Summary and conclusion

Studies on certain seaweed - bacterial interaction from Saurashtra coast
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The seaweeds in coastal ecosystem provide an ideal biotic surface for settlement of free floating microbes that subsequently develop biofilms through self-produced matrix “extracellular polymeric substances (EPS)” and QS signalling molecules following their successful colonization. Microbial communities living on the seaweed surface are highly complex, dynamic and consist of a consortium of organisms including bacteria, fungi, diatoms, protozoans, spores and larvae of marine invertebrates. However, bacteria are the most ubiquitous and present as pelagic, benthic, epi- and endophytic/zoic form in diverse living forms including seaweeds. The associated bacteria, while utilizing the extracellular substances released from seaweeds as a source of carbon, secretes a variety of signalling molecules and other substances including plant growth regulators that provide essential nutrients and stimulants required for sustainable growth and development in seaweeds. Thus, the study of seaweed-bacterial association has become a fascinating field of research for many investigators worldwide. Nevertheless, the study of seaweed-bacterial interaction is still in its infancy as compared to that of Rhizobacteria – legume plant, and other bacterial communities associated with marine diatoms, sponges and corals. The knowledge progressed on seaweed-bacterial interaction is far from elucidating the bacterial role in morphogenesis, growth, reproduction and development of seaweeds. In addition, there were no specific protocols existed for developing axenic plantlet and epi- and endophytic bacterial isolation.

The work presented in this thesis includes the development of standardized protocols for making axenic culture and subsequent epi- and endophytic bacterial isolation. Treatment of seaweed thallus with 1% detergent for 10 min, 1% betadine for 2 min and incubation with 1% antibiotic mixture for 24 h facilitated to obtain axenic explants of Ulva species while the 2% detergent [10 min], 2% betadine [2 min] and 1% antibiotic mixture [24 h] for obtaining axenic explants of Gracilaria species. A number of epi- and endophytic bacteria were isolated from both untreated and treated explants of Ulva and Gracilaria. In subsequent studies, the role of these bacteria in morphogenesis, growth, reproduction and development of seaweeds was investigated and attributed the same as a function of seaweed-bacterial association. The isolated epi- and endophytic bacterial strains belong to the orders Bacillales, Pseudomonadales, Alteromonadales and Vibrionales were commonest and prevailed in all three seasons (pre-monsoon, monsoon and post-monsoon). Contrary, the members of Actinomycetales and Enterobacteriales were found only in pre-monsoon and post-monsoon seasons respectively. Biodiversity parameters, OTU (32), Shannon–Weaver
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The studies indicate that maximum seaweed-associated bacterial diversity was observed during monsoon season. The maximum bacterial diversity observed among order *Vibrionadales*. It is evident from these findings that certain seaweeds harboured specific bacteria which are always associated with particular species indicating species-specific patterns. For example, it was found that *Vibrio parahaemolyticus* was always isolated from *G. corticata* while *Shewanella algae* and *P. aeruginosa* were from *G. dura*.

These associated bacteria were found to produce plant hormones, QS signals, bioactive compounds and some active molecules responsible for normal morphological structure and development of *U. fasciata* and *G. dura*. The role of gram negative bacteria associated with seaweeds has been reported for induction of morphogenesis in various seaweeds but the present study found that not only gram negative but also gram positive seaweed associated bacteria induced morphogenesis. For example, zoospore of *U. fasciata* treated separately with gram negative (*Marinomonas* sp.) and gram positive (*Bacillus* spp.) seaweed associated bacteria restored foliose morphology, development and reproduction when grown in axenic culture. However, the culture filtrates from these individual bacterial isolates and from a consortium of all five of them had less effect on morphogenesis and growth of *U. fasciata* than the bacteria themselves as compared to control. Additionally, it is for the first time found that physical association of bacterial cells to host plant showed a bearing on cell arrangement (shape), increasing cell area and zoospore induction. The increase in the cell size varied from 202.62 to 299.15 μm² with the *Marinomonas* and *Bacillus* species. Out of five putative morphogenesis inducing bacteria, maximum number of zoospores of 107700 zoospores g⁻¹ fresh weight of thallus were produced when incubated with *B. licheniformis* (GU723480) than other putative zoospore inducing bacteria (51000 to 105000 zoospores g⁻¹ fresh weight) in the axenic form over the control with production of 13300 zoospores g⁻¹ fresh weight.

The associated bacteria not only restored the normal morphogenesis in *U. fasciata* but also stimulated the bud formation and growth in red alga *G. dura*. The putative bud-inducing epiphytic *Exiguobacterium homiense* and endophytic *B. pumilus* and *B. licheniformis* were also examined for their atmospheric nitrogen fixing ability. The *B. pumilus*, *B. licheniformis* and *E. homiense* produced 445.5, 335 and 184.1 mg ml⁻¹ IAA and 12.51, 10.14 and 6.9 mg ml⁻¹ ammonium, respectively. New bud regeneration from plantlet was observed when axenic cultures supplemented with total protein of these bacteria. The
epi- and endophytic bacterial strains were able to induce 5 and 10 new buds per frond, respectively, in comparison to control. The combination of 25 ± 1 ºC and 30% salinity showed optimum condition for growth and bud induction. This finding for the first time revealed that IAA coupled with nitrogen fixation activity of these bacteria helped to regenerate new buds and their subsequent growth in *G. dura*.

The significance of exopolysaccharide between Rhizobacteria and legume plant is well established but the role of EPS in the bacteria-seaweed interaction is scantily studied till date. The EPS secreted by *B. flexus* (GU592213, an endophytic bacterium associated with *U. lactuca*) was obtained and investigated for settlement of zoospore in *U. fasciata*. The molecular weight of the EPS was estimated to be approximately 1528 and 33686 kDa with the elemental composition of Na, P, Mg, C, O, Cl and S. The ¹H NMR, FT-IR and UV–Vis spectrometric analysis of EPS confirmed the occurrence of different aliphatic and aromatic functional groups. The EPS was amorphous in nature with an average particle size of 13.969 µm (d 0.5) and roughness 193 nm. The EPS contained different monosaccharides such as fucose, ribose, xylose, galactose, mannose and glucose. The oligo and polysaccharides masses of the EPS were detected with MALDI TOF-TOF MS analysis. The zoospore settlement on EPS coated cover slips progressively increased with incubation time from 2 to 48 h over controls under axenic condition. The EPS, thus investigated in this study was found to facilitate the primary settlement of spores and subsequently other *U. fasciata* associated bacteria determined the further development and growth of the same and/or different plant.

QS signaling molecules are reported to influence the zoospore settlement in the *Ulva* species but significance of these molecules in the life cycle of *Gracilaria* species has not been reported so far. Thus, seaweed associated bacteria screened for AHLs production and seven gram negative bacterial strains were found to produce different AHLs. Out of them, *Shewanella algae* produced maximum number of AHLs viz. C₄-HSL, HC₄-HSL, C₆-HSL, 3-oxo-C₆-HSL and 3-oxo-C₁₂-HSL followed by *Photobacterium lutimaris* (JQ613504) that produced three kind of AHLs (C₄-HSL, HC₄-HSL and C6-HSL). The remaining five bacterial isolates, *P. aeruginosa*, *Photobacterium* and *Vibrio* species produced two types of AHLs. A positive correlation was found between bacterial AHLs production and carpospore liberation from *G. dura* over control. Carpospores liberation was the maximum with 179.625 ± 3.6 and 108.375 ± 21.62 mm² from the cystocarp bearing fragments of *G. dura* treated.
with putative AHLs producing bacteria *S. algae* and *P. aeruginosa* respectively. Similarly, 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 carpospores from the cystocarp bearing fragments of *G. dura*. On the other hand, standard C₄- (93.333 ± 15.33 mm²) and C₆- HSL (99.448 ± 30.94 mm²) also significantly induced the carpospore liberation, but C₈, C₁₀ and 3-oxo-C₁₂-HSL did not show any significant effect on carpospore liberation as compared to control (10.375 ± 5.105).

Conclusion

- A number of epi- and endophytic seaweed associated bacteria were isolated and identified using 16S rDNA gene sequence homology. The endophytic bacteria isolated in the present study were: *Allomonas enterica*, *V. parahaemolyticus*, *S. algae*, *P. aeruginosa*, *P. stutzeri*, *Micrococcus luteus*, *B. cereus*, *B. licheniformis*, *V. sinaloensis*, *V. nigripulchritudo* and *V. rotiferianus*. Of the total endophytic bacteria, ten bacterial strains were isolated from *Gracilaria* spp. while *B. cereus* was obtained from *U. fasciata* during monsoon and post-monsoon seasons. Additionally, some endophytic bacteria followed the patterns of species-specific. To the best of my knowledge, this is the first time a large number of endophytic bacteria were isolated and their phylogenetic affiliations were characterized.

- It was also become evident that both gram positive and negative seaweed associated bacteria induce morphogenesis, cell area, cell arrangement and zoospore formation in *U. fasciata*. Physical association of bacteria was found to be essential for foliose morphology and growth of *U. fasciata*, suggesting a symbiotic interaction between seaweed and bacteria.

- The bud induction and growth of *G. dura* was found to be due to total protein conjugated with IAA and nitrogen-fixing activity of seaweed associated bacteria. These findings helped us to understand the functional aspect of seaweed - bacteria association.

- It was found that the EPS obtained from *B. flexus* enhanced the zoospore settlement. Although, seaweed-bacteria interaction is an emerging area of research, the role of EPS has not been investigated before.
Seven gram negative bacterial strains were found to produce different AHLs. Out of them, *Shewanella algae* produced maximum number of AHLs viz. C$_4$-HSL, HC$_4$-HSL, C$_6$-HSL, 3-oxo-C$_6$-HSL and 3-oxo-C$_{12}$-HSL. All seven gram negative seaweed associated bacteria enhanced the spore liberation from cystocarp bearing fragment of *G. dura*. Standard C$_4$- and C$_6$-HSL significantly induced the carpospore liberation from *G. dura* while C$_8$, C$_{10}$ and 3-oxo-C$_{12}$-HSL did not show any significant effect. It may be due to small acyl chain AHL are more diffusible in the seawater than long acyl chain AHL.

The present study helped us to understand many features of seaweed – bacterial interaction such as role of bacteria in reproduction, development, growth and morphogenesis. While working on this topic, for the first time, it revealed many interesting facts such as seaweed associated bacteria enhancing cell arrangement and cell size as well as zoospore settlement on bacterial EPS. I have also found that gram positive bacteria induce foliose morphogenesis and zoospore formation in *U. fasciata*. Studies presented in this thesis will facilitate to micropropagation of these economic important seaweeds through spore seeding method. Additionally, the work carried out with bacterial EPS will open up a new field of research of seaweed-bacteria interaction and helps to unveiling concealed fact of this interaction in the natural environment. The involvement of QS signal in spore liberation from *G. dura* opens a new means to control the marine biofouling on marine structures and ships.