup> ± 0.01	530.43 <sup>c</sup> ± 0.07
DPP-4 inhibitory activity	7.00 <sup>a</sup> ± 0.03	354.12 <sup>b</sup> ± 0.02	769.63 <sup>c</sup> ± 0.09
Anti-hypertensive activity			
ACE inhibitory activity	77.39 <sup>a</sup> ± 0.01	448.57 <sup>b</sup> ± 0.05	638.74 <sup>c</sup> ± 0.03
Anti-hypercholesterolemic activity			
hMGCR inhibitory activity	0.07 <sup>a</sup> ± 0.06	17.30 <sup>b</sup> ± 0.01	74.60 <sup>b</sup> ± 0.01

The samples were analyzed in triplicates ( $n=3$ ) and expressed as mean ± standard deviation. Means followed by the different superscripts (a–c) within the same row indicate significant difference ( $p < 0.05$ )

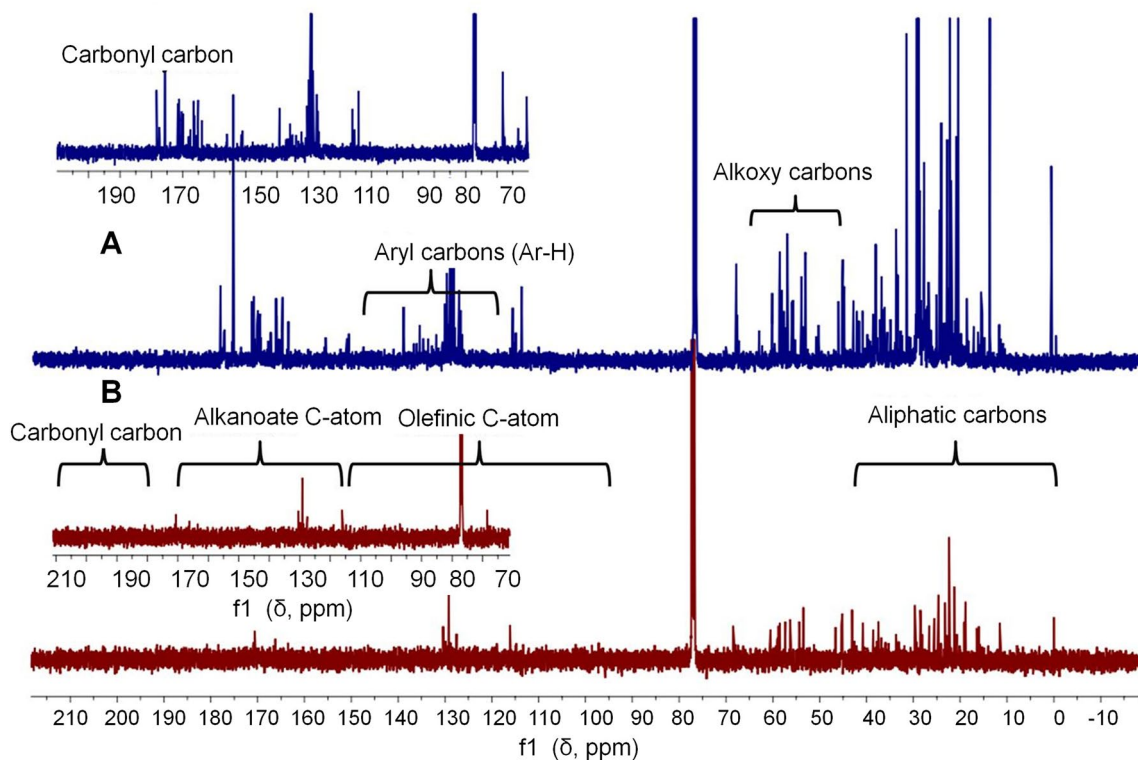
Other notations were as described in the text

<sup>a</sup>The standard antioxidant agent was α-tocopherol, whereas aspirin and acarbose were used as standard anti-inflammatory and anti-diabetic agent. Synthetic captopril and atorvastatin were used as standard ACE and hMGCR inhibitors, respectively





**Fig. 2** The stacked plot representing the  $^1\text{H}$  NMR spectra of EtOAc extracts of **(A)** *B. amyloliquefaciens* MTCC 12716 and **(B)** *S. algae* MTCC 12715



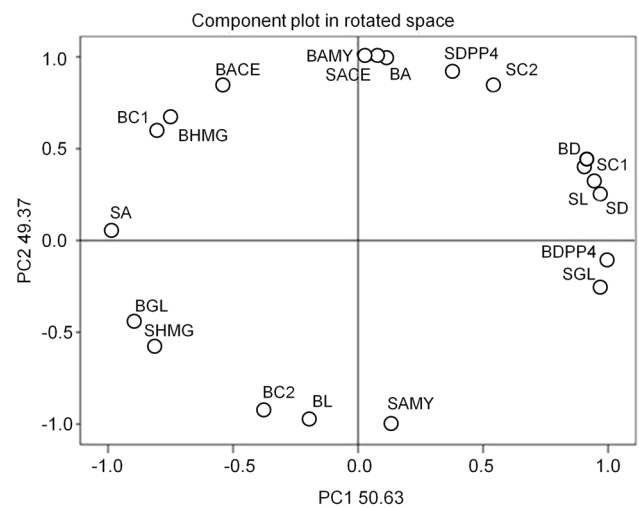
**Fig. 3**  $^{13}\text{C}$  NMR spectra of organic extracts of **(A)** *B. amyloliquefaciens* MTCC 12716 and **(B)** *S. algae* MTCC 12715. The chemical shift ( $\delta$ ) values were expressed in parts per million (ppm), and were referenced to the residual solvent signals of  $\text{CDCl}_3$

electronegative groups were apparent in that obtained from *S. algae* MTCC 12715 (Fig. 3). The  $^{13}\text{C}$  olefinic carbon traces at  $\delta_{\text{C}}$  110–140 were displayed by the EtOAc extract of *B. amyloliquefaciens* MTCC 12716 and *S. algae* MTCC 12715, although lesser proton integrals were recorded in the  $^1\text{H}$  NMR spectrum of the former, which might be attributed to the occurrences of quaternary olefinic carbons in the organic extract of *B. amyloliquefaciens*. The  $^{13}\text{C}$  NMR signals due to the alkoxy ( $-\text{CH}_2\text{O}-$ ;  $\delta_{\text{C}}$  45–70) group and those associated with the carbons of the hydride group of alkanooates ( $-\text{CH}_2\text{C}(=\text{O})\text{OMe}$ ,  $\delta_{\text{C}}$  75–80) were found to be greater in the EtOAc extract of *S. algae* MTCC 12715 than that recorded in the extract prepared from *B. amyloliquefaciens* MTCC 12716.

A sharp FTIR band at  $3700\text{ cm}^{-1}$  region represented free O–H stretching in the solvent extracts of *B. amyloliquefaciens* MTCC 12716 and *S. algae* MTCC 12715 (Fig. S5–S6). The broad bands in the region of  $3200\text{--}3600\text{ cm}^{-1}$  might represent the hydrogen bonded O–H stretching vibration. Intense IR bands at  $2928$  and  $2963\text{ cm}^{-1}$  might be related with the occurrences of alkyl C–H stretching and a variable frequency at  $1667$  and  $1662\text{ cm}^{-1}$  indicated alkenyl C=C stretching and/or aromatic C=C bending. Variable peaks at  $1449$  and  $1457\text{ cm}^{-1}$  were attributed to C–H bending of alkane groups.

### Principal component analysis

The resemblances and differences between crude extracts of the two bacterial species *B. amyloliquefaciens* MTCC 12716 and *S. algae* MTCC 12715 *vis-a-vis* the relationships among antioxidant activity assays and different enzyme inhibitory assays were statistically analyzed via PCA (principle component analyses) (Fig. 4). PCA was performed to corroborate the antioxidant and enzyme inhibitory activities contributing to the pharmaceutical potential of the titled marine macroalgae-associated symbionts. The loadings of PC1 and PC2 (first and second principal components) were added for 50.63 and 49.37% of the variance, respectively (Fig. 4). PC1 was primarily inclined by DPPH scavenging activity of the organic extracts of *B. amyloliquefaciens* MTCC 12716 and *S. algae* MTCC 12715 (BD and SD), COX-1 and  $\alpha$ -glucosidase inhibitory activities (BC1, SC1 and BGL, SGL), along with hMGCR (BHMg and SHMG) and DPP-4 inhibitory activities of *B. amyloliquefaciens* MTCC 12716 (BDPP4), ABTS and LOX-5 (SA and SL) activities of *S. algae* MTCC 12715. Whereas, DPP-4 inhibitory activities of *S. algae* MTCC 12715 (SDPP4) and ABTS radical scavenging activity of *B. amyloliquefaciens* MTCC 12716 (BA) were mostly contributed to PC2.



**Fig. 4** Loading plot diagram (various components namely, PC-1 and PC-2 in rotated space) of antioxidant activities *vis-a-vis* anti-inflammatory, anti-diabetic, anti-hypertensive and anti-cholesterol activities of different organic extracts of *B. amyloliquefaciens* MTCC 12716 and *S. algae* MTCC 12715. The abbreviations were explained in the text

### Discussions

Marine macroalgae were reported to possess chemical defense programmes against pathogenic microorganisms in oceanic ecosystem, and the significant share of marine macroalgae-associated heterotrophic bacteria with potential bioactive properties form a valuable source of pharmaceutical applications. Presently, we have isolated 148 heterotrophic bacterial species affiliated with 7 different species of marine macroalgae belonging to the classes *Chlorophyceae*, *Rhodophyceae*, and *Phaeophyceae* including brown algae (*Sargassum myricocystum*, *Padina gymnospora*, *Turbinaria ornata*), red algae (*Hypnea valentiae*, *Kappaphycus alvarezii*) and green algae (*Ulva reticulata*, *Caulerpa racemosa*). Major share (83%) of the bioactive isolates was the representatives of  $\gamma$ -*Proteobacteria* and *Firmicutes*. The bacteria selected for the current study were found to possess potential antibacterial properties against clinically important pathogens (MIC 6.25–25  $\mu\text{g}/\text{mL}$ ) *E. coli*, *S. pyogenes*, *E. tarda*, *V. parahemolyticus*, MRSA, and VRE. The strains were also examined for their pathogenic effects on blood agar plates and found to be non-pathogenic. It is widely accepted that the pathogenicity of disease-causing microorganisms could be evaluated by the production of certain extracellular products, such as hemolysins on blood agar plates (Gao et al. 2000).

Previous studies illustrated that the bacterial species in symbiotic association with eukaryotic host organisms appeared to display significantly greater bioactivities than the free-living microorganisms in the ocean and oceanic sediments (Zeng et al. 2005). The sponge-bacterial symbiotic

association has been considered as an important model system, and there have been several studies describing marine host associated heterotrophs and their potential bioactive properties (Quevrain et al. 2014).

It was reported that, among the phylum *Firmicutes*, *Bacillus* sp. were dominantly present as heterotrophs with various marine macroalgae (Lachnit et al. 2011), and were reported for potential antibacterial and antifungal activities (Penesyan et al. 2010; Chakraborty et al. 2014). Two of the most active cultures *B. amyloliquefaciens* MTCC 12716 and *S. algae* MTCC 12715 exhibited a broad inhibition spectrum, being active against the pathogens including multidrug-resistant pathogens, such as MRSA and VRE (MIC 6.25–25 µg/mL). Notably, *B. amyloliquefaciens* MTCC 12716 displayed significantly greater antibacterial activity than those exhibited by *S. algae* MTCC 12715. Vancomycin-resistant *Enterococci* (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) were reported to cause nosocomial infections, and have been connected with increased rates of illness and death (Amalaradjou and Venkitanarayanan 2014). In recent times, MRSA is a serious clinical problem attributed to the multidrug-resistance against variously available antibiotics. Although habitually opportunistic, many *S. aureus* strains are now recognized to be destructively pathogenic. It was assessed that *Staphylococci* permanently inhabit about 25% of adults permanently, and 75–80% transiently (Archer et al. 2011). The emergence of antibiotic-resistant bacteria led us to explore the new marine habitats for screening the production of novel anti-infective elements (Gram et al. 2010). It was found that the bacteria associated with brown alga *Sargassum* sp. had the antibacterial characteristic with inhibition zone ranging 3.6–4.9 mm against MRSA (Susilowati et al. 2015). Li et al. (2005) described the antibacterial activities of the solvent extract of *Aspergillus* sp., which was associated with red marine macroalga *Hypnea saiidana*, against MRSA. Heterotrophic *Pseudomonas* sp. isolated from *Ceratoduction spongiosum* (red marine macroalga) (Isnansetyo et al. 2001) was reported to inhibit the growth of MRSA. Antibacterial activity of *Bacillus licheniformis* in association with intertidal macroalgae *Fucus serratus* against MRSA and VRE were reported previously by Jamal et al. (2006). Thus, various studies on macroalgae-associated bacteria proved their antimicrobial potential against different pathogenic organisms (Penesyan et al. 2009; Chakraborty et al. 2014).

Marine microorganisms were poorly investigated for the development of drug candidates against common lifestyle disease targets, but might be of significant potential in the quest for new anti-diabetic, anti-inflammatory or anti-cholesterol drugs (Debbab et al. 2010). The marine macroalgae-associated heterotrophic bacteria *B. amyloliquefaciens* and *S. algae* exhibited significant antioxidative properties (Table 3). As compared to DPPH assay for determining antioxidant activity, ABTS assay was found to be

more prognostic for marine bacterial extracts (Al-Zereini 2014). The greater ABTS radical quenching activities of the organic extracts from these bacteria were observed than those recorded in DPPH scavenging properties, could be attributed to the statement that ABTS measures antioxidant ability of hydrophilic and hydrophobic fractions, whereas DPPH calculates antioxidant activity only for the hydrophobic antioxidants. Antioxidants were found to reduce or terminate the oxidation process of the reductants (oxidizable components), through free radical quenching and decreasing oxidative stress (disproportionate generation of reactive oxygen species and reactive nitrogen species) (Scott 2004). The resultant oxidation of various biomolecules might potentially lead to the development of various oxidative stress-induced disorders, such as hypertension, type-2 diabetes and arteriosclerotic vascular disease (Scott 2004). There has been increasing evidence that free radical-induced damage could play an important role in the onset of type-2 diabetes and impaired glucose tolerance (Wright et al. 2006) along with vascular injury and organ dysfunction due to hypertension (Elahi and Matata 2006).

*B. amyloliquefaciens* MTCC 12716 and *S. algae* MTCC 12715 showing potential antibacterial activities against clinically important pathogens were selected to evaluate their pharmacological properties associated with hypertension, inflammation, diabetes and hypercholesterolemia. Notably, Angiotensin Converting Enzyme (ACE) inhibitors prevent the vasoconstricting octapeptide angiotensin II conversion leading to the relaxation of blood vessels resulting in a lower blood pressure (Dostal and Baker 1999). ACE inhibitors can be the potential pharmacophores for the management of hypertensive disorders. However, the adverse reactions of synthetic drugs (Atkinson and Robertson 1979) have contributed to a greater interest in searching for natural ACE inhibitors. The organic extract of *B. amyloliquefaciens* MTCC 12716 displayed significantly greater ACE inhibitory activity (IC<sub>90</sub> 448 µg/mL) than that exhibited by *S. algae* (IC<sub>90</sub> 663 µg/mL). There were reports that the microbial peptides and secondary metabolites of actinomycetes (Manthey and Reuter 1989) were potent ACE inhibitors.

The  $\alpha$ -amylase and  $\alpha$ -glucosidase were reported to involve in the catabolism of ingested sugar moieties and their inhibition slows down the intake of glucose by acting as a promising approach in the treatment of type-2 diabetes (Tabatabaei-Malazy et al. 2015). The organic extract of *B. amyloliquefaciens* MTCC 12716 displayed significantly greater  $\alpha$ -glucosidase inhibitory activity (IC<sub>90</sub> 84 µg/mL) than that exhibited by *S. algae* (IC<sub>90</sub> > 600 µg/mL) and acarbose (IC<sub>90</sub> 645 µg/mL), the commonly prescribed drug for type-2 diabetes. Pandey et al. (2013) found that the marine sponge *Aka coralliphaga* associated heterotrophic *Proteobacteria* and *Firmicutes*, developed various glucosidase inhibitors.

The cyclooxygenase isoforms (constitutive COX-1 and inducible COX-2) and lipoxygenase (5-LOX) enzymes were reported to catalyze the synthesis of inflammatory lipid mediators, such as prostaglandins and leukotrienes (Charlier and Michaux 2003). Dual 5-LOX/COX-2 inhibitors are prospective anti-inflammatory candidates as they function by impeding the development of inflammatory prostaglandins and leukotrienes. Such combined inhibition of 5-LOX/COX-2 avoids the major drawbacks associated with COX-1 inhibitors and protects the gastrointestinal mucosa (Charlier and Michaux 2003). The organic extract of *B. amyloliquefaciens* MTCC 12716 displayed significantly greater COX-2 and 5-LOX inhibitory activities ( $IC_{90}$  29 and 6.06  $\mu\text{g/mL}$ , respectively) than those exhibited by *S. algae* ( $IC_{90}$  44 and 7.73  $\mu\text{g/mL}$ , respectively). Significant anti-inflammatory activities of marine symbiotic bacterial crude extracts were reported earlier by Kurian et al. (2015).

Hyperlipidemia (elevated cholesterol or triglyceride) has been recognized as one of the most important causes of atherosclerosis-related disorders, such as coronary heart disease and peripheral vascular disease. HMG-CoA reductase (hMGCR) was found to be the pivotal enzyme in the rate determining step of sterol biosynthesis and the statin-like drugs are inhibitors of human hMGCR (Maciejak et al. 2013). The organic extract of *B. amyloliquefaciens* MTCC 12716 displayed significantly greater hMGCR inhibitory activities ( $IC_{90}$  17  $\mu\text{g/mL}$ , respectively) than that exhibited by *S. algae* ( $IC_{90}$  75  $\mu\text{g/mL}$ ).

The greater proton integral due to saturated hydrocarbons (at  $\delta_{\text{H}}$  0.1–2.0) in  $^1\text{H}$  NMR spectrum of the organic extract of *S. algae* MTCC 12715 were attributed to associate with fatty acyl esters or fatty acid derivatives, phospholipids, sterols, or glycolipids. The presence of fatty acyl analogs could be corroborated by the higher proton integrals of the *S. algae* MTCC 12715 extract ( $\sum\text{H} \sim 24$ ) at  $\delta_{\text{H}}$  2–3.5 (attributed to the presence of acetyl and  $-\text{OCH}_3$  groups). Likewise, the higher proton integrals attributed to the acetyl or allylic functionalities ( $\sum\text{H} > 60$ ) in the extract prepared from *S. algae* MTCC 12715, and comparatively weaker proton integrals at this position for *B. amyloliquefaciens* MTCC 12716 extract ( $\sum\text{H} < 50$ ) appropriately supported these assignments. The greater proton integrals of the *S. algae* MTCC 12715 organic extract, at the characteristic olefinic region ( $\delta_{\text{H}}$  4.5–6) could thus be correlated with the occurrences of fatty acid derivatives with mono- and/or poly-unsaturations. Since the fatty acid derivatives were considered as the structural components of the organisms and did not attribute to the bioactive potential, the greater pharmacological properties of the EtOAc extract of *B. amyloliquefaciens* MTCC 12716 could thus be rationalized. Bioactive polysaccharides were found to occupy a predominant role in determining the pharmacological properties of the microorganisms (Rehm 2010), and

greater proton integrals ( $\sum\text{H} \sim 65$ ) at the anomeric region of the  $^1\text{H}$  NMR spectrum ( $\delta_{\text{H}}$  3.6–4.5) of the EtOAc extract of *B. amyloliquefaciens* MTCC 12716, than that obtained from *S. algae* MTCC 12715 extract ( $\sum\text{H} < 50$ ) attributed to the possible functional role of polysaccharide analogs in determining the bioactive properties of the former. The greater  $^1\text{H}$  NMR proton integral ( $\sum\text{H} \sim 29$ ) due to the aryl signals ( $\delta_{\text{H}}$  6.6–8.6), in the solvent extracts of *B. amyloliquefaciens* MTCC 12716 might be associated with the presence of aromatic centers, which were found to possess a significant role in determining the pharmacological properties of the said bacterium.

In this study, the organic extract of *B. amyloliquefaciens* MTCC 12716 was found to possess greater number of electronegative carbonyl and aryl carbons in the downfield space of the  $^{13}\text{C}$  NMR spectrum ( $\delta_{\text{C}}$  180–200 and 140–160, respectively) than that prepared from *S. algae* MTCC 12715, which apparently signified the presence of electron-rich centered bioactive principles of the former. The EtOAc extract of *S. algae* and *B. amyloliquefaciens* exhibited  $^{13}\text{C}$  NMR olefinic functionalities at the characteristic region of  $\delta_{\text{C}}$  110–140, although lower proton integrals in the  $^1\text{H}$  NMR spectrum of the latter assigned to the occurrences of quaternary olefinic carbons. The signal intensity and the number of  $^{13}\text{C}$  carbon atoms due to the alkoxy ( $\delta_{\text{C}}$  45–70) carbon and those associated with alkyl alkanoate groups ( $\delta_{\text{C}}$  75–80) were lesser in the organic extract of *B. amyloliquefaciens* MTCC 12716 than that from *S. algae* MTCC 12715. These results appropriately corroborated the presence of fatty acyl analogs (along with phospholipids), which were reported as the structural components of the bacterium, and possessed meager functional role towards the bioactivities and pharmacological properties, due to the *S. algae* MTCC 12715 extract.

Marine *Bacillus* species was reported to produce promising bioactive metabolites, such as aromatic polyketides (with multiple carbonyl groups bearing aromatic functionalities), bacteriocins, and other unique compounds of potential clinical significance (Armstrong et al. 2001). In this study, PCA were performed on the basis of the covariance structure of the data, which convert the means across the originally measured numerical information into new variables, the principal components (PCs), which were orthogonal (Maisuthisakul et al. 2008). PCA appropriately corroborated the antioxidant *vis-a-vis* enzyme inhibitory activities contributing to the various pharmaceutical potential of the studied marine macroalgae-associated symbionts, and revealed the loadings of PC1 and PC2 for the studied bioactivities. The organic extract of *B. amyloliquefaciens* displayed significantly greater antioxidative properties ( $IC_{90} < 1$   $\text{mg/mL}$ ) and the activities showed considerable positive correlation ( $r^2 > 0.8$ ,  $P < 0.05$ ) with the inhibitory activities against ACE-1,



pro-inflammatory biocatalysts, DPP-4 and hMGCR, which were associated with hypertension, inflammation, diabetes, and hypercholesterolemia, respectively. Specifically, the loadings of PC1 were also inclined towards the positive correlation between the bioactivities, which implied that the functional groups present in the extract prepared from *B. amyloliquefaciens* MTCC 12716 were endowed with the potential pharmacological properties with regard to antioxidant (DPPH scavenging), anti-inflammatory (anti-cyclooxygenase), anti-diabetic (DPP-4 inhibitory), and anti-hypercholesterolemic (hMGCR inhibitory) activities. These results appropriately revealed the promising therapeutic potential of marine macroalga-associated heterotrophic bacterium *B. amyloliquefaciens* MTCC 12716.

The present work revealed pharmaceutical potentials of the organic extracts derived from the heterotrophic bacteria in association with marine macroalgae. The heterotrophs predominantly belonging to  $\gamma$ -*Proteobacteria* and *Firmicutes* comprised greater than 50% of culturable bacteria, and were found to possess potential antibacterial properties (MIC 6.25–25  $\mu\text{g}/\text{mL}$ ) against the clinically important pathogens including MRSA and VRE. Heterotrophic *B. amyloliquefaciens* MTCC 12716 and *S. algae* MTCC 12715 displayed potential anti-infective properties and their ethyl acetate extracts were assayed for their pharmacological properties using various in vitro models. The organic extract of *B. amyloliquefaciens* MTCC 12716 displayed significantly greater ACE-1, pro-inflammatory COX-2/5-LOX,  $\alpha$  glucosidase/DPP-4 and hMGCR-inhibitory activities associated with hypertension, inflammation, diabetes, and hypercholesterolemia, respectively.  $^1\text{H}$  NMR fingerprint analyses of the prominent functional groups in the organic extracts of the symbionts revealed the presence of greater proton integral of the same derived from *B. amyloliquefaciens* MTCC 12716 at the downfield region of  $\delta_{\text{H}}$  2.0–3.5 and 4.5–8.5, which could be responsible for the potential bioactivities. The heterotrophic marine macroalga-associated *B. amyloliquefaciens* MTCC 12716 might, therefore, serve as a potential therapeutic candidate to develop microbial products with wide pharmaceutical and biotechnological applications.

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## Compliance with ethical standards

**Research involving human or animal participants** This article does not contain any studies with human participants or animals performed by any of the authors.

**Conflict of interest** No potential conflict of interest was reported by the authors.

## References

- Ademiluyi AO, Oboh G (2013) Soybean phenolic-rich extracts inhibit key-enzymes linked to type-2 diabetes ( $\alpha$ -amylase and  $\alpha$ -glucosidase) and hypertension (angiotensin-I converting enzyme) *in-vitro*. *Exp Toxicol Pathol* 65:305–309
- Ali AIB, Bour ME, Ktari L, Bolhuis H, Ahmed M, Boudabbous A, Stal LJ (2012) *Jania rubens*-associated bacteria: molecular identification and antimicrobial activity. *J Appl Phycol* 24:525–534
- Al-Zereini WA (2014) Bioactive crude extracts from four bacterial isolates of marine sediments from Red Sea, Gulf of Aqaba, Jordan. *Jordan J Biol Sci* 7:133–137
- Amalaradjou MAR, Venkitanarayanan K (2014) Antibiofilm effect of octenidine hydrochloride on *Staphylococcus aureus*, MRSA and VRSA. *Pathogens* 3:404–416
- Archer NK, Mazaitis MJ, Costerton JW (2011) *Staphylococcus aureus* biofilms: properties, regulation, and roles in human disease. *Virulence* 2:445–459
- Armstrong E, Yan L, Boyd KG, Wright CP, Burgess JG (2001) The symbiotic role of marine microbes on living surfaces. *Hydrobiol* 461:37–40
- Atkinson AB, Robertson JI (1979) Captopril in the treatment of clinical hypertension and cardiac failure. *Lancet* 2:836–839
- Balouiri M, Sadiki M, Ibsouda SK (2016) Methods for *in vitro* evaluating antimicrobial activity: a review. *J Pharm Anal* 6:71–79
- Baylac S, Racine P (2003) Inhibition of 5-lipoxygenase by essential oils and other natural fragrant extracts. *Int J Aromather* 13:138–142
- Brand-Williams W, Cuvelier M, Berset C (1995) Use of free radical method to evaluate antioxidant activity. *LWT - Food Sci Technol* 28:25–30
- Chakraborty K, Thilakan B, Raola VK (2014) Polyketide family of novel antibacterial 7-*O*-methyl-5'-hydroxy-3'-heptenoate macro-lactin from seaweed-associated *Bacillus subtilis* MTCC 10403. *J Agric Food Chem* 62:12194–12208
- Chakraborty K, Thilakan B, Raola VK (2017a) Antimicrobial polyketide furanoterpenoids from seaweed-associated heterotrophic bacterium *Bacillus subtilis* MTCC 10403. *Phytochemistry* 142:112–125
- Chakraborty K, Thilakan B, Chakraborty RD, Raola VK, Joy M (2017b) *O*-heterocyclic derivatives with antibacterial properties from marine bacterium *Bacillus subtilis* associated with seaweed, *Sargassum myriocystum*. *Appl Microbiol Biotechnol* 101:569–583
- Charlier C, Michaux C (2003) Dual inhibition of cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) as a new strategy to provide safer non-steroidal anti-inflammatory drugs. *Eur J Med Chem* 38:645–659
- Davidson BS (1995) New dimensions in natural products research: cultured marine microorganisms. *Curr Opin Biotechnol* 6:284–291
- Debbab A, Aly AH, Lin WH, Proksch P (2010) Bioactive compounds from marine bacteria and fungi. *Microb Biotechnol* 5:544–563
- Dostal DE, Baker KM (1999) The cardiac renin-angiotensin system: conceptual, or a regulator of cardiac function? *Circ Res* 85:643–650

- Elahi MM, Matata BM (2006) Free radicals in blood: evolving concepts in the mechanism of ischemic heart disease. *Arch Biochem Biophys* 450:78–88
- Gao DQ, Huang XQ, Lu CP, Wu SY (2000) Characteristic of the hemolysin of *Edwardsiella tarda*. *Chin J Zoon* 16:53–55
- Gram L, Melchiorson J, Bruhn JB (2010) Antibacterial activity of marine culturable bacteria collected from a global sampling of ocean surface waters and surface swabs of marine organisms. *Mar Biotechnol* 12:439–451
- Hollants J, Leliaert F, Clerck OD, Willems A (2012) What we can learn from sushi: a review on seaweed-bacterial associations. *FEMS Microbiol Ecol* 83:1–16
- Holmquist B, Bunning P, Riordan JF (1979) A continuous spectrophotometric assay for angiotensin converting enzyme. *Anal Biochem* 95:540–548
- Isnansetyo A, Horikawa M, Kamei Y (2001) *In vitro* anti-methicillin-resistant *Staphylococcus aureus* activity of 2,4-diacetylphloroglucinol produced by *Pseudomonas* sp. AMSN isolated from a marine alga. *J Antimicrob Chemother* 47:724–725
- Jamal MT, Morris PC, Hansen R, Jamieson DJ, Burgess GJ, Austin B (2006) Recovery and characterization of a 30.7-kDa protein from *Bacillus licheniformis* associated with inhibitory activity against methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococci*, and *Listeria monocytogenes*. *Mar Biotechnol* 8:587–592
- Kennedy J, Baker P, Piper C, Cotter PD, Walsh M, Mooij MJ, Bourke MB, Rea MC, O'Connor PM, Ross RP, Hill C, O'Gara F, Marchesi JR, Dobson AD (2009) Isolation and analysis of bacteria with antimicrobial activities from the marine sponge *Haliclona simulans* collected from Irish waters. *Mar Biotechnol* 11:384–396
- Krieg NR, Holt JG (1984) Bergey's manual of systematic bacteriology. Williams and Wilkins Co., Baltimore
- Lachnit T, Meske D, Wahl M, Harder T, Schmitz R (2011) Epibacterial community patterns on marine macroalgae are host-specific but temporally variable. *Environ Microbiol* 13:655–665
- Larsen LN, Dahl E, Bremer J (1996) Peroxidative oxidation of leucodichlorofluorescein by prostaglandin H synthase in prostaglandin biosynthesis from polyunsaturated fatty acids. *Biochim Biophys Acta* 1299:47–53
- Li JW, Vederas JC (2009) Drug discovery and natural products: end of an era or an endless frontier? *Science* 325:161–165
- Li G-Y, Li B-G, Yang T, Yin J-H, Qi H-Y, Liu G-Y, Zhang G-L (2005) Sesterterpenoids, terretonins A-D, and an alkaloid, asterrelenin, from *Aspergillus terreus*. *J Nat Prod* 68:1243–1246
- Maciejak A, Leszczynska A, Warchol I, Gora M, Kaminska J, Plochocka D, Kapcinska MW, Tulacz D, Siedlecka J, Swiezewska E, Sojka M, Danikiewicz W, Odolczyk N, Szkopinska A, Sygitowicz G, Burzynska B (2013) The effects of statins on the mevalonic acid pathway in recombinant yeast strains expressing human HMG-CoA reductase. *BMC Biotechnol* 13:68
- Maisuthisakul P, Pasuk S, Ritthiruangdej P (2008) Relationship between antioxidant properties and chemical composition of some Thai plants. *J Food Compos Anal* 21:229–240
- Tabatabaei-Malazy O, Larijani B, Abdollahi M (2015) Targeting metabolic disorders by natural products. *J Diabetes Metab Disord* 14:57
- Manthey A, Reuter G (1989) Microbial synthesis of metabolites with antihypertensive activity: aspects of fermentation derived inhibitors of angiotensin-converting enzyme ACE. *J Basic Microbiol* 29:623–639
- Molinski TF, Dalisay DS, Lievens SL, Saludes JP (2009) Drug development from marine natural products. *Nat Rev Drug Discov* 8:69–85
- National Research Council (1999) From monsoons to microbes: understanding the ocean's role in human health. The National Academies Press, Washington, DC. <https://doi.org/10.17226/6368>
- Kurian NK, Nair HP, Bhat HG (2015) Evaluation of anti-inflammatory property of melanin from marine *Bacillus* spp. BTCZ31. *Asian J Pharm Clin Res* 8:251–255
- Okamura M, Shimazaki K, Nakata T, Chida N, Miyatake T, Maemoku H, Tsutsumi H, Nakamura T, Yamaguchi C, Ogawa M (1992) Submarine active faults in the northwestern part of Beppu Bay, Japan—On a new technique for submarine active fault survey. *Mem Geol Soc Jpn* 40:65–74
- Pandey S, Sree A, Dash SS, Sethi DP, Chowdhury L (2013) Diversity of marine bacteria producing beta-glucosidase inhibitors. *Microb Cell Fact* 12:35
- Paul VJ, Ritson-Williams R, Sharp K (2011) Marine chemical ecology in benthic environments. *Nat Prod Rep* 28:345–388
- Penesyan A, Marshall-Jones Z, Holmstrom C, Kjelleberg S, Egan S (2009) Antimicrobial activity. *FEMS Microbiol Ecol* 69:113–124
- Penesyan A, Kjelleberg S, Egan S (2010) Development of novel drugs from marine surface associated microorganisms. *Mar Drugs* 8:438–459
- Quevrain E, Roue M, Bourguet-Kondracki IM (2014) Assessing the potential bacterial origin of the chemical diversity in calcareous sponges. *J Mar Sci Tech* 22:36–49
- Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice Evans C (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic Biol Med* 26:1231–1237
- Rehm BHA (2010) Bacterial polymers: biosynthesis, modifications and applications. *Nat Rev Microbiol* 8:578–592
- Scott J (2004) Pathophysiology and biochemistry of cardiovascular disease. *Curr Opin Genet Dev* 14:271–279
- Susilowati R, Sabdono A, Widowati I (2015) Isolation and characterization of bacteria associated with brown algae *Sargassum* spp. from Panjang Island and their antibacterial activities. *Procedia Environ Sci* 23:240–246
- Tabbene O, Gharbi D, Slimene IB, Elkahoui S, Alfeddy MN, Cosette P, Mangoni ML, Jouenne T, Limam F (2012) Antioxidative and DNA protective effects of bacillomycin D-like lipopeptides produced by b-38 strain. *Appl Biochem Biotechnol* 168:2245–2256
- Tang JS, Zhao F, Gao H, Dai Y, Yao ZH, Hong K, Li J, Ye WC, Yao XS (2010) Characterization and online detection of surfactin isomers based on HPLC-MS(n) analyses and their inhibitory effects on the overproduction of nitric oxide and the release of TNF- $\alpha$  and IL-6 in LPS induced macrophages. *Mar Drugs* 8:2605–2618
- Thilakan B, Chakraborty K, Chakraborty RD (2016) Antimicrobial properties of cultivable bacteria associated with seaweeds in Gulf of Mannar of South East Coast of India. *Can J Microbiol* 62:668–681
- Turick CE, Caccavo F Jr, Tisa LS (2008) Pyomelanin is produced by *Shewanella algae* BrY and affected by exogenous iron. *Can J Microbiol* 54:334–339
- Webster NS, Taylor MW (2012) Marine sponges and their microbial symbionts: love and other relationships. *Environ Microbiol* 14:335–346
- Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* 173:697–703
- Wiese J, Thiel V, Nagel K, Staufenberg T, Imhoff JF (2009) Diversity of antibiotic-active bacteria associated with the brown alga *Laminaria saccharina* from the Baltic Sea. *Mar Biotechnol* 11:287–300
- Wright E Jr, Scism-Bacon JL, Glass LC (2006) Oxidative stress in type 2 diabetes: the role of fasting and postprandial glycemia. *Int J Clin Pract* 60:308–314
- Zeng L, Han X, Chen HM, Lin W, Yan XJ (2005) Marine bacteria associated with marine macro organisms: the potential antimicrobial resources. *Ann Microbiol* 55:119–124
- Zhu K, Ding X, Julotok M, Wilkinson BJ (2005) Exogenous isoleucine and fatty acid shortening ensure the high content of anteiso-C15:0 fatty acid required for low-temperature growth of *Listeria monocytogenes*. *Appl Environ Microbiol* 71:8002–8007