6.0. DISCUSSION

Endophytes are a poorly investigated group of microorganism that represent an abundant and dependable source of bioactive and chemically novel compounds with potential for exploitation in a wide variety of medical, agricultural, and industrial arenas. The mechanisms through which endophytes exist and respond to their surroundings must be better understood in order to be more predictive about which higher plants to seek study and spend time for isolating micro floral components. This may facilitate the product discovery processes. Although work on the utilization of this vast resource of poorly understood microorganisms has just begun, it has already become obvious that an enormous potential for organism product and utilitarian discovery in this field holds exciting promise. This is witnessed by the discovery of a wide range of products and microorganisms that already hold linking for future prospects as detailed in the review.

Certainly, one of the major problems facing the future of endophyte biology and natural-products discovery is the rapid diminishment of rainforests which hold the greatest possible resource for acquiring novel microorganisms and their products. The total land mass of the world that currently supports rainforests is about equal to the area of the United States
Each year, an area the size of Vermont or larger is lost to clearing, harvesting fire, agricultural development, mining or other human-oriented activities. It is estimated that, only 12 to 15% of what were the original rainforests in the “hot spots of biodiversity” existing 1,000 to 2,000 years ago, are currently present on the earth. Few have ever expressed information or opinions about what is happening with regard to the potential loss of microbial diversity as entire plant species disappear. It can only be guessed that, this loss is also happening. Perhaps with the same frequency as the loss of mega-life forms, especially since certain microorganisms may have developed unique specific symbiotic relationships with their plant hosts. Thus, when a plant species disappears so too does its entire suite of associated endophytes. Multistep processes are needed now to secure information and life forms before they continue to be lost. Areas of the planet that represent unique places housing biodiversity need immediate preservation. Countries need to establish information bases of their biodiversity and at the same time begin to make national collections of microorganisms that live in these areas.

The isolation methods affect the species composition of the endophyte assemblage in a given host. The number of endophyte taxa isolated from a host species is usually large; however, only few, normally host specific species or strains are dominant. Endophyte assemblages are specific at the
host species level, but species composition and frequencies are significantly affected by site-specific conditions. Moreover, the relative importance and number of endophytic species vary among individuals within sites.

Investigation on the distribution of endophytic microorganisms are carried out enormously but, such studies are lacking behind in marine halophytes. Hence, the present study was made an attempt to findout the biodiversity of endophytic microorganisms in mangrove plants. Mangrove plants are specially adopted group of woody plants found in between land and sea. They have special adaptation such as salt excreting gland, stilt root, prop root, pneumatophores, high content of phenolic compounds and more UV-absorbing compounds (Kathiresan, 2005). The present study has collected apical buds from 11 mangrove plant species which include salt extruders and salt intruders for the isolation of endophytic bacteria and fungi. Among the plant species, the *Bruguiera cylindrica* harboured maximum counts of endophytic bacteria and *Avicennia marina* harboured minimum counts. The presence of maximum counts in *B. cylindrica* might be due to the reason that, the plant species always inhabited in water logged condition but the *A. marina* prefer to inhabit in water less habitat (Field observation). While in the case of endophytic fungi, the counts were found maximum in the mangrove plant species of *Ceriops decandra* but found minimum in *Bruguiera cylindrica*. Eventhough, both the plant species are
belonging to the same family, the density of bacterial and fungal flora is different from each other. It is clearly reveals that, the variation in the population is not only due to the habitat but also due to the age of the plant and collection site. It is true that, the apical bud from 11 mangrove plant species are not collected from a single collection site but from 4 different geographical locations. So it is evident that, the population density of endophytic microflora may vary with the geographical location, soil nutrients and other non living contaminants too. Spiering et al., (2005) reported that, the endophyte ratio increased with leaf age. Liu et al., (2007) reported that mangrove, a kind of special host plants, is a resource of abundant endophytic fungi. More than 200 species of endophytic fungi are isolated and identified from mangrove, being the second largest community of marine fungi. The reported endophytic fungi of mangrove are mainly Alternaria, Aspergillus, Cladosporium, Colletotrichum, Fusarium, Paecilomyces, Penicillium, Pestalotiopsis, Phoma, Phomopsis, Phyllosticta and Trichoderma. Most endophytic fungi have wide range of hosts, and a few only have single host. The colonization of endophytic fungi always varies with different parts (leaves, twigs, stems) and age of host plants and with seasons.

Verma et al., (2007) reported that, a systematic study was made on the endophytes of Azadirachta indica A. Juss (the neem tree) growing in several of its natural habitats in India. A total of 233 isolates of endophytic fungi
representing 18 fungal taxa were reported from segments of bark, stem, and leaves of this tree. Hyphomycetes (62.2%) were the most prevalent followed by the Coelomycetes (27.4%) and *Mycelia Sterilia* (7.7%). As mathematically determined, the maximum species richness and frequency of colonization of endophytes appeared in leaf segments rather than stem and bark tissues from each location. Endophytic colonization frequency was also greater in leaves (45.5%) than bark (31.5%). The leaf samples from all locations were nearly constant in their endophytic composition, whereas bark samples showed maximum diversity at different locations. The dominant endophytic fungi isolated were *Phomopsis oblonga*, *Cladosporium cladosporioides*, *Pestalotiopsis sp.*, *Trichoderma sp.*, *Aspergillus sp.*, *Periconia*, *Stenella*, and *Drechslera* are reported here for the first time as endophytes from this host plant. This report illustrates the value of sampling different tissues of a given plant in several locations to obtain the greatest species diversity of endophytes.

The present study also analysed the distribution pattern of endophytic microflora among the chosen mangrove plants belonging to different families. It reveals that, the bacterial counts were found maximum in Combretaceae family members and the fungal counts were found maximum in Avicenniaceae family members. This clearly indicates that, the distribution of endophytic microflora have their balance in keeping the
bacterial and fungal density i.e. if the bacterial density is more in one plant species in which the fungal density is less. Ananda and Sridhar (2002) reported that, mangrove plant species are a valuable source of useful metabolites, their endophytes have gained more importance. The richness of endophytic fungal species from whole root segments after direct plating and damp chamber incubation was greatest for *R. mucronata* than for other plants. Incubation of whole root segments in bubbling chambers yielded conidia of two freshwater hyphomycetes. The greatest number of isolates, species richness, and diversity of fungi were shown by the whole root segments of *R. mucronata* from the mid-tide level.

Krechel *et al.* (2002) reported that, the abundance and diversity of bacteria isolated from the rhizosphere, phyllosphere, endorhiza, and endosphere of field grown potato. The endophytic populations averaged $10^3$ and $10^5$ CFU.g$^{-1}$ (fresh wt.) for the endosphere and endorhiza respectively, which were lower than those for the ectophytic microenvironments, with $10^5$ and $10^7$ CFU.g$^{-1}$ (fresh wt.) for the phyllosphere and rhizosphere, respectively. Zinniel *et al.* (2002) isolated 853 endophytic strains from aerial tissues of four agronomic crop species and 27 plant species. Host range greenhouse studies demonstrated that, 26 of 29 endophytes were recoverable from at least one host other than corn and sorghum at levels of up to $5.8 \log_{10}$ CFU.g$^{-1}$ (fresh weight).
The isolated endophytic bacterial and fungal strains were further subjected for screening against chosen chicken pathogens, human pathogens and shrimp pathogens by cross streak assay. The reason for selecting these pathogens are (1) Poultry production/shrimp production in India particularly in Tamil Nadu state have been facing serious economic loss due to microbial diseases which cause severe mortality (2) Use of chemotherapeutic agents for the treatment of microbial diseases now lead to antibiotic resistance and hence current therapy could not help to reduce the mortality (3) Alternative therapy is necessary particularly from the microorganisms. Likewise, the existing therapy for the treatment of diseases in humans could not help in future because of developing resistance and newer bacterial pathogens. The cross streak assay reveals that, the isolated endophytic bacterial and fungal strains did not showed any antibacterial sensitivity against chosen human and chicken pathogens but 2 endophytic bacterial strains showed sensitivity against 2-3 shrimp pathogens. It is very clear from the present study that, all the endophytic microflora does not have the antibacterial property particularly endophytes from mangrove plants. The inhibitory effect of extracellular secondary metabolites from the endophytic bacteria (MB4 and MB8) may contain unique chemical moiety which could only can show the antibacterial activity against some shrimp pathogens. Ellaiah (2002) reported that, the preliminary study for
antimicrobial activity by cross streak method indicated that 16 isolates (53.3%) have excellent antagonistic properties. All these 16 isolates were subjected to detailed submerged fermentation studies. It was observed that, 12 isolates (40.0%) exhibited antibacterial activity, 9 isolates (30.0%) showed antifungal activity while 5 isolates (16.6%) showed both antibacterial and antifungal activities. Comparatively, the percentage of sensitivity was found minimum in the present study. Krechel et al. (2002) measured the antagonistic potential of potato-associated bacteria, a total of 440 bacteria were screened by dual testing for in vitro antagonism towards the soilborne pathogens Verticillium dahliae and Rhizoctonia solani. The proportion of isolates with antagonistic activity was highest for the rhizosphere (10%), followed by the endorhiza (9%), phyllosphere (6%), and endosphere (5%). It was also reported that, B. thuringiensis significantly decreased the incidence of E. carotovora infection and symptom development of potato soft rot caused by the pathogen (Dong et al., 2004; Senthilkumar et al., 2009; Aravind et al., 2009; Shittu et al., 2009; Wicklow and Poling, 2009; Smith et al., 2008; Lian et al., 2008; Naik et al., 2009). A total of 35 bacteria were screened by dual testing for in vitro antagonism towards the fungal and bacterial pathogens. The proportion of isolates with antagonistic activity was highest against Streptomyces sp. (43%) followed by those against Xanthomonas sp. (29%). The biotechnological potential of endophytic isolates assessed by their
antagonistic activity and by *in vitro* production of enzymes, antibiotics, siderophores, and the plant growth hormone indole-1,3-acetic acid was generally high. Seven endophytes were found to antagonize fungal as well as bacterial pathogens and showed a high production of active compounds and were therefore considered promising biological control agents (Sessitsch *et al.*, 2004).

Owen and Hundley (2004) reported that, endophytes are microbial entities that live within living tissues of plants. In most cases their relationship with the host plant is symbiotic and probably mutualistic. Many are capable of synthesizing bio-active compounds that can be used by the plant for defence against pathogenic fungi and bacteria. Some of these compounds have proven useful for novel drug discovery. By encouraging the endophytes to grow outside the plant in nutrient rich media, it is possible to harvest the bio-active compounds that they produce. However, much needs to be discovered and understood about the host/endophyte relationship before we can fully utilize endophytes in the discovery of medicinally important compounds. The present study also made an attempt to find out the effectiveness of the extracts from 2 promising bacterial endophytes against the chosen shrimp pathogens at different concentrations. It reveals that, the crude secondary metabolites from *Bacillus pumilus* showed sensitivity against *Bacillus subtilis* (250µg.ml⁻¹), *Serratia sps.*, (250µg.ml⁻¹),
Vibrio harveyii (125µg.ml⁻¹), Aeromonas hydrophila (250µg.ml⁻¹) and the crude secondary metabolites from Bacillus thuringensis showed sensitivity against Bacillus subtilis (250µg.ml⁻¹), Serratia sps., (250µg.ml⁻¹), Vibrio harveyii (250µg.ml⁻¹), Aeromonas hydrophila (500µg.ml⁻¹). The minimum bactericidal concentration (MBC) reveals that, the crude secondary metabolites from Bacillus pumilus showed sensitivity against Bacillus subtilis (500µg.ml⁻¹), Serratia sps. (500µg.ml⁻¹), Vibrio harveyii (500µg.ml⁻¹), Aeromonas hydrophila (500µg.ml⁻¹) and the crude secondary metabolites from Bacillus thuringensis showed sensitivity against Bacillus subtilis (500µg.ml⁻¹), Serratia sps. (500µg.ml⁻¹), Vibrio harveyii (500µg.ml⁻¹), Aeromonas hydrophila (500µg.ml⁻¹).

Phongpaichit et al., (2006) reported that, antimicrobial activity in cultures of endophytic fungi isolated from Garcinia species. Garcinia plants having 5 different species, G. atroviridis, G. dulcis, G. mangostana, G. nigrolineata and G. scortechinii, found in southern Thailand. Fermentation broths from 377 isolated fungi were tested for antimicrobial activity by the agar diffusion method. Minimum inhibitory concentrations (MICs) were obtained for crude ethyl acetate extracts. Seventy isolates (18.6%) displayed antimicrobial activity against at least one pathogenic microorganism, such as Staphylococcus aureus, a clinical isolate of methicillin-resistant S. aureus, Candida albicans and Cryptococcus neoformans. The results revealed that 6-10%, 1-2% and 18% of the crude ethyl acetate extracts inhibited both strains of
S. aureus (MIC 32-512µg.ml⁻¹), Ca. albicans and Cr. neoformans (MIC 64-200 µg.ml⁻¹) and Microsporum gypseum (MIC 2-64 µg.ml⁻¹), respectively. Isolates D15 and M76 displayed the strongest antibacterial activity against both strains of S. aureus. Isolates M76 and N24 displayed strong antifungal activity against M. gypseum. Fungal molecular identification based on internal transcribed spacer rRNA gene sequence analysis demonstrated that isolates D15 (DQ480353), M76 (DQ480360) and N24 (DQ480361) represented Phomopsis sp., Botryosphaeria sp. and an unidentified fungal endophyte, respectively. These results indicate that, some endophytic fungi from Garcinia plants are a potential source of antimicrobial agents. Shin et al. (2007) reported that endophytic bacteria associated with the roots of coastal sand dune plants. Testing for antagonism towards plant pathogenic fungi, 25 strains were antagonistic towards Rhizoctonia solani, 57 strains were antagonistic towards Pythium ultimum, 53 strains were antagonistic towards Fusarium oxysporum, and 41 strains were antagonistic towards Botrytis cinerea.

The present study also made an important finding that, the most promising endophytic bacterial strains were identified as Bacillus pumilus and Bacillus thuringiensis through 16S rRNA sequencing. Mehnaz et al. (2001) reported that, the isolation of plant growth promoting rhizobacteria (PGPR) from rice (variety NIAB IRRI-9) and the beneficial effects of these inoculants on two Basmati rice varieties. Nitrogen-fixing activity (acetylene-reduction
activity) was detected in the roots and submerged shoots of field-grown rice variety NIAB IRRI-9. Three isolates S1, S4, and R3 reduced acetylene to ethylene in nitrogen-free semi-solid medium. Morphological and physiological characteristics of the isolates indicated that, three nitrogen-fixing isolates S1, S4, and R3 belonged to the genus Enterobacter, while the non-fixing isolate R8 belonged to the genus Aeromonas. 16S rRNA sequence of one isolate from root (R8) and one isolate from shoot (S1) was obtained which confirmed identification of the isolates as Aeromonas veronii and Enterobacter cloacae.

Kang et al. (2007) reported that, 150 bacterial isolates collected from healthy stems of peppers cultivated in the Chungcheong and Gyeongsang provinces of Korea, 23 putative endophytic isolates that were considered to be predominating and representative of each pepper sample were selected. By phenotypic characterization and partial 16S rDNA sequence analysis, the isolates were identified as species of Ochrobacterium, Pantoea, Pseudomonas, Sphingomonas, Janthinobacterium, Ralstonia, Arthrobacter, Clavibacter, Sporosarcina, Acidovorax, and Brevundimona. Reiter et al. (2002) reported that, the cultivation-independent terminal restriction fragment length polymorphism analysis of 16S ribosomal DNA directly amplified from plant tissue DNA was used in combination with molecular characterization of isolates to examine the influence of plant stress, achieved by infection with
the blackleg pathogen *Erwinia carotovora* sub sp. *atroseptica*, on the endophytic population in two different potato varieties. Partial sequencing of the 16S rRNA genes of isolated endophytes revealed a broad phylogenetic spectrum of bacteria, including members of the alpha, beta, and gamma subgroups of the Proteobacteria, high- and low-G+C-content gram-positive organisms, and microbes belonging to the Flexibacter-Cytophaga-Bacteroides group.

It is important to noticed that, all the previous works have done up to the level of isolation and molecular identification of endophytic microbial flora and their bioactive potential against some of the pathogens but the present study is the first time to prove the effectiveness of the extracts under *in vivo* trials so as to determine the commercial/field applicability. For that, the present study supplemented the mass cultivated crude extracts along with the shrimp pathogen *Bacillus thuringensis* and it showed a encouraging results that, the extract added with the shrimp pathogens showed 100% survival but the shrimps added with only shrimp pathogen showed 20% survival. And also, the bioenergetics of shrimps kept under various treatments reveals that, the shrimp treated with the shrimp pathogen along with the extract have more promotory effect and also less FCR. This is the first attempt made in the field of bioactive natural products from endophytic microorganisms.