Chapter 6

Seasonal bacterial diversity, quorum sensing signal production and spore release in *Gracilaria* spp.
6.1. Introduction

The extracellular substances released from macroalgal communities serve as a feed for diverse micro-organisms in coastal ecosystems (Armstrong et al., 2001; Lane & Kubiczek, 2008). The macroalgal surface also provides an ideal substrate for settlement of microbes and subsequently the formation of biofilms and/or facilitating further microbial buildup (Weinberger, 2007; Joint et al., 2007). Microbial communities living on macroalgal surfaces are highly complex, dynamic and consist of a consortium of micro-organism including bacteria, fungi, diatoms, protozoa, spores and larvae of marine invertebrates (Holmström et al., 2002). However, bacteria are the most ubiquitous and occur on external surfaces as well as in internal tissues of algae, and impart a variety of effects on host plant development including reproduction.

The earlier studies have shown that seaweed-associated bacteria play an important role in controlling the life cycle process including spore settlement, foliose structure and overall development (Joint et al., 2002; Matsuo et al., 2003; Chapter 3 & 4). Seaweeds-associated bacteria including epi- and endophytic in origin are known to produce IAA that in turn regulate the morphogenesis pattern and growth in Ulva spp. (Maruyama et al., 1988) and Gracilaria dura (Chapter 4). It has also been established that seaweed-associated bacterial strains produce QS molecules such as AHLs facilitating the zoospore settlement in the Ulva spp. (Joint et al., 2002). Weinberger et al. (2007) reported that AHLs induce the spore liberation in the epiphytic red alga Acrochaetium sp. found on Gracilaria chilensis. Weinberger et al. (2007) demonstrated that bacterial antibiotics have reduced the spore liberation phenomenon but did not study the phylogenetic characterization of bacterial strains affiliated with this seaweeds. The spore liberation in general is an important event of life cycle of macroalgae and the role of QS signals in release of spores is hardly investigated particularly for red seaweeds.

The QS investigations began with the finding that AHLs (a most studied QS molecules) signals when present at threshold concentration lead to the induction of luminescence in a marine symbiotic bacterium Vibrio fischeri, (Nealson & Hastings, 1979; Bassler, 1999). The expression of luciferase in the V. fischeri was controlled by LuxI (an autoinducer synthase) and LuxR (a transcriptional regulator) proteins (Engebrecth &
Silverman, 1984). AHLs consist of five-membered lactone rings and varied amide linked acyl side-chains. AHLs are classified based on the length of the N-linked acyl chains (4 to 18 carbons long chains) and substituted on the C3 carbon of the N-linked acyl chain, usually with hydroxy or oxo group (Chhabra et al., 1993). AHLs are now known to modulate expression of a huge variety of genes involved in biofilm formation, motility, antibiotic production, the exchange of genetic material through the accumulation of diffusible extracellular signals (Williams, 2007). Putative AHLs producing bacteria play an important role in the field of plant-bacterial interaction and cystic fibrosis (Joint et al., 2002; Williams, 2007). The studies of Joint et al. (2002) showed that AHLs are involved in attraction of Ulva zoospores to bacterial biofilms. AHLs are secreted only by gram-negative bacteria of the phylum Proteobacteria till date (Hense et al., 2007; Williams, 2007).

*Ulva* and *Gracilaria* species are the most common macroalgae and grow abundantly in intertidal region of coastal habitats worldwide (Chapter 3 & 4). In this chapter, it is investigated the epibenthic and endophytic bacteria associated with both *Ulva* and *Gracilaria* species from two different locations over three different seasons. All the bacterial isolates obtained in this finding were identified by 16S rRNA gene sequencing. Further, the bacterial strains were screened for the production of AHLs and subsequently determined using LC-ESI-MS/MS- CID analysis. The spore liberation property of AHLs was also investigated using red alga *Gracilaria dura*.

### 6.2. Materials and methods

#### 6.2.1. Chemicals

The selected *N*-acyl-homoserine-lactone, *N*-butanoyl- (C4-HSL), *N*-3-hydroxybutanoyl- (HC4-HSL), *N*-hexanoyl- (C6-HSL), *N*-heptanoyl- (C7-HSL), *N*-octanoyl- (C8-HSL), *N*-decanoyl- (C10-HSL), *N*-dodecanoyl- (C12-HSL), *N*-3-oxo-hexanoyl- (3-oxo-C6-HSL), *N*-3-oxo-octanoyl-(3-oxo-C8-HSL) and *N*-3-oxo-dodecanoyl-(3-oxo-C12-HSL) homoserine lactone were purchased from Sigma Aldrich (Buchs, Switzerland). Analytical grade acetonitrile and formic acid were purchased from Sisco Research Pvt. Lit. (India). Standard samples were prepared by dissolving the AHLs in acetonitrile at a concentration of 1 mg ml\(^{-1}\) and stored at −20°C.
6.2.2. Sample collection, epi and endophytic bacterial isolation

The *Ulva fasciata, U. lactuca, Gracilaria dura* and *G. corticata* had been collected from Veraval (N 20° 54.87’, E 70° 20.83’) and *U. fasciata* and *G. dura* from Okha Port Harbour (22° 28’ 22” N and 69° 05’ 03” E), India, during low tide at three different seasons in the year of 2011. *U. lactuca* and *G. corticata* were not found at Okha Port Harbour location. Both sites are located 250 km away from each other (Chapter 2, fig. 2.1). Different parameter including average pH, temperature and salinity has been measured during collection time (Table 6.1).

**Table 6.1.** Different parameter including average pH, temperature and salinity were measured during collection time.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pre-monsoon</th>
<th>Monsoon</th>
<th>Post-monsoon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Veraval</td>
<td>Okha</td>
<td>Veraval</td>
</tr>
<tr>
<td>pH</td>
<td>8.2</td>
<td>8.2</td>
<td>8.1</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>29</td>
<td>32</td>
<td>29</td>
</tr>
<tr>
<td>Salinity (‰)</td>
<td>36</td>
<td>39</td>
<td>22</td>
</tr>
</tbody>
</table>

Three individual plantlets of a species were collected from different tidal pools, located at < 25 m apart from one another but same location at different seasons. Processing of samples and isolation of epi- and endophytic were carried out according to chapter 2. In present finding Marine agar 2216, Simmons citrate, TCBS, XLD and pseudomonas agar media were used instead of those media described in chapter 4. A number of bacterial strains isolated from different location, seasons and diverse seaweeds are depicted in figure 6.1.
Fig. 6.1. A number of bacterial strains isolated from different location at different season as well as from different microbiological media.

6.2.3. 16S rRNA gene amplification and sequencing

Bacterial genomic DNA was extracted using the CTAB buffer method (Chen & Kuo, 1993). Purification of genomic DNA band was confirmed with 0.8% agarose gel electrophoresis. The universal bacterial primer 27F and 1492R (Lane, 1991) were used for the PCR amplification of the 16S rRNA gene sequences. The reaction mixture and PCR condition were maintained according previous chapter 3. Forward and reverse rDNA sequencing reactions of PCR amplification product were carried out with above mentioned primers. Sequencing of PCR purified products was done by the Macrogen (South Korea). The sequences were manually trimmed and their homology matched at http://www.ncbi.nlm.nih.gov/BLAST/cgi.
6.2.4. Phylogenetic analysis of 16S rRNA gene sequences, taxonomic classification and biodiversity indices

16S rRNA bacterial sequence alignments were carried out by Clustal-W software, http://www.ebi.ac.uk/Tools/msa/clustalw2/ and clustered into OTUs at 0.03 cut off values using sequence homology. Aligned 16S rRNA bacterial sequences were used to construct the phylogenetic trees with neighbour joining method by using the MEGA-5 software (Tamura et al., 2011). Bootstrap test was performed with 1,000 replicates in the phylogenetic trees. Sequences were taxonomically classified using the Ribosome Database Project (RDP) using Naive Bayesian rRNA Classifier Version 2.4 (Wang et al., 2007) with 80% confidence threshold. Similarity in the nucleotide sequences were analyzed using Venn diagram, http://bioinfogp.cnb.csic.es/tools/venny/index.html.

Shannon–Weaver and Chao 1 biodiversity indices were calculated for each season sample using FastGroupII software, http://biome.sdsu.edu/fastgroup/fg_tools.htm (Yu et al., 2006). The Shannon–Weaver index is nonparametric diversity index that combines estimates of evenness and richness of OTUs (Shannon & Weaver, 1963). A more even distribution of species within communities has a higher index while communities with one dominant species has low index. Chao 1 is estimator of minimum richness of calculated OTUs and is based on the number of rare OTUs, present within a sample (Chao, 1987). One way ANOVA was used to analyze the significant differences of OTUs, Chao 1 and Shannon–Weaver index at p > 0.01.

6.2.5. Extraction for AHLs production and its identification

For extraction of AHL, different species of negative bacterial strains were grown overnight in Zobell marine broth. Bacterial culture filtrates were obtained by centrifugation of 50 ml cultures at 4000 rpm for 15 min. Thereafter, supernatant was decanted and adjusted to pH 2.5 using 1 N HCl to prevent additional hydrolysis of AHLs then extracted twice with an equal volume of ethyl acetate. Upper organic layer was washed with an equal amount of Milli-Q water and solvent evaporated under nitrogen gas (Bainton et al., 1992; Shaw et al., 1997; Cataldi et al., 2009). The residues were dissolved in 1 ml of 25% methanol and 0.1% acetic acid and stored for subsequent experiment.
Preliminary screening of all the bacterial strains (collected at different seasons) for AHL producing activity was screened with electrospray ionization mass spectrometry (ESI-MS), followed and confirmed with LC-ESI-MS/MS-CID. The characteristics for these product ions were proposed on the basis of low-resolution MS/MS spectra (Morin et al., 2003). Spectra of LC-ESI-MS/MS were recorded from 0 to 300 m/z to get definite identification of these product ions for their accurate mass values. It is possible to obtain high-resolution tandem mass spectra by using CID as the fragmentation tool (Decho et al., 2009). The theoretical masses of the most likely AHL fragments in the protonated form were calculated and compared with standards. ESI-MS and LC-ESI-MS/MS-CID were performed using Waters Micromass Q Tof micro™ mass spectrometer connected to Waters alliance HPLC and equipped with electrospray ionization source. Sample residues were dissolved in 1 ml of acetonitrile supplemented with 0.1% acetic acid to facilitate electrospray ionization and analysed in ESI-MS (ES⁺) mode using direct injection technique at 20 µl/min of flow rate. Capillary voltage sample cone and extraction cone were maintained at 2.5 KV, 25 V and 1.5 V respectively throughout the ESI-MS analysis. For LC-ESI-MS/MS-CID analysis, samples were prepared in methanol and analysed in coupled HPLC-ESI-MS/MS mode. 20 µl volumes were injected onto a C18 column (Phenomenex, 150 × 4.6 mm) and fractionated using solvent gradient (Table 6.2). Tandem MS analysis was performed using ultra high purity argon gas as collision source.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>A (Water + 0.1% acetic acid, v/v)</th>
<th>B (Methanol + 0.1% acetic acid, v/v)</th>
<th>Flow rate (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>95</td>
<td>5</td>
<td>0.30</td>
</tr>
<tr>
<td>5</td>
<td>95</td>
<td>5</td>
<td>0.30</td>
</tr>
<tr>
<td>30</td>
<td>5</td>
<td>95</td>
<td>0.30</td>
</tr>
<tr>
<td>40</td>
<td>5</td>
<td>95</td>
<td>0.30</td>
</tr>
<tr>
<td>41</td>
<td>95</td>
<td>5</td>
<td>0.30</td>
</tr>
<tr>
<td>60</td>
<td>95</td>
<td>5</td>
<td>0.30</td>
</tr>
</tbody>
</table>

6.2.6. Effect of different AHLs on carpospore production from *G. dura*

Healthy and mature cystocarpic thalli of *G. dura* were collected from intertidal region of Veraval, West coast of India. The plantlets were immediately transported to the laboratory using plastic bags containing seawater and segments of thalli were cleaned and made axenic.
according to above stated protocol. The axenic cultures were maintained with sterilized MP 1 medium in 150 ml conical flasks at 25 ± 1°C under daylight white fluorescent lamps at 15 μ mol photon m⁻² s⁻¹ irradiance with 12:12 h light: dark photoperiod. The axenicity of the algal cultures was tested as above described. Thalli bearing mature cystocarp were cut into smaller fragments. The fragment bearing 5 cystocarps were placed in Petri plates containing 15 ml of 30% autoclaved seawater and allowed the liberation of carpospores naturally. Once the liberation of carpospore was stopped naturally, cystocarp bearing fragments were used for AHLs treatment. To examine AHL’s influence on carpospore liberation, Plates were supplemented with standard AHLs of different form (C₄, C₆, C₈, C₁₀ and 3-oxo-C₁₂-HSL) at the concentration of 10 μM each. The different concentration (2, 4, 6, 8 and 10 μM) of C₄ and C₆-HSL were also used to evaluate the effect on carpospore liberation.

Simultaneously different gram-negative bacterial and Bacillus flexus supernatant were also used to examine the effect for carpospore liberation which were able to produce AHLs. All experiments were carried out in triplicates. The plant containing Petri plates without supplementation of AHLs were treated as control. The experiment set up was maintained at 25°C under daylight white fluorescent lamps at 15 μ mol photon m⁻² s⁻¹ irradiance with 12:12 h light: dark photoperiod. Fragments were transferred to new Petri plates at every 24 h interval and liberated carpospores were counted manually. The data was represented in average release per mm². One way ANOVA and Dunnett’s post hoc analysis were used to analyze the carpospore liberation capacity of standard as well as bacterial AHLs and significant differences were determined at p > 0.01.

6.2.7. Accession numbers

The bacterial sequences reported in the present chapter were submitted to GenBank with the following accession numbers: JQ665283-JQ665389, JN996469, JQ408391, JQ408396, JQ613503- JQ613504 and JQ613506, for the 16S rRNA gene sequences.

6.3. Results

6.3.1. Epi- and endophytic bacterial isolation
A total of 113 bacterial strains were isolated where major proportion of 102 strains belonged to epiphytic and small fraction of 11 considered as endophytic bacteria. All isolated bacteria were belonging to six orders (Actinomycetales, Alteromonadales, Bacillales, Enterobacteriales, Pseudomonadales and Vibrionadales). Epiphytic bacterial communities related to Vibrionadales which were harbored onto all macroalgae either collected in different seasons or locations. During pre-monsoon and monsoon seasons members of Bacillales were also present on all macroalgae. However, epiphytic bacteria belong to Bacillales did not found during post-monsoon. Bacterial strains of Pseudomonadales and Alteromonadales were affiliated with G. dura as on/in either epi or endophytic form, collected from Veraval coast while Actinomycetales and Enterobacteriales were affiliated with G. corticata collected from Okha coast.

The endophytic bacteria are: Allomonas enterica (JQ665324), Vibrio parahaemolyticus (JQ665335), Shewanella algae (JN996469), Pseudomonas aeruginosa (JQ665348), P. stutzeri (JQ665358), Micrococcus luteus (JQ665283), Bacillus cereus (JQ665291), B. licheniformis (JQ665350) V. sinaloensis (JQ665310), V. nigrilachritudo (JQ665360) and V. rotiferianus (JQ665367). Of all endophytic bacteria, ten bacterial strains were isolated from Gracilaria spp. while Bacillus cereus (JQ665291) was obtained from U. fascita during monsoon and post-monsoon seasons. V. parahaemolyticus was always isolated from G. corticata whereas S. algae and P. aeruginosa from G. dura and these followed the patterns of species-specific.

6.3.2. Taxonomic classification, biodiversity indices and phylogenetic analysis

The present findings did not include any short, chimeric and repeated sequences. Thus, all bacterial nucleotide sequences were used for construction of the phylogenetic trees. Most dynamic seaweeds associated bacterial communities belong to Firmicutes phylum. However, members of Gammaproteobacteria were consistently observed during all three seasons. A greater proportion of sequences were related to the Gammaproteobacteria, particularly Vibrionales, followed by Bacillales during pre-monsoon and monsoon seasons. However, 87.87% proportion of bacteria collected during post-monsoon season was only belonging to Vibrionaceae family (Fig. 6.2). Sequences of Marine bacterium (JQ665296) and Bacterium strain (JQ665312) were unclassified with Ribosome Database Project while neighbour-joining phylogenetic trees of 16S rRNA demonstrated their relationship with Vibrio species
which were cultured during pre-monsoon season (Fig. 6.2 A). Similarly, some of bacterial strains isolated between monsoon and post-monsoon seasons were unclassified (Fig. 6.2 B and C).

A total of 77 OTUs (≥97% sequence identity) were obtained for all bacterial nucleotide sequences over three seasons. These 77 OTUs represent 6 orders from 3 bacterial phyla including *Proteobacteria*, *Firmicutes* and *Actinobacteria* (Fig. 6.3). The majority of the 16S rRNA gene sequences occurred within the *Gammaproteobacteria* particularly in *Vibrionadales* (Fig. 6.2 A). The bacterial strains of different order including *Bacillales*, *Pseudomonadales*, *Alteromonadales* and *Vibrionales* were found over three seasons. Contrary, the member of *Actinomycetales* (*Micrococcus luteus*) and *Enterobacteriales* (*Klebsiella pneumoniae*) were found between pre-monsoon and post-monsoon respectively (Fig. 6.2 B and C). However, diverse bacterial strain communities relative to *Vibrionales* were dominant throughout collection periods. There was no significant variation in bacterial communities among different samples collected from two different locations.
Studies on certain seaweed-bacterial interaction from Saurashtra coast

Fig. 6.2A
Studies on certain seaweed - bacterial interaction from Saurashtra coast

Fig. 6.2B
Fig. 6.2C

Fig. 6.2. Evolutionary relationships of taxa between pre-monsoon (6.3A), monsoon (6.3B) and post-monsoon (6.3C). Neighbor-Joining method of partial 16S rRNA gene sequences
Studies on certain seaweed - bacterial interaction from Saurashtra coast

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(Saitou & Nei, 1987). Bootstrap test was performed with 1,000 replicates in the phylogenetic trees (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura-2-parameter method (Kimura, 1980) and were in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 5 for pre-monsoon, post-monsoon and 2 for monsoon season). Evolutionary analyses were conducted in MEGA-5 (Tamura et al., 2011).

Fig. 6.3. Percentage composition of 16S rRNA gene sequences established from epi- and endobacterial communities of Ulva fasciata, U. lactuca, Gracilaria dura and G. corticata at different sampling times based on order level.

Bacterial strain biodiversity estimators were computed for all seasons using OTUs at 0.03% cut off value (97%). The OTUs for pre-monsoon, monsoon and post-monsoon were 20, 32 and 27 respectively. Shannon–Weaver diversity indices the relative OTUs abundance, for pre-monsoon (3.545), monsoon (3.7212) and post-monsoon (3.4545) which indicate that all seasonal communities were approximately equally diverse despite the OTUs were highly variable for different seasons community. Chao 1 is nonparametric estimator of the minimum species richness (number of ribotypes) within a sample. The Chao 1 for pre-monsoon, monsoon and post-monsoon were 613, 442 and 513 respectively. Shannon–Weaver diversity and Chao 1 and OTUs indicate that monsoon communities were more diverse including species richness and evenness as compared to pre and post–monsoon communities. Venn-diagrams show numbers of unique and shared nucleotide sequences from the three seasons based on 16S rRNA gene homology (Fig. 6.4). One way ANOVA
analysis revealed the significant differences of OTUs, Chao 1 and Shannon–Weaver index at p > 0.01.

**Fig. 6.4.** Venn-diagram showing numbers of unique and shared nucleotide sequences from all the three seasons samples by 16S rRNA gene analysis.

### 6.3.3. Screening for AHLs production

In the MS/MS analysis activated natural compound [M + H] + ions derived from AHLs decomposed into specific ions, including the [M + H - C₄H₇NO₂ or M + H -101] + ion resultant from the neutral loss of homoserine lactone and an ion at m/z 102 corresponding to the protonated lactone (Table 6.3). Among gram-negative bacteria only seven strains were found potent for the production of AHLs. *S. algae* (JN996469) was found the most potent bacterial strain and produced C₄-HSL, HC₄-HSL, C₆-HSL, 3-oxo-C₆-HSL and 3-oxo-C₁₂-HSL as shown in fig. 6.5 (A, B, C, D and H). After this, *Photobacterium lutimaris* (JQ613504) produced three AHLs (C₄-HSL, HC₄-HSL, C₆-HSL) and remaining bacterial isolates produced two types of AHLs as shown in the Table 6.3 and figure 6.5, along with their parent and fragmentation ions patterns. This experiment was repeated three times and found that data were reproducible.
Table 6.3. AHL autoinducers detectable using mass spectrometry (MS/MS) in sample extracts from gram-negative bacteria

<table>
<thead>
<tr>
<th>AHLS</th>
<th>Parent ion</th>
<th>Fragmentation ions</th>
<th>Frequency of occurrence in 3 replicates</th>
<th>Bacterial strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[M+H] +</td>
<td>[M+H-chain]+</td>
<td>[M+H-lactone ring]+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>m/z</td>
<td>m/z</td>
<td>m/z</td>
<td></td>
</tr>
<tr>
<td>C4-HSL</td>
<td>172.21</td>
<td>102.16</td>
<td>71.08</td>
<td>*** + + + + + +</td>
</tr>
<tr>
<td>HC4-HSL</td>
<td>173.12</td>
<td>102.01</td>
<td>72</td>
<td>+ - - + - - -</td>
</tr>
<tr>
<td>C6-HSL</td>
<td>200</td>
<td>102.01</td>
<td>99.04</td>
<td>*** + - + + + -</td>
</tr>
<tr>
<td>3-oxo-C_6^-HSL</td>
<td>214</td>
<td>102.05</td>
<td>113.06</td>
<td>** + + - - - -</td>
</tr>
<tr>
<td>C7-HSL</td>
<td>213.98</td>
<td>101.93</td>
<td>113.05</td>
<td>* - - - - - + -</td>
</tr>
<tr>
<td>C8-HSL</td>
<td>228.08</td>
<td>102.05</td>
<td>127.08</td>
<td>** - - - + + -</td>
</tr>
<tr>
<td>C10-HSL</td>
<td>256.28</td>
<td>102.16</td>
<td>155.37</td>
<td>*** - - - - - +</td>
</tr>
<tr>
<td>3-oxo</td>
<td>C12-HSL</td>
<td>298.17</td>
<td>197.44</td>
<td>** + + - - - -</td>
</tr>
</tbody>
</table>

Using CID, the [M+H]+ ion, derived from the parent AHL molecule, decomposes into two ‘fragmentation ion’ products corresponding to the lactone (m/z 102) and the acyl-chain moiety [M+H-101]+. 

m/z = mass/charge ratio. (+) indicates the detection of AHL and (-) absences of AHL. Except Shewanella algae, all the bacteria isolated were of epiphytic origin.
Fig. 6.5A

Fig. 6.5B
Fig. 6.5C

Fig. 6.5D
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Fig. 6.5. AHL are detectable using LC-MS/MS-CID in sample extracts from different gram-negative bacterial strains. Using CID, the [M+H]$^+$ ion, derived from the parent AHL
molecule, decomposes into two ‘fragmentation ion’ products corresponding to the lactone (m/z 102) and the acyl-chain moiety [M+H-101]+. (A) MS /MS spectra for C₄-HSL at 172.21 m/z. (B) MS /MS spectra for HC₄-HSL at 173.04 m/z. (C) MS /MS spectra for C₆-HSL at 200.05 m/z. (D) MS /MS spectra for 3-oxo-C₆-HSL at 214.01 m/z. (E) MS /MS spectra for C₇-HSL at 213.98 m/z. (F) MS /MS spectra for C₈-HSL at 228.08 m/z. (G) MS /MS spectra for C₁₀-HSL at 256.28 m/z. (H) MS /MS spectra for 3-oxo-C₁₂-HSL at 298.17 m/z. m/z = mass/charge ratio. % - Relative intensities.

6.3.4. Effect of different AHLs on carpospore spore liberation from G. dura

The AHL putative producing bacterial strains and standard of C₄- and C₆-HSL were found to have significant effect on spore liberation in G. dura in comparison with control. The positive correlation was found between different concentration (2, 4, 6, 8 and 10 μM) of C₄ and C₆-HSL and carpospore liberation from G. dura (Fig. 6.6 A). Carpospores liberation were maximum as 179.625 ± 3.6 and 108.375 ± 21.62 mm² from the cystocarp bearing fragments treated with AHLs putative producing S. algae and P. aeruginosa respectively. Similarly, the other five bacterial strains, Photobacterium sp. (76.66 ± 5.07 mm²), P. lutimaris (66.87 ± 28.97 mm²), V. gallicus (44.26 ± 6.06 mm²), V. fluvialis (50.58 ± 3.74 mm²) and V. parahaemolyticus (62.83 ± 6.34 mm²) liberated carpospore from the cystocarp bearing fragments. On the other hand, carpospore liberation was also influenced by standard C₄- (93.33 ± 15.33 mm²) and C₆- HSL (99.448 ± 30.94 mm²). The C₈, C₁₀, 3-oxo-C₁₂-HSL and Bacillus flexus did not show any significant effect on carpospore liberation as compared to control (10.375 ± 5.105) (Fig. 6.6B). 1 way ANOVA and Dunnett’s post hoc analysis showed significant differences at p > 0.01 for standard as well as bacterial AHLs.
Fig. 6.6. Effect of different AHLs on the spore liberation in *Gracilaria dura*. Effect of different concentration of C₄- and C₆-HSL on spore liberation from *Gracilaria dura* (A). Labels indicate the length of N-acyl side chains increasing from C₄-HSL to 3-oxo-C₁₂-HSL and effect of different gram-negative bacterial strains. The spore settlement on glass substrata were quantified after an exposure of 24 h. Bars indicates minima and maxima of 3 replicates. Effect was significant at p ≤ 0.01 (B).
6.4. Discussion

Present chapter reports, the surface of *Ulva* and *Gracilaria* species are covered with diverse groups of bacterial phyla including *Proteobacteria*, *Firmicutes* and *Actinobacteria*. The members of *Gammaproteobacteria* were consistently observed between all the three seasons onto seaweed surface which confers the abundance of *Gammaproteobacteria* in marine environment. It was observed that the surface of green alga *Enteromorpha* and *Ulva* species occupied with the predominance of epiphytic bacteria belonging to *Gammaproteobacteria* isolated from Wembury beach, Devon, UK (Patel *et al.*, 2003; Tait *et al.*, 2009). Similarly, abundance of *Gammaproteobacteria* were also isolated from the Australian red macroalgae *Amphiroa aniceps* while *Bacteroidetes* and *Gammaproteobacteria* isolated from another red alga *Corallina officinalis* (Huggett *et al.*, 2006). In contrast to this, *Gammaproteobacteria*, *Actinobacteria*, *Planctomycetes* and *Bacillales* have a global distribution in oceanic waters (Cottrell & Kirchman, 2000; Venter *et al.*, 2004; Giovannoni & Stingl, 2005; Rusch *et al.*, 2007). Thus, phylogenetic studies of epiphytic bacteria concluded that recruitment of different bacterial community members onto macroalga is most likely to come from the seawater.

A few studies have been reported for endophytic bacteria isolated from different seaweeds. Some of endosymbiotic bacteria isolated from *Caulerpa*, *Codium*, *Bryopsis* and *Penicillus* spp. could not be characterized for their phylogenetic relevance (Turner & Friedmann, 1974, Dawes & Lohr, 1978, Rosenberg & Paerl, 1981). Recently, Hollants *et al.* (2011) isolated endophytic bacteria belonging to *Flavobacteriaceae*, *Bacteroidetes* and *Phyllobacteriaceae* affiliated with siphonous green seaweed *Bryopsis hypnoides* whereas *Xanthomonadaceae*, *Gammaproteobacteria*, *Epsilonproteobacteria* and a new *Arcobacter* species found associated with *Bryopsis pennata*. On the contrary, present finding first time reports the endophytic bacteria isolated from different species of *Ulva* and *Gracilaria* as well as characterized their phylogenetic affiliation.

The abundance of *Gammaproteobacteria* among all bacterial strains could be attributed to their tendency to form biofilms (Tait *et al.*, 2009). Consequently, they are providing substrata for spore settlement, attachment followed by successful germination and development (Patel *et al.*, 2003). Subsequently, some of *Gammaproteobacteria* entered in
the plantlet’s cells during development and form endo-bacterial consortia. It could be the reason to find same bacterial strains as epi- and endophytic. During monsoon season, intertidal Oceanic waters were contaminated with anthropogenic waste carried by runoff from coastal region and accounts for high bacterial diversity. It was also observed that mixing of runoff water with intertidal Oceanic water leads to decrease in the salinity of intertidal region which caused bleaching of seaweed, even some times washed off. Thereafter, new plantlets grow on rocks at the intertidal habitat and surface of the plantlet would be occupied by same or different bacterial communities depending on the lottery hypothesis (Sale, 1976; Burke et al., 2011). Attachment of different bacterial communities but sharing similar tropic abilities (same niche) to newly plantlet surface follows stochastic recruitment. It means whichever bacterial species, first encounter and occupies the surface of alga that will colonize on it. Similarly, newly growing plantlet’s surface are associated with less bacterial communities than old plantlet’s surface as found between pre-monsoon and monsoon seasons (Goecke et al., 2010). Sometimes variation in associated bacterial communities of seaweed may be either due to presence of different bacterial strains in surrounding waters or the ability of bacterial strains to attach with the algal surface. Seaweed associated bacterial communities also determined the post- associating micro-organism including bacteria (Holmström et al., 2002).

Plantlets age is also considered to be a significant inherent source of variation in spatial and temporal associated bacterial communities (Baker, 1998; Goecke et al., 2010). Recently, it has been demonstrated that bacterial communities of young meristem and cauloid sections of brown alga Laminaria saccharina of different plants were more similar to each other than ageing phyloid section of same plant (Staufenberger et al., 2008). Present study also confirmed the temporal variability between different seasons. During, post-monsoon season lower bacterial diversity was observed as compared to pre-monsoon and monsoon seasons (Fig. 6.3). During pre-monsoon and monsoon seasons seaweed surface was also occupied by other biofilms forming bacteria such as Firmicutes communities. Considering this fact, present findings revealed that Firmicutes are highly variable where as Gammaproteobacteria communities are seaweed loving but temporally variable. Despite levels of variability, the epi- and endophytic community includes a sub-population of bacteria that were consistently associated with Ulva and Gracilaria species. The consistent detection of this sub-population may have an important role in the function of the bacterial community on Ulva and Gracilaria species. Similar findings have recently been reported for
bacterial communities associated with marine diatoms (Grossart et al., 2005) and sponges (Taylor et al., 2005; Lee et al., 2009). Such type of studies will increasingly add to understanding of marine bacterial - host interactions.

It is well established that QS signaling (AHLs) produced from gram negative biofilm forming bacteria enhanced the settlement of zoospore of Ulva species (Joint et al., 2002; Tait et al., 2005; Wheeler et al., 2006; Joint et al., 2007). Although, preliminary worked on carpospore liberation from red algae has been done but still so novel. Weinberger et al. (2007) first time studied the phenomenon of spore liberation from cystocarp of Acrochaetium sp. and concluded that spore liberation might be depended on different AHLs. Thereafter, this study provides the evidence for this phenomenon. In the present study S. algae produced various types of AHLs (C4-, HC4-, C6-, 3-oxo-C6- and 3-oxo-C12-HSL) and were found potent for carpospore liberation from G. dura as compared to P. aeruginosa, Vibrio and Photobacteria species. Weinberger et al. (2007) reported that C4-HSL potentially influenced the carpospore liberation capacity in Acrochaetium sp. Contrary, present study found that C4- and C6-HSL were required for carpospore liberation from G. dura. Positive correlation between different concentration of C4- and C6-HSL and carpospore liberation revealed that increasing the concentration upto 10 μM enhanced the carpospore liberation. However, present study could not identify the amount of AHL produced by these gram negative bacteria but generally reported that 0.1 to 30 μM of AHLs is produced by gram negative biofilm forming bacteria (Ahlgren et al., 2011). The C8, C10, 3-oxo-C12-HSL and gram positive B. flexus did not influence the spore liberation and provides evidence that this phenomenon is controlled by AHLs. The effect of bacterial AHLs on liberation of carpospore could not be assigned to species or even genus level as different bacterial strain within species and genera showed dissimilar effects. The diffusion ability, stability and availability of QS signals around the plant are an important factor for carpsoe liberation in natural environments and prokaryotic-eukaryotic cell signaling. Tait et al. (2005) reported that short acyl chain molecules (C6-HSL and 3-hydroxy-C6-HSL) diffused more quickly from agarose gel than the longer acyl chain molecules such as 3-oxo-C10-HSL and more. Additionally, C10-HSL and longer acyl side chain of lactone ring are less soluble in seawater and may not necessarily have any significant effect on carpospore liberation. Apparent molecular mechanism of AHLs production and structure of AHLs produced from seaweed-associated bacteria represented in figure 6.7.
Macroalgal surface are living hosts and perform essential roles in coastal ecosystems (Alongi, 1998; Schiel & Lilley, 2007; Burke et al., 2011). Firmicutes were found the second most abundant bacteria on these algal surfaces and contributed to approximately 15-30% of the dimethylsulfoniopropionate assimilation (Tujula et al., 2010). The high variability of bacterial communities associated with different species of Ulva and Gracilaria or even among same species suggests that functional redundancy exists within these communities. This conclusion follows the redundancy hypothesis which presumes that more than one species are capable of performing a specific role within an ecosystem (Naeem, 1998). Seaweeds-associated epi- and endophytic bacterial strains help in the settlement of spore, morphogenesis and development in the Ulvaceae family. Members of Ulvaceae lose their typical foliose thallus morphology when cultured axenically in defined synthetic media (Provasoli & Pintner, 1980; Chapter 3). The aberrant morphology of Ulvaceae is successfully reversed to the foliose thallus following the inoculation of appropriate morphogenesis inducing seaweed associated bacteria to the culture media (Nakanishi et al., 1996; Matsuo et al., 2003). In addition to this, they are known for fix atmospheric nitrogen and produce IAA which regulates growth of G. dura (Chapter 4).
The present finding identified the epi- and endophytic bacterial communities associated with different seaweeds. It will enable us to the identification of novel microbial interactions through the characterization of similar signal transduction pathways in other eukaryotes. Consequently, elucidating this pathway may enable us to develop the methods for cultivating this economically important alga.