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Bioactive potential of seagrass bacteria against human bacterial pathogens

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Abstract: *Study of marine organisms for their bioactive potential, being an important part of marine ecosystem, has picked up the rhythm in recent years with the growing recognition of their importance in human life. Investigation was carried out to isolate 32 strains of endo and epiphytic bacteria in 2 seagrass species viz., Syringodium isoetifolium and Cymodocea serrulata. Morphologically different bacterial strains were tested against 5 antibiotic resistant human bacterial pathogens, of which 10 associated bacteria shown inhibitory activity against one or more bacterial pathogens. Minimum inhibitory concentration (MIC) and Minimum bacterial concentration (MBC) determination with extracellular bioactive compounds from the associated bacteria reveals that, the strain ENC 5 showed inhibitory activity against all the bacterial pathogens with the maximum sensitivity against Pseudomonas aeruginosa at the MIC value of 500 μg ml^{-1}.*

Key words: *Antibiotic resistant bacteria, Antimicrobial activity, Bioactive compounds, Sea grass, Symbiotic bacteria*
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Introduction

New trends in drug discovery from natural sources emphasize on investigation of the marine ecosystem to explore numerous complex and novel chemical entities. These entities are the source of new lead for treatment of many diseases such as cancer, AIDS, inflammatory condition, arthritis, malaria and large variety of viral, bacterial, fungal diseases (Prakash Williams *et al.,* 2007; Nazar *et al*., 2009). Because of the highly chemical and physical harsh condition in marine environment, the organisms produce a variety of molecules with unique structural features and exhibit various biological activities. Majority of the marine natural products have been isolated from sponges, coelenterates (sea whips, sea fans and soft corals) tunicates, opisthisbranch mollusks, echinoderms, sea grass, bryozoans and wide variety of marine micro organisms in their tissues (Prakash Williams *et al.,* 2007). Despite the fact that, the biodiversity in marine environment for exceeds that of the terrestrial environment, research in to the use of marine natural products has pharmaceutical agents is still in its infancy (Rajeev Kumar and Xu Zi-rong, 2004). Given that chemically mediated disease resistance is well documented among terrestrial plants (Hammerschmidt, 1999), marine plants are known to produce a large number of structurally diverse secondary metabolites (Faulkner, 2000; Blunt *et al.,* 2004).

Sea grasses are the marine flowering plants. They are the only angiosperms that successfully growth in tidal and sub tidal marine environment. Sea grass belongs to the families Hydrocharitaceae and Potamogetonaceae and they are in no way related to the terrestrial grass of Poaceae. There are 13 genera and 58 species available all over the world of these six genera (*Amphibolis, Heterzostera, Phyllospadi, Posidonia, Pseudalthenia* and *Zostera*) are mostly restricted to temperate seas and the remaining seven genera (*Cymodocea, Enhalus, Halodula, Halophila, Syringodium, Thalasia* and *Thalassodendeon*) are distributed in tropical seas. Several species of sea grass have obligate microbial populations inhabiting their roots, leaves and rhizomes. A variety of medicines and chemical are also prepared from sea grass and their associates (Ravikumar *et al.,* 2005). The effect of marine secondary metabolites on co-occurring micro organisms remain largely undocumented. Perhaps of greater concern than resistance to single antibiotics is the development of bacteria resistant to multiple antibiotic types. As antibiotic resistance has developed, medical researchers have fought back with alternative antibiotics and combination therapy, but constant over use of antibiotics in humans and livestock means that strains of bacteria resistant to nearly all antibiotics are now known. The resistance problem demand that renewed effort be made to seek antibacterial agents effective against pathogenic bacteria resistant to current antibiotics.

Materials and Methods

Isolation of bacteria: Seagrass species of *Syringodium isoetifolium* and *Cymodocea serrulata* were collected during monsoon month (January) from Palk Strait (Lat 99°44' and 79°45' E), South East Coast of India were brought to the laboratory in sterilized containers and washed thoroughly with sterile distilled water for the isolation of epiphytic and endophytic heterotrophic

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bacteria. Leaves and stems were carefully separated and surface sterilized with disinfectant (2% sodium hypochlorite containing 0.1% Tween 20). To remove the disinfectant, samples were rinsed five times with de-ionized distilled water followed by sterile water. Approximately 1g of samples were surface washed with sterile distilled water and 1 ml of samples were plated onto Zobell Marine Agar 2216e (Hi-media) to recover epiphytic bacteria. Further, the same samples were crushed by using mortar and pestle for the isolation of endophytic bacteria. 1ml of the serially diluted samples were plated on to the same agar media and incubated at 37±2°C for 24 hrs. After obtaining visible growth, morphologically different epi and endophytic bacterial colonies were restreaked thrice onto the same medium to obtain pure culture. The endophytic strains were annotated with EN and epiphytic strains were annotated with EP and numbered (Kang *et al*., 2007).

Antibacterial sensitivity assay: *In vitro* antibacterial sensitivity was carried out with morphologically different 32 strains of endo and epiphytic bacteria against 5 antibiotic resistance human pathogens *viz*., *Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella sp., Streptococcus pneumoniae, Streptococcus aeruginosa* (IMTECH, Chandigarh, India) by Cross streak assay (Sivakumar *et al*., 2005). Single streak of the isolated strains was done on sterile Muller Hinton Agar plates followed by overnight culture of antibiotic resistant human pathogens were streaked at perpendicular to the original streak of isolates and incubated at 37±2°C. Bacterial strains showed maximum inhibitory effect against tested pathogens were subjected for mass cultivation in broth and was filtered by using Millipore filter. Filtrate was mixed with equal volume of ethyl acetate (v/v) in separating funnel and shaken well and then allowed to stand without any disturbance for 15 minutes. After that, the lower aqueous phase was discarded and the upper solvent phase was concentrated in a vacuum evaporator at room temperature for 24 hr to obtain powder form of crude extract and stored in a refrigerator for further analysis (Kang *et al.,* 2007).

Minimum inhibitory concentration (MIC) was carried out with the extracts from chosen bacteria. 0.5ml of various concentration (31, 62, 125, 250, 500, 1000, 1500, 2000 µg ml^{-1}) of extracts was prepared with Dimethyl sulphoxide (DMSO) and mixed with 50µl of 24 hr old bacterial inoculum and allowed to grow overnight at 37°C for 48 hr. To calculate the MIC, turbidity due to bacterial growth was observed in each concentration. To avoid the possibility of mis-interpretations due to the turbidity of insoluble compounds, the minimum bactericidal concentration (MBC) was determined by sub culturing the MIC dilutions on to the sterile agar plates. The lowest concentration of the extracts which inhibits the growth of tested bacteria are observed and tabulated.

Results and Discussion

The earliest bioactive marine metabolite, isolated by Burkholder, was the highly brominated antibiotic, 2, 3, 4-tribromo-5-(1'hydroxyl-2', 4'-dibromophenyl) pyrrole. The compound showed *in vitro* properties against Gram positive bacteria, with minimum inhibitory concentration (MIC) ranging from 0.0063-0.2 mg ml^{-1} (Burkholder, 1966). Based on the morphological characters, 32 strains of endo and exo symbiotic heterotrophic bacteria were isolated by the present study. All the isolated strains were tested for their antagonistic activity against five antibiotic resistance human pathogens. Among 32 strains, 10 strains were shown antagonistic activity against one or more antibiotic resistance human pathogens. Balagurunathan and Subramanian (2001) reported that, out of 57 isolates from the littoral sediments of Parangipettai coastal water, 8 strains showed very promising antibiotic activity against pathogenic bacteria and fungi.

The strain ENS3, ENS13 has MIC value of 125µg against *P. aeruginosa*, the ENS 5, EPC 10 showed MIC value of 250 µg ml^{-1} against *P. aeruginosa*, the ENC 5 showed MIC value of 250 µg ml^{-1} against *S.aureus* and *Klebsiella sp., S.pneumoniae, S. aeruginosa* but the MIC value was found lower by 125 µg against *P.aeruginosa*. The ENC7 showed MIC value of 250 µg against *P.aeruginosa* and 500µg against *S. pneumoniae*, the stain no EPC 20, ENS 10

Table - 1: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) (µg ml^{-1}) of the extracts from epi and endophytic bacteria against chosen antibiotic resistant bacteria

Strain no.	*Staphylococcus aureus*		*Klebsiella* sp.		*Pseudomonas aeruginosa*		*Streptococcus pneumoniae*		*Streptococcus aeruginosa*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
ENS 3	-	-	-	-	125	500	-	-	-	-
ENS 5	-	-	-	-	250	-	-	-	-	-
ENC 5	250	500	250	500	125	500	250	-	250	-
ENC 7	-	-	-	-	250	-	500	-	-	-
EPC10	-	-	-	-	250	-	-	-	-	-
ENS10	-	-	-	-	250	-	-	-	-	-
ENS11	-	-	-	-	125	1000	-	-	-	-
ENS13	-	-	-	-	125	500	-	-	-	-
EPS20	-	-	-	-	250	-	-	-	-	-
EPS18	-	-	-	-	125	-	-	-	-	-

ENS = Endophytic *Syringodium,* EPS = Epiphytic *Syringodium,* ENC = Endophytic *Cymodocea,* EPC = Epiphytic *Cymodocea*

showed MIC value of 125 µg against *P. aeruginosa* (Table 1). The strain ENS3 and ENS13 showed MBC value of 500 µg against *P. aeurginosa*, the strain ENS11 showed MBC value of 1000 µg against *P. aeruginosa*. Surprisingly, the strain ENC 5 showed MBC value of 500 µg to three of the pathogenic bacteria *viz.*, *S.aereus*, *Klebsiella* sp. and *P. aeurginosa* (Table 1). However, the other strains did not show any sensitivity against the tested human pathogens. It is interesting to notice that, the concentration of the extract is minimum when compared to the previous reports. For instance, the minimum inhibitory concentration of *M. jodocodo* against *E.coli* was 2.75 mg ml^{-1} while that of *T. robustus* against *M. bourtardi* was 15.75 mg ml^{-1} (Jonathan Gbolagade *et al.,* 2007). Likewise, Liasu and Ayandele (2008) reported that, the minimum inhibitory concentration of the ethanolic plant extract ranged from 0.01 mg ml^{-1} to 100 mg ml^{-1} against pathogenic bacteria and fungi. Gandhimathi *et al.* (2008) reported that the endosymbiotic marine actinomycetes from sponges exhibited potent antimicrobial activity against the growth of human pathogens. Generally, the endophytic bacteria isolated from seagrasses showed maximum sensitivity against several human bacterial pathogens compared with the epiphytic bacteria. Moreover, the bioactive compounds from endophytic bacteria showed maximum sensitivity with minimum concentration than the bioactive compounds from epiphytic bacteria and other biological origin. Hence, steps have been undertaken to find out the reason for the maximum activity of endophytic bacteria from seagrasses.

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