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# Probiotic bacteria promote the growth of associating host(red seaweed, *Gracilaria edulis*) also synthesize antibacterial protein

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ABSTRACT

Those plant associated bacteria supporting the growth, reproduction and yield by synthesizing phytohormones, antibiotics and lytic enzymes are called plant probiotics. In this study, isolates of Gram positive *Lysinibacillus xylanilyticus* associated with red seaweed species were evaluated for antibacterial activity against plant pathogenic bacterium *X. oryzae* pv. *oryzae* which cause bacterial blight in rice using disc and well diffusion assays. The isolate HVT234 among the isolates of *L. xylanilyticus* associated with red seaweed species exhibit high antibacterial activity. The optimum level of extracellular antibacterial protein synthesize in HVT234 was recorded when cultured in 1.5% inoculum in seawater medium with lactose carbon and ammonium chloride nitrogen at pH 7.0 at 35 °C for 48 h. The antibacterial substance extracted in ethyl acetate from the culture supernatant of *L. xylanilyticus* HVT234 was isolated and separated as 66 kDs protein. The *L. xylanilyticus* synthesizing antibacterial substance elicited by some non-active co-associating bacteria promote the host *Gracilaria edulis* growth is considered as probiotic because another associated *Bacillus cereus* produce antibacterial protein did not support the host growth. This chemical elicitor protein most probably a quorum signal *N*-acyl homoserine lactone of probiotic *L. xylanilyticus* which support the host *G. edulis* growth reported for the first time from this study has immense value in the seaweed mariculture because this species is one of the major biomass feedstocks for agar production.

## 1. Introduction

Marine macroalgae(seaweeds) are commercially important biomass feedstock occurring along the tidal, intertidal and subtidal regions of the coastal waters exploited for industrially valuable compounds such as agar, carrageenan, alginate, fucoidan, pigments etc., that are being used for preparing several commodities like human food, medicine, fertilizers and fuel as well (Teas, 2007). The marine environment has a huge diversity of life forms and the water column of the oceans contains approximately $10^6$ bacterial cells per millilitre(Hagström et al., 2002). These marine bacteria are recently being evaluated for the source of biologically active compounds (Debbab et al., 2010) because of growing demand for novel compounds of natural origin that have potential applications in pharmaceutical and other allied industries (Singh et al., 2014). As bacteria living in association with seaweeds as epibionts or endobionts which experience highly competitive environment for space and access to host nutrients (Lemos et al., 1986; Suvega and Arunkumar, 2014), they synthesize wide range of enzymes/compounds in order to absorb the nutrient from the host seaweeds. This marine algae contains unique polysaccharides and other substances that are not at all present in other marine as well as terrestrial plants and animals (Popper et al., 2011). Hence these seaweed associated bacteria can be considered as a potential source for specific enzymes and active compounds (Zheng et al., 2005; Suvega and Arunkumar, 2014; Satheesh et al., 2016).

Studies show the seaweed associated bacteria as epibionts and endobionts displaying various biological activities like antifouling, antimicrobial and cytotoxicity mainly belong to *Alphaproteobacteria, Gammaproteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Planctomycetes, Pseudomonas, Stenotrophomonas, Vibrio, Alteromonas, Shewanella, Streptomyces,* and *Bacillus* are isolated from the members of *Rhodophyceae*(red), *Chlorophyceae*(green) and *Phaeophyceae*(brown) (Armstrong et al., 2000; Egan et al., 2001; Dobretsov and Qian, 2002; Harder et al., 2004; Rajasree et al., 2012; Janaki Devi et al., 2013; Hong and Cho, 2013; Singh et al., 2014; Suvega and Arunkumar, 2014). This seaweed-bacteria association has been ascertained as beneficial, harmful or neutral and obligate to facultative (Goecke et al., 2010). And the chemical compounds exerted by the associating bacteria reported as promoting the host seaweed growth through development, morphogenesis and reproduction (Singh et al., 2011a, 2014) have been

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identified as hormones, growth regulators, quorum sensing signal molecules etc., (Burke et al., 2011; Singh et al., 2011b, 2014; Lachnit et al., 2011). Further there are substances produced by the seaweed associated bacteria involve nitrogen fixation (Singh et al., 2011b) and spores liberation (Joint et al., 2007). Such associated bacteria beneficial to the host recently ascertained as plant probiotics (Rahman et al., 2018) are belong to *Bacillus, Paraburkholderia, Pseudomonas, Acinetobacter, Alcaligenes, Arthrobacter* and *Serratia* (Bashan and Holguin, 2008; Sawana et al., 2014; Borriss et al., 2017).

One of the reasons in the loss of agriculture production has recently been identified as by pathogens developing resistance against chemical pesticides because of their indiscriminate applications. The Gram-negative bacterium *Xanthomonas oryzae* pv. *oryzae* (Ishiyama) Dye causes blight in rice results up to 50% yield loss (González et al., 2012). The commercial agents of natural as well as synthetic origin available in the market to control this disease are either ineffective or hazardous to environment (Zhu et al., 2013). There are studies which screened the seaweed associated bacteria for antimicrobial activity against pathogens cause diseases in animal and human (Vijayalakshmi et al., 2008; Debbab et al., 2010; Singh et al., 2014). In our previous study, red seaweeds associated bacteria isolates GT132/JQ677989, GT134/JQ677988, GT154/JQ677990 of *Gracilaria edulis*; GSTP512/JQ677987 of *Grateloupia filicina* and HVT234/JQ739716 of *Hypea valentiae* identified as *Lysinibacillus xylanilyticus* exhibiting antimicrobial activities against plant pathogens are reported (Suvega and Arunkumar, 2014). In this article, probiotic isolates of *Lysinibacillus xylanilyticus* synthesize extracellular antibacterial protein against the plant pathogenic bacterium *Xanthomonas orzae* pv. *oryzae* also promote the host seaweed *Gracilaria edulis* growth is presented.

## 2. Materials and methods

### 2.1. Isolation and characterization of antibacterial protein from the red seaweed associated bacteria isolates

#### 2.1.1. Screening L. xylanilyticus isolates for antibacterial activity

The *L. xylanilyticus* isolates namely GT132/JQ677989, GT134/JQ677988, GT154/JQ677990, GSTP512/JQ677987 and HVT234/JQ739716 isolated from red seaweeds by our previous study available in the Department of Botany laboratory (Suvega and Arunkumar, 2014) were grown in 5 mL Zobell marine broth (Hi Media, India) in the test tube for 4 days and centrifuged at 15,000 × *g* for 10 min at 25 °C. The supernatant was filter-sterilized using 0.22 μ filter. For the disc diffusion assay, 50 μL of culture supernatant aseptically saturated with sterile 6.0 mm diameter Whatman No. 1 filter paper was impregnated onto Petri plates containing 15 mL of 1.5% peptone sucrose agar (PSA) medium (g $L^{-1}$; peptone 10, sucrose 10, sodium glutamate 1.0, agar 20.0) at pH 7.2. It was swapped with 50 μL of 24 h old culture of test pathogen *Xanthomonas oryzae* pv. *oryzae* and incubated at 25 °C. The well diffusion assay was performed by transferring 50 μL of culture supernatant into 6 mm diameter well made at the centre of the Patri plate contain 1.5% PSA medium. Antibacterial activity was measured as mm diameter zone of inhibition in and around the disc after 48 h of incubation.

#### 2.1.2. Antibacterial substance extraction from the L. xylanilyticus isolate HVT234/JQ739716

On screening, extracellular substance synthesized by the HVT234 among the isolates of *L. xylanilyticus* showing maximum antibacterial activity was chosen for this study. Antibacterial activity of crude substance (25, 50, 75 and 100 μg) extracted in ethyl acetate from the culture supernatant of HVT234 (centrifuged at 15,000 × *g* for 10 min at 25 °C) grown in 100 mL conical flasks contain 25 mL of seawater medium (SWM) for 4 days (Romanenko et al., 2008) was evaluated by disc and well assay as mentioned in the 2.1.1 section. The crude substance was extracted in ethyl acetate and culture supernatant in 1: 5 ratio (v/v) using separating funnel by vigorous shaking for 10 min at least thrice. The upper ethyl acetate layer was concentrated using Rotary evaporator under reduced pressure at 40 °C.

#### 2.1.3. Optimization of antibacterial substance synthesis

The isolate HVT234 was grown in SWM broth at different inoculum concentration (0.5, 1, 1.5 and 2%), incubation period (24, 48 and 72 h), pH (5, 6, 7, 8 and 9) and temperature (30, 35, 40 and 45 °C). For optimizing nutrient source, the isolate was grown separately in SWM broth with uniform amount of glycerol, lactose, mannitol and sucrose for carbon source and for nitrogen $NH_4Cl$, $(NH_4)_2SO_4$, $KNO_3$ and urea at pH 7.0 at 35 °C for 48 h. The crude substance was extracted as described in the section 2. 1.2 and antibacterial activity was conducted as described in the section 2. 1.1.

### 2.2. Isolation and partial characterization of antibacterial substance

The extracellular antibacterial crude protein was extracted as described in the section 2. 1.2 from the isolate HVT234 grown in 100 mL SWM broth containing lactose carbon and ammonium chloride nitrogen at pH 7.0 in 35 °C for 2 days. The crude protein was further freeze-dried and dialyzed against 0.1 M NaCl using dialysis (Spectra/Por 1 6–8 kDs MWCO). The dialysed crude protein was applied onto a DEAE Cellulose 52 column (1 × 9 cm) using 20 mM sodium phosphate buffer and eluted using 10 mL of 0.1–1 M NaCl in 20 mM sodium phosphate buffer at a flow rate of 3 $mL^{-1}$ minute. The active fractions (against *Xanthomonas oryzae* pv. *orzae*) were pooled, dialyzed against 0.1 M NaCl and freeze-dried. For identifying the antibacterial protein molecular weight, SDS 10% polyacrylamide gel electrophoresis was performed along with low range standard proteins (1610304,BIO-RAD) as described by Laemmli (1970) and was visualized by silver staining.

### 2.3. Gracilaria edulis associated bacteria growth under in vitro individually and consortia

Fresh healthy thallus weighing 1.0 g of *G. edulis* collected along the coast of Thondi (9.7438° N, 79.0185° E; Bay of Bengal, India) was used for isolating endobiotic bacteria (Suvega and Arunkumar, 2014). Twelve endobiotic bacteria isolates associated with red seaweed *G. edulis* collected from the Thondi coast screened against phytopathogen *X. oryzae* pv. *oryzae* found three active (GT 119,128 and 132) and others 9 non-active (GT 117,120, 125, 126, 129, 130, 134, 139 and 144) (Table 1) were grown in test tubes containing 5 mL Zobell marine broth inoculated individually (100 μL) or consortia (active 50 μL + non-active 50 μL) using exponential culture (1.0 OD at 660 nm) and incubated at 27 °C. Bacterial growth was measured by recording the OD of culture broth at 660 nm on day 1, 2 and 3 after inoculation. The antibacterial activity of culture broth and extract of cell pellet of individual and consortia was assayed by agar well and disc diffusion methods as described in the section 2. 1.1 against phytopathogen *X. oryzae* pv. *oryzae*. The individual culture broths were cross-streaked on Zobell marine agar for confirming the antagonistic effects between isolates of consortia by visual observation.

### 2.4. Effect of associated bacteria on the growth of host G. edulis under in vivo

The young and healthy 10 cm G. *edulis* thalli cut at the tip collectively weighing 250 g collected in plastic bag along with seawater from the Thondi coast were immediately brought to laboratory. Thalli weighing 1 g was used for isolating associated bacteria (Suvega and Arunkumar, 2014) and the remaining specimens were grown in the enriched natural seawater medium at 3.5% salinity (Guillard, 1973) using 1 L conical flask under 12:12 h Light/Dark regime at 25 °C at pH 8.5 under 29.67–40.34 μmol$m^{-2}$ $s^{-1}$ illumination. The medium was replenished at 5 days interval.

**Table 1**
Screening 12 bacteria isolates associated with the red seaweed *Gracilaria edulis* collected along the coast of Thondi, India for antibacterial activity against plant pathogenic bacterium *Xanthomonas oryzae* pv. *oryzae*.

| S.No | GT Isolates /consortia | Bacteria | Antibacterial activity (zone of inhibition mm diameter) | |
|---|---|---|---|---|
| | | | Culture supernatant | Cell Pellet |
| 1 | 117 | *Corynebacterium* sp | - | - |
| 2 | 119 | *Bacillus megaterium* | 17.0 ± 2.0 | 10.0 ± 1.0 |
| 3 | 120 | *Klebsiella oxytoca* | - | - |
| 4 | 125 | *Corynebacterium* sp | - | - |
| 5 | 126 | *Bacillus pasteurii* | - | - |
| 6 | 128 | *Bacillus cereus* GT 128,HM573311 | 21.0 ± 3.0 | 13.0 ± 2.0 |
| 7 | 129 | *Aeromonas* sp. | - | - |
| 8 | 130 | *Corynebacterium* sp | - | - |
| 9 | 132 | *Lysinibacillus xylanilyticus* GT132,JQ677989 | 25.0 ± 4.0 | 15.0 ± 2.0 |
| 10 | 134 | *Lysinibacillusxylanilyticus* GT134, JQ677988 | 14.0 ± 3.0 | 9.0 ± 1.0 |
| 11 | 139 | *Lactobacillus casei* | - | - |
| 12 | 144 | *Aeromonas hydrophila* | - | - |
| 13 | 119 + 117 | | 20.0 ± 3.0 | 12.0 ± 2.0 |
| 14 | 119 + 120 | | 17.0 ± 2.0 | 11.0 ± 1.0 |
| 15 | 128 + 125 | | 17.0 ± 3.0 | 12.0 ± 1.0 |
| 16 | 128 + 129 | | 14.0 ± 2.0 | 13.0 ± 1.0 |
| 17 | 128 + 139 | | 30.7 ± 5.4 | 14.0 ± 2.0 |
| 18 | 128 + 144 | | 24.4 ± 3.6 | 13.4 ± 2.7 |
| 19 | 132 + 120 | | 31.7 ± 2.4 | 16.0 ± 2.0 |
| 20 | 132 + 117 | | 26.0 ± 2.0 | 13.0 ± 2.0 |

S. No 13 to 20 consortium bacteria exhibit high growth in Zobel marine broth as shown Fig. 3 were taken up for evaluating antibacterial assay.

For axenization, acclimatized thalli weighing 100 g was soaked in filtered (0.45 μm Nitrocellulose) sterile seawater (SSW) for 5 h. Then the specimen was immersed in 100 mL of 1.0% Povidone-iodine for 2 min and again transferred to fresh SSW for 1 h. Subsequently the thalli were incubated in 100 mL of SSW containing antibiotics (g $l^{-1}$; Penicillin 0.5, Chloramphenicol 0.5, Cefotaxime 0.3 and Ofcoxacin 0.3) for 48 h. Thalli were re-soaked in 100 mL SSW for 3 h to remove the traces of chemicals if any. The axenicity was ensured by rubbing the thalli on the Zobell marine agar (HiMedia) plates which did not show any bacteria growth. The viability of the thalli was checked by staining with 0.25% Evans blue dye which coloured the dead tissue. The axenized live thalli measuring 5 cm length from the tips were taken up for further experiment.

Experiment was conducted using 5 cm axenized thalli by growing in 50 mL enriched SSW medium using 250 mL Erlenmeyer conical flask under laboratory condition as mentioned above. The isolated bacteria were re-introduced in to the axinized *G. edulis* thalli by soaking in 10 mL of sterilized seawater with equal volume of exponential bacterial broth of 1.0 OD at 660 nm for 20 min. The experiments were conducted as mentioned in the Table 2 to assess the effect of associating bacteria on the growth of host *G. edulis* by recording relative growth rate(RGR) at 5 day interval for 45 days. The RGR was estimated using the formula $[(W_t - W_o)/t] \times 100$, where $W_o$ and $W_t$ are initial and final fresh weights of the alga, respectively; t is the time of culture in days. The excess water was removed by blotting the samples with soft absorbent filter paper before recording the fresh weight. The RGR was expressed as % increase in fresh weight biomass per day (% $d^{-1}$). All the experiments were carried out in triplicates and mean and standard deviations were expressed; and treatments among each experiment were grouped based Duncan Multiple Range Test (DMRT) using SPSS 14.

## 3. Results and discussion

### 3.1. Characterization of antibacterial protein from the G. edulis associated bacteria

The seaweed associated bacteria as epibiotics and endobiotics screened for antimicrobial activities wherein the activity was mostly tested against animal and human pathogens (Burke et al., 2011; Lachnit et al., 2011) nevertheless, only our previous study was tested against plant pathogens (Suvega and Arunkumar, 2014). The major *Phylla* of seaweed associated bacteria identified are belong to *Alteromonas, Pseudoalteromonas, Vibrio, Bacillus*, *Firmicutes, Proteobacteria,* and *Gammaproteobacteria* (Thilakan et al., 2016; Satheesh et al., 2016). As seaweed species contain specific biochemical constituents, associated bacteria establish unique chemical relationship with host by producing varied enzymes and compounds for effective colonization. This was evident from our previous study by varied antimicrobial activity of *Lysinibacillus xylanilyticus* isolates such as GT132, HVT234 and GSTP512 associated with different red seaweed hosts such as *Gracilaria edulis*, *Hypnea valentiae* and *Grateloupia filicina,* respectively (Suvega and Arunkumar, 2014). In this study, culture supernatant of bacteria isolates exhibit more antibacterial activity than the cell extracts. Among the *L. xylanilyticus* isolates, HVT234 isolated from host red seaweed *Hypnea valentiae* exhibit significantly maximum antibacterial activity followed by GT132, GR154, GSTP512 and GT134 associated with other red seaweed species (Table 3) reveal as *L. xylanilyticus* isolates endured by synthesizing array of chemical substances in response to associating host seaweed species that lead to varied antibacterial potential (Ali et al., 2012). As reported by Ismail et al. (2016), higher bioactivity in the culture supernatant than cell pellet indicates the extracellular production of active substance by *L. xylanilyticus* and the low bioactivity observed in the cell pellet extract was by the cell held substance (Table 3). The loss of activity as a result of proteinase *K* treatment confirmed that the extracellular substance is protein (Suvega and Arunkumar, 2014). Nevertheless the isolate *L. xylanilyticus* HVT234 exhibit varying antibacterial activity while grown at different physiochemical and nutritional regimes and significantly maximum activity was recorded by 1.5% inoculum in seawater medium with lactose carbon and ammonium chloride nitrogen at pH 7.0 in 35 °C for 48 h (Fig. 1).

Studies pertain to isolate the seaweed associated culturable bacteria are more relevant rather than metagenomic approach for searching novel antibacterial compounds (Yung et al., 2011; Singh et al., 2015) because the successful seaweed associated bacterial communities are endured with array of compounds for sustaining in the competition to obtain nutrients from the seaweed host (Penesyan et al., 2009). On screening, only some seaweed associated bacteria are identified as producing antimicrobial properties ((Lemos et al., 1986; Kanagasabhapathy and Nagata, 2008; Wiese et al., 2009; Penesyan et al., 2009; Janaki Devi et al., 2013). In our previous study, 40.2% *Bacillus* species (39.54% epibiotics and 40.74% endobiotics) among the 673 isolates belong to 27 bacterial genera associated with 11 seaweeds were active against plant pathogenic bacteria (*X. axonopodis* pv. *citri*, *X. oryzae* pv. *oryzae*) and fungus *Ustilaginoidea virens* (Suvega and Arunkumar, 2014). Several secondary metabolites and protein peptides possessing antimicrobial activity characterized from the seaweed associated bacteria (Goecke et al., 2010; Debbab et al., 2010) were found active against animal and human pathogens. The *N*-acyl homoserine lactones elicited by plant host associating bacteria through quorum signals possess bioactivity (Grazia et al., 2007; Goecke et al., 2010). The molecular weight of this signal substance isolated from the seaweed associated bacterium *Shewanella algae* is ranged from 50 to 60 kDs protein (Singh et al., 2015). In this present study, quorum elicitor substance active against plant pathogenic bacterium *Xanthomonas oryzae* pv. *oryzae* isolated from the red seaweed *Hypnea valentiae* associated bacterium *Lysinibacillus xylanilyticus* HVT234 was partially

**Table 2**
Relative growth rate (RGR) of *Gracilaria edulis* cultured under laboratory condition re-inoculated with and without associated bacteria.

| Sl.No | *G. edulis* grown in enriched seawater medium (SM) | RGR (%) of *G. edulis* day after inoculation with associated bacteria | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 5 | 10 | 15 | 20 | 25 | 30 | 35 | 40 | 45 |
| 1 | Axenic alga in unsterilized-SM | 0.211 ± 0.012 | Decayed | | | | | | | |
| 2 | Axenic alga in sterilized-SM | $0.271^{A}$ ± 0.025 | $0.071^{C}$ ± 0.010 | $0.201^{B}$ ± 0.002 | $0.309^{A}$ ± 0.015 | $0.271^{A}$ ± 0.010 | Decayed | | | |
| 3 | Non-axenic alga in unsterilized-SM | $0.100^{B}$ ± 0.005 | $0.368^{A}$ ± 0.050 | $0.375^{A}$ ± 0.005 | $0.044^{B}$ ± 0.020 | $0.052^{B}$ ± 0.025 | $0.172^{B}$ ± 0.010 | Decayed | | |
| 4 | Non-axenic alga in sterilized- SM | $0.271^{D}$ ± 0.002 | $0.041^{E}$ ± 0.042 | $0.391^{C}$ ± 0.022 | $0.472^{B}$ ± 0.024 | $0.481^{B}$ ± 0.020 | $0.582^{A}$ ± 0.025 | $0.273^{D}$ ± 0.010 | $0.265^{D}$ ± 0.004 | $0.318^{C}$ ± 0.015 |
| 5 | Axenic alga in sterilized -SM with active GT132 + non-active 9 isolates. | $0.287^{C}$ ± 0.005 | $0.386^{C}$ ± 0.035 | $0.292^{C}$ ± 0.021 | $0.140^{D}$ ± 0.02 | $0.286^{C}$ ± 0.010 | $0.311^{C}$ ± 0.010 | $0.357^{C}$ ± 0.025 | $0.522^{B}$ ± 0.025 | $1.070^{A}$ ± 0.012 |
| 6 | Axenic alga in sterilized-SM with active GT128 + non-active 9 isolates | $0.375^{B}$ ± 0.015 | $0.256^{C}$ ± 0.015 | $0.164^{D}$ ± 0.021 | $0.291^{C}$ ± 0.002 | $0.121^{D}$ ± 0.004 | $0.491^{A}$ ± 0 .002 | $0.182^{D}$ ± 0.025 | Decayed | |
| 7 | Axenic alga in sterilized-SM with active GT119 + non-active 9 isolates. | $0.211^{D}$ ± 0.015 | $0.242^{D}$ ± 0.012 | $0.285^{D}$ ± 0.011 | $0.371^{C}$ ± 0.015 | $0.412^{C}$ ± 0.020 | $0.566^{B}$ ± 0.015 | $0.515^{B}$ ± 0.014 | $0.526^{B}$ ± 0.020 | $0.639^{A}$ ± 0.022 |
| 8 | Axenic alga in sterilized-SM with non-active 9 isolates | $0.875^{A}$ ± 0.020 | $0.639^{B}$ ± 0.015 | $0.477^{C}$ ± 0.011 | $0.180^{D}$ ± 0.020 | $0.132^{D}$ ± 0.011 | $0.072^{DE}$ ± 0.002 | Decayed | | |
| 9 | Axenic alga in sterilized-SM with active isolates GT119,128,132 + non-active 9 isolates | $0.723^{A}$ ± 0.015 | $0.521^{B}$ ± 0.002 | $0.317^{D}$ ± 0.012 | $0.376^{D}$ ± 0.020 | $0.491^{BC}$ ± 0.020 | $0.507^{B}$ ± 0.025 | $0.529^{B}$ ± 0.005 | $0.558^{B}$ ± 0.025 | $0.775^{A}$ ± 0.120 |

Mean values follow with different alphabets in each row significantly different ($P < 0.05$ level).

**Table 3**
Antibacterial activity of isolates of *Lysinibacillus xylanilyticus* associated with red seaweeds.

| S.No | Bacteria isolates | Red seaweed | Antibacterial activity (zone of inhibition mm diameter) | |
|---|---|---|---|---|
| | | | Culture supernatant | Cell Pellet |
| 1 | GT132,JQ677989 | *Gracilaria edulis* | $29.0 \pm 2.0^{B}$ | $10.0 \pm 1.0^{C}$ |
| 2 | GT134,JQ677988 | *G. edulis* | $15.0 \pm 3.0^{D}$ | $09.0 \pm 1.0^{C}$ |
| 3 | GR154,JQ677990 | *G. edulis* | $19.0 \pm 1.0^{C}$ | $13.0 \pm 2.0^{B}$ |
| 4 | GSTP512,JQ677987 | *Grateloupia filicina* | $16.0 \pm 1.0^{D}$ | $10.0 \pm 1.0^{C}$ |
| 5 | HVT234,JQ739716 | *Hypnea valentiae* | $39.0 \pm 2.0^{A}$ | $17.0 \pm 3.0^{A}$ |

Mean values follow with different alphabets in each column significantly different ($P < 0.01$ level).

characterized as 66 kDs protein (Fig. 2) presumably a *N*-acyl homoserine lactone like compound shows significant increase in the bioactivity by increasing purity (Table 4). The purified protein 7 μg/mL exhibit minimum inhibitory concentration (MIC) against the test pathogen *X. oryzae* pv. *oryzae* under *in vitro* in broth assay.

### *3.2. Antibacterial substance synthesized by G. edulis associated bacteria elicited by co-associating bacteria*

Bacteria in the colony coordinate and/or communicate to others by synthesizing chemical signals which elicit or suppress the growth of other co-associating bacterial species resulted synergistic or antagonistic implications. Such a chemical signals are quorum sensing signal compounds (Joint et al., 2002, 2007; Tait et al., 2009). As seaweed associated bacteria combat/coordinate with other associating bacteria for space and nutrient in the host, there is an immense potential to tap the bacterial signal compounds for various applications (Singh et al., 2015). Among the 12 isolates associated with *Gracilaria edulis,* 3 isolates are considered active as their extracellular substances showing inhibitory property against plant pathogens tested whereas other 9 isolates are non-active as they did not posses bioactivity (Suvega and Arunkumar, 2014). In this study, 12 isolates were grown individually and consortia (one active + one non-active) in Zobell marine broth (Fig. 3). The growth of non-active isolates GT120 and 129 was comparatively higher than other active and non-active isolates when grown individually but their growth was suppressed in the consortia with active isolates (GT 119,128 and 132). On the other side, growth and antibacterial substance production of three active isolates were higher when grown in consortia with some non-active isolates (119 + 117; 128 + 144; 132 + 120 and 132 + 117; Fig. 3 and Table 1). The active isolates GT 119, 128 and 132 are *Bacillus megaterium, B. cereus* and *Lysinibacillus xylanilyticus,* respectively. In consortia, active substance synthesis in *Bacillus megaterium* GT 119 and *L. xylanilyticus* GT132, JQ677989 was elicited by non-active *Corynebacterium* sp GT117. Beside the active GT132 also elicited by non-active *Klebsiella oxytoca* GT120 whereas non-active *Aeromonas hydrophila* GT144 for active isolate *Bacillus cereus* GT 128,HM573311. But non-active GT117 (*Corynebacterium* sp.) with active GT128, the growth and active substance synthesis were suppressed whereas the growth was promoted with non-active isolates GT144(*Bacillus* sp.), GT129 (*Aeromonas* sp.), GT139(*Bacillus* sp.) and GT125(*Corynebacterium* sp.) (Table 1). This observations therefore conclude that active isolates GT119 and GT132 synthesize one type of bioactive substances which is elicited through quorum sensing by non-active isolates GT117-*Corynebacterium* sp. and GT120-*Klebsiella* sp. whereas active compound of GT128 was different type as it is elicited by different non-active isolates GT125, GT129, GT139 and GT144 (Fig. 3, Tables 1 and 3). It suggests that the host *Gracilaria edulis* associated bacteria interaction for space and nutrient are mediated by chemical signal molecules which exhibit antibacterial activity against phytopathogens (Joint et al., 2002, 2007; Tait et al., 2009; Malmstrom et al., 2004; Burke et al., 2011) such signal compounds are potential for various therapeutics.

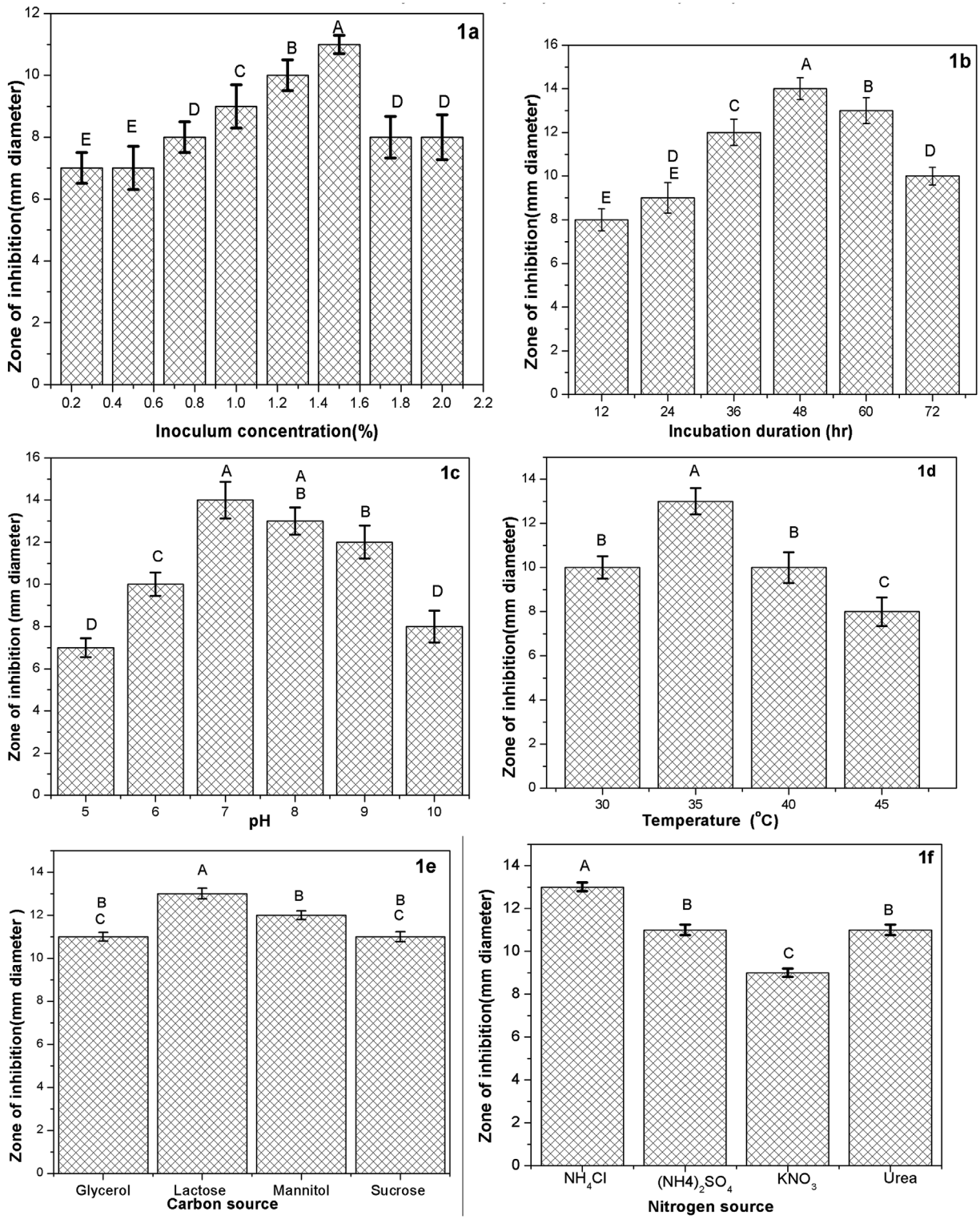


**Fig. 1.** Optimizing the physico-chemical conditions of medium such as 1a) inoculum concentration; 1b) duration of incubation; 1c) pH; 1d) temperature; 1e) carbon source; and 1f) nitrogen source for maximum antibacterial substance synthesis by *Lysinibacillus xylanilyticus* isolate HVT234. Mean values follow with different alphabets among bars in each figure significantly different ($P < 0.01$ level).

### *3.3. Associated bacteria role on the growth of host seaweed Gracilaria edulis*

The twelve associated bacteria isolates(3 active and 9 non-active) reintroduced into the axenized host *Gracilaria edulis* grown in the enriched seawater medium are presented in the Table 2 and the growth was recorded on the basis of relative growth rate (RGR) with and without associated bacteria. High RGR in seaweed with active isolate GT132 + 9 non-active isolates was recorded followed by with active GT119 + 9 non-active and 12 isolates (3 active + 9 non-active) and this three experiments seaweed maintained progressive growth as like ocean collected unaxinized seaweed. Whereas other experiments

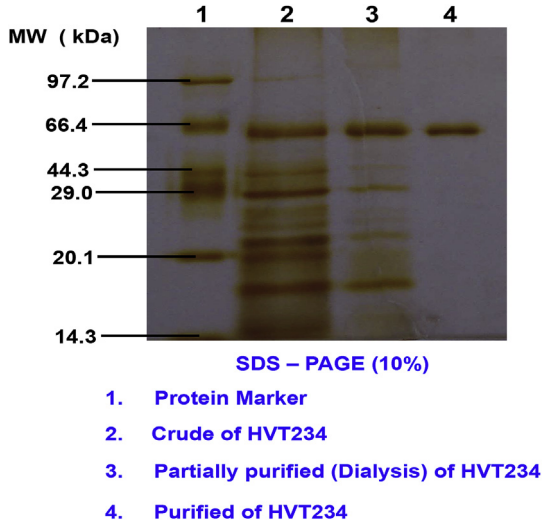


**Fig. 2.** Antibacterial protein profile on SDS PAGE isolated from the culture supernatant of *Lysinibacillus xylanilyticus* isolate HVT234.

**Table 4**
Protein content at various level of separation of antibacterial culture supernatant of *L. xylanilyticus* HVT234/JQ739716.

| Antibacterial substance | OD at 590 nm | Protein (µg/ml crude) | Zone of inhibition (mm diameter) |
|---|---|---|---|
| Crude | 0.659 | 323.157 | 14.0 ± 1.02$^{C}$ |
| Dialysed | 0.238 | 113.03 (34.97) | 16.0 ± 1.24$^{B}$ |
| Purified | 0.143 | 70.58 (21.84) | 28.0 ± 2.02$^{A}$ |

Values in parenthesis are percent pure protein in the crude.
Mean values follow with different alphabets in each column significantly different ($P < 0.01$ level).

including seaweed grown with active GT128 + 9 non-active isolates and with only 9 non-active isolates were bleached(see Table 2). This shows that growth of seaweed was supported by associating active isolates GT119 and 132 only and not by another active isolate GT 128. This growth promoting active isolates *B. megaterium* GT119 and *L. xylanilyticus* GT132 are considered as probiotics to the red seaweed *Gracilaria edulis* (Rahman et al., 2018). This increasing antibacterial substance synthesize by active isolates (GT119 and 132) in consortia with selected non-active isolates (GT120 and 117) suggest that this non-active competitive bacteria combated effectively by eliciting signal compounds by probiotic active isolates which support the host growth(*G. edulis*). On the other hand, the isolate GT128 synthesize only antibacterial substance elicited by non-active isolates GT125, 129 and 139 in consortia but not supporting the host seaweed growth was evident as potential colonizer among the host associated bacteria only for space and food (Wietz et al., 2013). Nevertheless, in this study, as reported by Singh et al. (2015) protein isolated from the *L. xylanilyticus* GT132J, Q677989 presumably a quorum sensing signal compound *N*-acyl homoserine lactones exhibit the antibacterial activity is found responsible for promoting the growth of red seaweed *G. edulis*.

## 4. Conclusion

It is concluded that extracellular substance active against plant pathogenic bacterium *Xanthomonas oryzae* pv. *oryzae* synthesized by *Lysinibacillus xylanilyticus* HVT234 associated with red seaweeds was partially characterized as 66 kDs protein peptide *N*-acyl homoserine lactone like compound has potential application in agriculture and therapeutics. Among the associated bacteria, growth promoting isolates *B. megaterium* GT119 and *Lysinibacillus xylanilyticus* GT132 synthesize bioactive signal substances are probiotics to red seaweed *Gracilaria edulis* reported first time in this study has potential value in the seaweed mariculture.

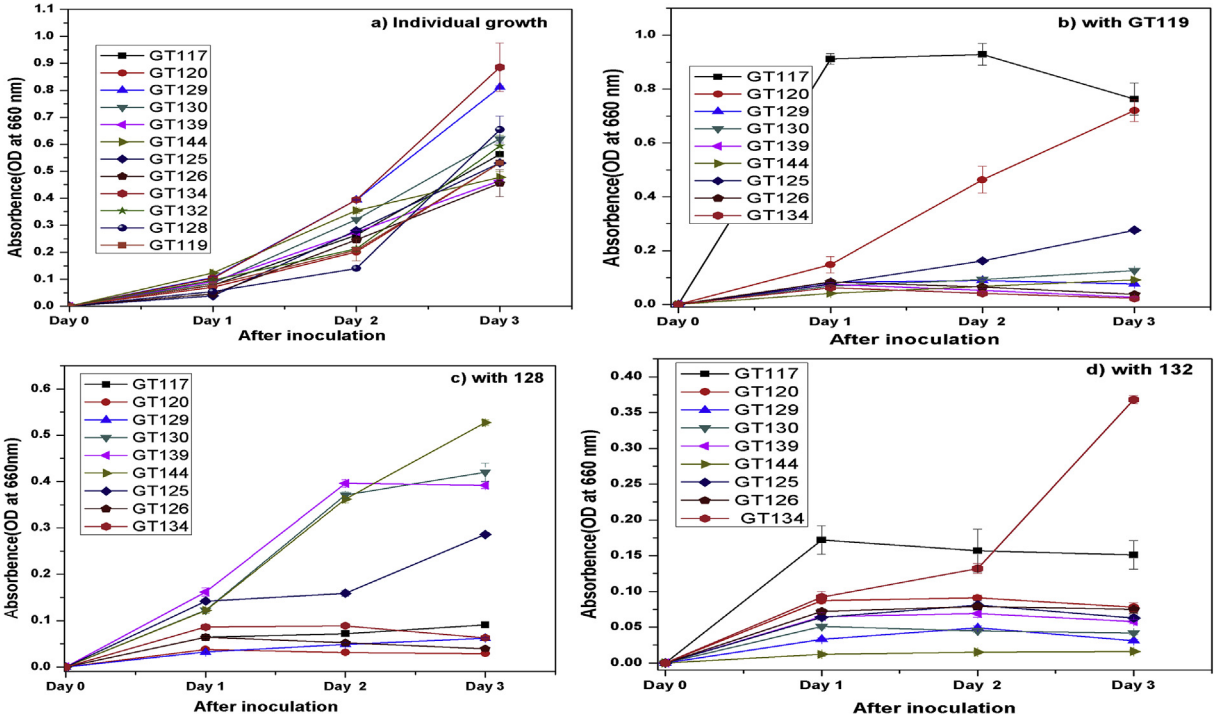


**Fig. 3.** *In vitro* growth of 12 bacteria isolates associated with red seaweed *Gracilaria edulis* cultured in Zobell marine broth a) individually and in consortia with three active isolates (**b-** GT119 + 9 non-active, **c**- GT128 + 9 non-active; and **d**- 132 + 9 non-active). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

## Conflicts of interest

We declare no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101136.

## References


Ali, A.I.B., El Bour, M., Ktari, L., Bolhuis, H., Ahmed, M., Boudabbous, A., 2012. *Jania rubens* associated bacteria, molecular identification and antimicrobial activity. J. Appl. Phycol. 24, 525–534. https://doi.org/10.1007/s10811-011-9758-0.

Armstrong, E., Boyd, K.G., Pisacane, A., Peppiatt, C.J., Burgess, J.G., 2000. Marine microbial natural products in antifouling coatings. Biofouling 16, 215–224. https://doi.org/10.1080/08927010009378446.

Bashan, Y., Holguin, G., 2008. Proposal for the division of plant growth-promoting rhizobacteria into two classifications: biocontrol-PGPB (plant growth-promoting bacteria) and PGPB. Soil Biol. Biochem. 30, 1225–1230.

Borriss, R., 2017. Phytostimulation and biocontrol by the plant-associated Bacillus amyloliquefaciens FZB42: an update. In: Islam, M.T., Rahman, M., Pandey, P., Jha, C.K., Aeron, A. (Eds.), Bacilli and Agrobiotechnology. Springer International Publishing, pp. 163–184.

Burke, C., Steinberg, P., Rusch, D., Kjelleberg, S., Thomas, T., 2011. Bacterial community assembly based on functional genes rather than species. Proc. Natl. Acad. Sci. U. S. A 108, 14288–14293.

Debbab, A., Aly, A.H., Lin, W.H., Proksch, P., 2010. Bioactive compounds from marine bacteria and fungi. Microb. Biotechnol. 3, 544–563. https://doi.org/10.1111/j.1751-7915.2010.00179.x.

Dobretsov, S., Qian, P.Y., 2002. Effect of bacteria associated with the green alga *Ulva reticulata* on marine micro- and macrofouling. Biofouling 18, 217–228. https://doi.org/10.1080/08927010290013026.

Egan, S., James, S., Holmstrom, C., Kjelleberg, S., 2001. Inhibition of algal spore germination by the marine bacterium *Pseudoalteromonas tunicate*. FEMS Microbiol. Ecol. 35, 67–73.

Goecke, F., Labes, A., Wiese, J., Imhoff, J.F., 2010. Chemical interactions between marine macroalgae and bacteria. Mar. Ecol. Prog. Ser. 409, 267–300. https://doi.org/10.3354/meps08607.

González, J.F., Degrassi, G., Devescovi, G., De, V.D., Höfte, M., Myers, M.P., Venturi, V.A., 2012. Proteomic study of *Xanthomonas oryzae* pv. *oryzae* in rice xylem sap. J. Proteom. 75, 5911–5919. https://doi.org/10.1016/j.jprot.2012.07.019.

Grazia, L., Iris, B., Steindler, L., Bella, P., Venturi, V., Catara, V., 2007. *Pseudomonas corrugata* contains a conserved *N*-acyl homoserine lactone quorum sensing system; its role in tomato pathogenicity and tobacco hypersensitivity response. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Ecol. 61 (2), 222–234. https://doi.org/10.1111/j.1574-6941.2007.00338.x.

Guillard, R.R.L., 1973. In: In: Stein, Janet R. (Ed.), Handbook of Phycological Methods: Culture Methods and Growth Measurements, vol. 1. Cambridge University Press, Cambridge, United Kingdom, pp. 289–311.

Hagström, A., Pommier, T., Rohwer, F., Simu, F., Stolte, W., Svensson, D., Zweifel, U.L., 2002. Use of 16S ribosomal DNA for delineation of marine bacterioplankton species. Appl. Environ. Microbiol. 68, 3628–3633.

Harder, T., Dobretsov, S., Qian, P.Y., 2004. Water borne polar macromolecules act as algal antifoulants in the seaweed *Ulva reticulata*. Mar. Ecol. Prog. Ser. 274, 133–141. https://doi.org/10.3354/meps274133.

Hong, Y.K., Cho, J.Y., 2013. Effect of seaweed epibiotic bacterium *Streptomyces violaceoruber* SCH-09 on marine fouling organisms. Fish. Sci. 79, 469–475. https://doi.org/10.1007/s12562-013-0604-y.

Ismail, A., Ktari, L., Ahmed, M., Bolhuis, H., Boudabbous, A., Stal, L.J., El Bour, M., 2016. Antimicrobial activities of bacteria associated with the Brown alga *Padina pavonica*. Front. Microbiol. 7, 1072. http://doi.org/10.3389/fmicb.2016.01072.

Janaki Devi, V., Yokesh Babu, M., Umarani, R., Kumarguru, A., 2013. Antagonistic activity of seaweed associated bacteria against human pathogens. Int. J. Cur. Micobiol. App. Sci. 2, 140–147.

Joint, I., Tait, K., Wheeler, G., 2007. Cross-kingdom signalling: exploitation of bacterial quorum sensing molecules by the green seaweed *Ulva*. Phil. Trans. R. Soc. B. 362, 1223–1233. https://doi.org/10.1098/rstb.2007.2047.

Joint, I., Tait, K., Callow, M.E., Callow, J.E., Milton, D., Williams, P., 2002. Cell-to-cell communication across the procaryote – eucaryote boundary. Science 298, 1207. https://doi.org/10.1126/science.1077075.

Kanagasabhapathy, M., Nagata, S., 2008. Cross-species induction of antibacterial activity produced by epibiotic bacteria isolated from Indian marine sponge *Pseudocratina purpurea*. World J. Microbiol. Biotechnol. 24, 687–691.

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.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of head of bacteriophage T4. Nature 227, 680–685.

Lemos, M.L., Toranzo, A.E., Barja, L.J., 1986. Antibiotic activity of epiphytic bacteria isolated from intertidal seaweeds. Microb. Ecol. 11, 149–163 doi:101007/BF02010487.

Malmstrom, R., Kiene, R.P., Kirchman, D.L., 2004. Identification and enumeration of bacteria assimilating dimethyl sulfonio propionate (DMSP) in the North Atlantic and Gulf of Mexico. Limnol. Oceanogr. 49, 597–606. https://doi.org/10.4319/lo.2004.49.2.0597.

Penesyan, A., Marshall-Jones, Z., Holmstrom, C., Kjelleberg, S., Egan, S., 2009. Antimicrobial activity observed among cultured marine epiphytic bacteria reflects their potential as a source of new drugs. FEMS Microbiol. Ecol. 69, 113–124.

Popper, Z.A., Michel, G., Herve, C., Domozych, D.S., Willats, W.G.T., Tuohy, M.G., Kloareg, B., Stengel, D.B., 2011. Evolution and diversity of plant cell walls: from algae to flowering plants. Annu. Rev. Plant Biol. 62, 567–590.

Rahman, M., As Sabir, A., Mukta, J.A., Khan, M.M.A., Mohi-Ud-Din, M., Miah, M.G., Rahman, M., Islam, M.T., 2018. Plant probiotic bacteria Bac*illus* and Par*aburkholderia* improve growth, yield and content of antioxidants in strawberry fruit. Sci. Rep. 8, 2504.

Rajasree, V., Satheesh, S., Vincent, S.G.P., 2012. Antifouling activity of marine epibiotic bacterium from the seaweed *Sargassum wightii*. Thalassas 28, 37–44.

Romanenko, L.A., Uchino, M., Tebo, B.M., Tanaka, N., Frolova, G.M., Mikhailov, V.,V., 2008. *Pseudomonas marincola* sp. Nov. isolated from marine environments. Int. J. Syst. Evol. Microbiol. 58, 706–710.

Satheesh, S., Ba-akdah, M.A., Al-Sofyani, A.A., 2016. Natural antifouling compound production by microbes associated with marine macroorganisms-A review. Electron. J. Biotechnol. 21, 26–35.

Sawana, A., Adeolu, M., Gupta, R.S., 2014. Molecular signatures and phylogenomic analysis of the genus *Burkholderia*: proposal for division of this genus into the emended genus *Burkholderia* containing pathogenic organisms and a new genus *Paraburkholderia* gen. nov. harbouring environmental species. Front. Genet. 5, 429. https://doi.org/10.3389/fgene.2014.00429(2014).

Singh, R.P., Mantri, V.A., Reddy, C.R.K., Jha, B., 2011a. Isolation of seaweed associated bacteria and their morphogenesis inducing capability in axenic cultures of the green alga *Ulva fasciata*. Aquat. Biol. 12, 13–21. https://doi.org/10.3354/ab00312.

Singh, R.P., Bijo, A.J., Baghel, R.S., Reddy, C.R.K., Jha, B., 2011b. Role of bacterial isolates in enhancing the bud induction in the industrially important Red alga *Gracilaria dura*. FEMS Microbiol. Ecol. 76, 381–392. https://doi.org/10.1111/j.1574-6941.2011.01057.x.

Singh, R.P., Baghel, R.S., Reddy, C.R.K., Jha, B., 2015. Effect of quorum sensing signals produced by seaweed-associated bacteria on carpospore liberation from *Gracilariadura*. Front. Plant Sci. 6, 117. https://doi.org/10.3389/fpls.2015.00117.

Singh, R.P., Kumari, P., Reddy, C.R.K., 2014. Antimicrobial compounds from seaweeds associated bacteria and fungi. Appl. Microbiol. Biotechnol. 99 (4), 1571–1586. https://doi.org/10.1007/s00253-014-6334-y.

Suvega, T., Arunkumar, K., 2014. Antimicrobial activity of bacteria associated with seaweeds against plant pathogens on par with bacteria found in seawater and sediments. Br. Microbiol. Res. J. 4, 841. https://doi.org/10.9734/BMRJ/2014/8678. 85.

Tait, K., Williamson, H., Atkinson, S., Williams, P., Camara, M., Joint, I., 2009. Turnover of quorum sensing signal molecules modulate cross kingdom signalling. Environ. Microbiol. 11, 1792–1802. https://doi.org/10.1111/j.1462- 2920.2009.01904.x.

Teas, J., 2007. Seaweed and soy: companion foods in asian cuisine and their effects on thyroid function in American women. J. Med. Food 10, 90–100.

Thilakan, B., Chakraborty, K., Chakraborty, R.D., 2016. Antimicrobial properties of cultivable bacteria associated with seaweeds in the Gulf of Mannar on the southeast coast of India. Can. J. Microbiol. 62, 668–681. https://doi.org/10.1139/cjm-2015-0769.

Vijayalakshmi, S., Ramasamy, M.S., Murugesh, S., Murugan, A., 2008. Isolation and screening of marine associated bacteria from Tamil Nadu, Southeast coast of India for potential antibacterial activity. Ann. Microbiol. 58, 605–609. https://doi.org/10.1007/BF03175564.

Wiese, J., Thiel, V., Nagel, K., Staufenberger, T., Imhoff, J.F., 2009. Diversity of antibiotic active bacteria associated with the brown alga *Laminaria saccharina* from the Baltic Sea. Mar. Biotechnol. 11, 287–300.

Wietz, M., Duncan, K., Patin, N.V., Jensen, P.R., 2013. Antagonistic interactions mediated by marine bacteria: the role of small molecules. J. Chem. Ecol. 39, 879–891.

Yung, P.Y., Burke, C., Lewis, M., Kjelleberg, S., Thomas, T., 2011. Novel antibacterial proteins from the microbial communities associated with the sponge *Cymbastela concentrica* and the green alga *Ulva australis*. Appl. Environ. Microbiol. 77, 1512–1515.

Zheng, L., Han, X., Chen, H., Lin, W., Yan, X., 2005. Marine bacteria associated with marine macroorganisms the potential antimicrobial resources. Annal. Microbial. 5, 119–124.

Zhu, X.F., Xu, Y., Peng, D., Zhang, Y., Huang, T.T., Wang, J.X., Zhou, M.G., 2013. Detection and characterization of bismerthiazol-resistance of *Xanthomonas oryzae* pv. *oryzae*. Crop Prot. 47, 24–29. https://doi.org/10.1016/j.cropro.2012.12.026.