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Antimicrobial Activity of *Ulva reticulata* and Its Endophytes

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Abstract Seaweeds are known to exhibit various antimicrobial properties, since it harbours an enormous range of indigenous bioactive compounds. The emergence of drug resistant strains has directed to the identification of prospective metabolites from seaweed and its endophytes, thereby exploiting the properties in resisting bacterial diseases. The current study was aimed to assess the antimicrobial activity of extracts obtained from *Ulva reticulate*, for which metabolites of *Ulva reticulata* and its endophytes were extracted and assessed against human pathogens like *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, and *Bacillus subtilis*. It was observed that the hexane extract of isolate VITDSJ2 was effective against all the tested pathogens but a significant inhibition was observed for *Staphylococcus aureus* and *Escherichia coli.* Further, Gas chromatography coupled with Mass spectroscopy (GC-MS) revealed the existence of phenol, 3, 5-bis (1, 1-dimethylethyl) in the crude hexane extract which is well-known to possess antibacterial activity. The effective isolate VITDSJ2 was identified to be the closest neighbour of *Pseudomonas stutzeri* by phenotypic and genotypic methods. The crude extracts of the seaweed *Ulva reticulata* was also screened for antibacterial activity and the hexane extract was effective in showing inhibition against all the tested pathogens. The compound in the crude extract of *Ulva reticulata* was identified as hentriacontane using GC-MS. The extracts obtained from dichloromethane did not show significant activity in comparison with the hexane extracts. Hence the metabolites of *Ulva reticulata* and the bacterial secondary metabolites of the endophytes could be used in the treatment of bacterial infections.

Key words endophytic bacteria; anti-microbial; macroalgae; bioactive compounds; GC-MS

1 Introduction

Algae are simple heterogeneous population of plants composed of single cell or occur in colonies or as organisms with many cells, sometimes associating together as simple tissue (Bold *et al*., 1985). One of the major reasons attributed to the potential uses of marine macroalgae is the endophytic and epiphytic microbial populations associated with them (Mercado, 2012; Bacon, 2000). Endophytes are the population of bacteria which colonize the interior of the seaweed by garnering entry through the damaged algal cell wall; other mechanisms include regeneration of macroalgae from protoplast which facilitates the environmental uptake of bacteria into cell; also the endogenous bacteria can persist by vertical inheritance through gametes. Several reports have been made on the presence of bacteria as endophytes from various macroalgae. Singh *et al*., (2011) have reported endophytic *Bacillus* sp from *Gracilaria.* Most of the endophytic bacteria can produce biologically active compounds or secondary metabolites which has numerous applications in pharmaceutical chemistry (pharmacology) with therapeutic intent as well as in various other industrial fields (British Mycological Society Symposium Society Proceedings 2001; Strobel, 2003).

List of secondary metabolites produced by endophytes includes alkaloids, benzopyranones, chinones, cytochalasines, depsipeptides, enniatines, flavonoids, furandiones, isocumarines, peptides, polyketones, phenols, quinols, terpenoids, tetralones and xanthones which have been reported to elicit a number of pharmacological effects (Gamal, 2009). Nagaraj and Osborne, (2014) has reported the presence of alkaloids, saponins and terpenoids in *Caulerpa racemosa.* The above bioactive compounds are known to elicit anti-cancerous, anti-oxidant, anti-viral, anti-insecticidal, immunosuppressant (Lee *et al*., 2005), anti-microbial (Roy *et al*., 2010), anti-malarial and anti-mycobacterial (Zhang *et al*., 2005) property. Anticancer drugs like vincristine, vinblastine, taxol, camptothecine, *etc*., (Shweta *et al*., 2013) obtained from higher plants were used in chemotherapy treatment. Since the above mentioned compounds show secondary toxicity or resistance it has led to further discovery of novel bioactive compounds from various marine microorganisms.

In the recent years microorganisms are developing resistance to the existing pharmaceuticals, so there is an urge for identifying sources of novel and bioactive compounds. Traditionally, the medicinal plants were used for the treatment of various infections and diseases. Although

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there is an immense diversity existing in the terrestrial environment, diversity prevalent in the oceans is extraordinarily rich offering an infinite resource for novel compounds (Tziveleka *et al*., 2013). About 70% of the world's surface is covered with ocean consisting of more than 300 000 species of plants and animals awaiting discovery for the treatment of infectious diseases. The production of metabolites from microorganisms, especially fungi and bacteria, is a rapidly growing field. *Microdiplodia* sp., an endophytic fungus derived from seaweed, produced the tetramic acid derivative Sch210972 which showed inhibition of human leucocyte elastase (HLE) with an IC_{50} of 1.04 μg mL^{-1} (Nicolaouk *et al*., 2007). Among mangrove actinomycetes, the genus *Streptomyces* and *Micromonospora* are predominant producers of natural products such as indole alkaloids including indolo sesquiterpenes, indolocarbazoles, macrolides, benzene derivatives and alkaloids, sesquiterpenes respectively (Xie *et al*., 2012). Marine cyanobacteria are also a promising source of novel compounds with potential anticancer activity (Costa *et al*., 2013).

Seaweeds have promoted the interest in biotechnological, medical and pharmaceutical areas because of the novel and bioactive compounds in them which serve various potentials such as anti-tumoral, anti-microbial, anti-viral, and anti-inflammatory activity. Marine algae like *Codium fragile*, *Sargassum muticum*, *Endarachne binghamiae*, *Centroceras clavulatum* and *Laurencia pacifica* possess compounds having antibacterial activity against Gram negative bacteria *Proteus mirabilis*. Paula *et al*., (2012) found Phaeophyta be the most effective group of algae representing higher amount of active compounds like phloroglucinol, mannitol, fatty acids, fucosterol and proved them to be potent radical scavengers and cholinesterases inhibitors thereby elaborating their potentials as additives in food products, nutraceuticals and pharmaceutical preparations.

The current study was focused on the isolation of bacterial endophytes colonizing the marine microalgae *Ulva reticulate*, for previous reports have shown the presence of endophytes in *Gracilaria* spp (Sing *et al*., 2011). There had been several studies on the various marine algae, the present study is the first on extracts of *Ulva reticulta* capable of showing antibacterial activity. *Ulva reticulata* is then a green algae (division Cholorophycota, class-Ulvophyceae, order-Ulvales) belonging to the family of Ulvaceae.

2 Materials and Methods

2.1 Sample Collection

The sample *Ulva reticulata* was collected from Mandapam near Rameshwaraman coast, Gulf of Mannar, India (Fig.1). The raw sample was rinsed carefully with water and transferred into a sterile polyethylene bag. The sample was transported to laboratory and was processed immediately (Fig.2).

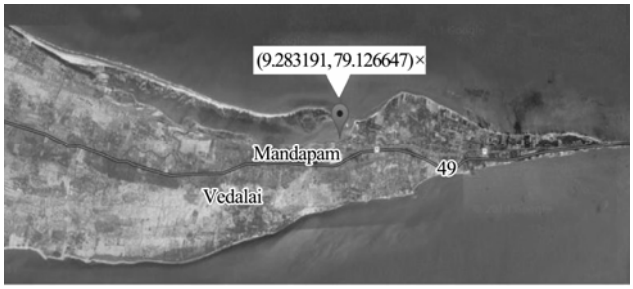

Fig.1 Map of sample collection site Mandapam near Rameshwaram coast.

Fig.2 Marine macroalgae *Ulva reticulate*.

2.2 Surface Sterilization

The seaweed obtained was surface sterilized using 70% ethanol (3 min) which acts as a dehydrating agent and Sterile distilled water (5 min), likewise even 3% of sodium hypochlorite (4–5 min) can be used. Five petriplates were taken which contained sterile distilled water in the 1st, 3rd and 5th plate and 70% ethanol in the 2nd and 4th plate. The final wash with sterile distilled water ensured that no residual sterilizing agent (ethanol or sodium hypochloride) was left on surface of algae (Schulz, 1993; Silvani *et al*., 2008). The algae were transferred to sterile dry petriplate.

2.3 Isolation of Endophytic Bacteria on Zobell Marine Agar Medium

The medium used for isolation of endophytes from algae was Zobell marine agar 2216 (Marhaeni *et al*., 2010) as it mimics the composition of sea water and thus enhances growth of marine bacteria. The agar plate was divided into two sections where one was used for control and another for the isolation of endophytes. In the control section of the agar plate, both sides of the surface sterilized algae were impregnated to assess the efficiency of the surface sterilization (Schulz *et al*., 1993). The sample was sectioned into two pieces using sterile surgical blade and the cut pieces were placed on section exclusive for isolation. The plate was incubated at room temperature for a period of 24–48 h with periodic examination to check bacterial growth. Colonies obtained from the exudate were further sub-cultured using appropriate streaking technique.

2.4 Screening for Bioactive Compounds

A patch culture of VITDSJ1, VITDSJ2 and VITDSJ3 was performed to assess the antimicrobial activity of the pure isolates against the pathogens: *Escherichia coli* MTCC No. 9721, *Staphylococcus aureus* MTCC No. 3160, *Pseudomonas aeruginosa* MTCC No. 10462, *Salmonella typhi* MTCC No. 8587 and *Bacillus subtilis* NCIM No. 2547. The pathogens were selected based on their grams nature and pathogenicity. Seed cultures of the pathogenic strains were swabbed onto the surface of Luria Bertani Agar medium and a patch of VITDSJ1, VITDSJ2 and VITDSJ3 was inoculated onto the surface. The plate was incubated at room temperature and was assessed for zone of inhibition around the patch after 24 h.

2.5 Extraction of Bacterial and Algal Metabolites

The pure isolates from the sub-cultured plates were mass multiplied using Zobell marine broth (350 mL). After 3 days of incubation liquid-liquid extraction was carried out using polar (dichloromethane) and non-polar solvents (hexane) 100 mL each. Following these procedures, the culture extracts were concentrated at their respective boiling points using a rotary evaporator (Vairappan, 2001). 25 g of washed algae sample was shade dried and powdered. The powdered algal sample was soaked in 100 mL of methanol and was kept under shaker conditions for 3 d. Then filtration was done and the residue was further treated with 100 mL of hexane. The filtrates obtained at end of each step were concentrated at their respective boiling points using a rotary evaporator and the solvent extracts were stored at −18℃ until screening (Stephen *et al*., 1989).

For the calculation of the percentage of bioactive compounds the following formula was used (Nagaraj and Osborne, 2014):

% of extraction = weight of extract after evaporation of solvent/weight of dried algal material) × 100.

2.6 Antibacterial Testing

Agar diffusion assay was performed to assess the antibacterial activity of the culture and algal extracts against the human bacterial pathogens *Escherichia coli* MTCC No. 9721, *Staphylococcus aureus* MTCC No. 3160, *Pseudomonas aeruginosa* MTCC No. 10462, *Salmonella typhi* MTCC No. 8587 and *Bacillus subtilis* NCIM No. 2547. Seed cultures of the pathogenic strains were swabbed onto Muller Hinton Agar medium. Wells were made and 30 μL of the extracts were added to the wells. A well containing solvent alone was considered as negative control and disc impregnated with ampicillin was used as positive control. The zones of inhibition were measured after incubating at 37℃ for 24 h.

2.7 GC-MS Analysis for the Crude Extracts

Crude extracts of the isolate VITDSJ2 and algae for the detection of various components were given for GC-MS analysis. GC-MS analysis was performed using Perkin Elmer GC model (30 m×0.25 mm×0.25μm) Clarus 680 (Mass spectrometer Clarus 600 EI) which uses helium, at a constant flow rate of 1 mL min^{-1}. One microliter of the samples were injected and oven temperature was programmed from 60℃ to 300℃ for 2 min at the rate of 10℃ min^{-1} and then isothermally held for 6 min until the analysis was completed.

2.8 Characterization of the Isolates

2.8.1 Morphological and biochemical characterization

The characterization of pure isolates was done by performing grams staining, IMViC, TSI and motility tests (Gupta *et al*., 2013).

2.8.2 Molecular characterization

The characterization was performed by amplification of 16S rRNA sequence using universal primers 27F (5-AGAGTTTGATCCTGGCTCAG-3) and 1492R (5-GGTTACCTTGTTACGACTT-3). The DNA was extracted from cells and the 16S rRNA was sequenced by the fluorescent dye terminator method using the sequencing kit (ABI Prism Big dye terminator cycle sequencing ready reaction kit v.3.1). The product was run on an ABI13730XL capillary DNA sequencer (ABI Prism 310 genetic analyser, Tokyo, Japan). The sequence thereby obtained was aligned using Clustal W. software and phylogenetic tree was constructed by neighbour join method employing MEGA4 software (Poonguzhali *et al*., 2009).

3 Results

3.1 Isolation of Endophytic Bacteria on Zobell Marine Agar Medium

After surface sterilization and incubation of the cut sections of the algae onto the surface of Zobell marine agar, morphologically distinct colonies VITDSJ1, VITD SJ2 and VITDSJ3 (pigmented and non-pigmented) were obtained from the exudate which were further purified and stored in glycerol stocks.

3.2 Screening for Bioactive Compounds

The VITDSJ2 and 3 showed better zone of inhibition around the patch than VITDSJ1, so for the further assessment of the activity VITDSJ2 and VITDSJ3 were mass multiplied.

3.3 Extraction of Bacterial and Algal Metabolites

The percentage of extracts obtained from solvent ex-

Table 1 Percentage of secondary metabolites obtained from fractions of VITDSJ2 and VITDSJ3

Culture	Percentage of secondary metabolites	
	Hexane	DCM
VITDSJ2	2.48%	1.04%
VITDSJ3	1.52%	0.96%

Table 2 Percentage of secondary metabolites obtained from dried algae after solvent extraction

S. No	Percentage of secondary metabolites	
Dried algae	Methanol 1.44%	Hexane 5.76%

traction of *Ulva reticulata* and the culture isolates VITDSJ2 and VITDSJ3 is summarized in Table 1 and Table 2.

3.4 Antibacterial Testing

Antimicrobial assay performed for the culture and algal extracts revealed measurable zones of inhibition against human pathogens *Escherichia coli*, *Salmonella typhi*, *Bacillus subitils* and *Pseudomonas aeruginosa.* The maximum zone of inhibition was obtained with hexane extracts of VITDSJ2 against *Staphylococcus aureus*, which was 14 mm followed by *Escherichia coli* (12 mm) (Figs.3, 4). Both hexane and DCM extracts of VITDSJ2 showed greater antimicrobial activity compared to that of VITDSJ3 (Table 3 and 4). A minimum zone of inhibition of 11mm was seen against *Bacillus subtilis* and *Pseudomonas aeruginosa* with hexane extracts of VITDSJ2.

The algal extracts assessed using the agar diffusion assay showed a maximum zone of inhibition of 16 mm against *Staphylococcus aureus* (Fig.5) using hexane extracts of *Ulva reticulata*, followed by 15 mm and 14 mm against *Pseudomonas aeruginosa* and *Bacillus subtilis* respectively. The methanolic extracts of the algae showed maximum zone of inhibition of 13 mm against *Pseudomonas aeruginosa* followed by *Bacillus subtilis* and *Staphylococcus aureus* (12 mm) and a minimum zone of inhibition of 11 mm was seen against *Salmonella typhi* (Table 5).

Fig.3 Zone of inhibition against *S. aureus* using hexane extracts of VITDSJ2 & VITDSJ3.

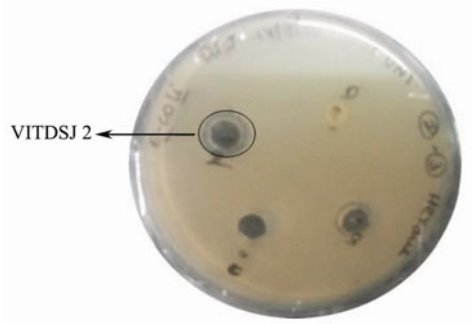

Fig.4 Zone of inhibition against *E. coli* using hexane extracts of VITDSJ2.

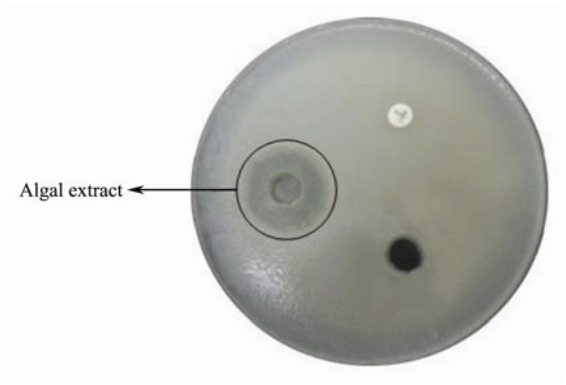

Fig.5 Zone of inhibition against *S. aureus* using hexane extracts of algae.

Table 3 (A) Antibacterial activity of isolated endophytic bacterial strains VITDSJ2 and VITDSJ3 with hexane extracts

Strain	Zone of inhibition of hexane extracts (in mm)				
	1	2	3	4	5
VITDSJ2	11	11	–	14	13
VITDSJ3	11	–	–	13	12

Notes: Human bacterial pathogen: 1. *Bacillus subtilis*; 2. *Pseudomonas aeruginosa*; 3. *Salmonella typhi*; 4. *Staphylococcus aureus*; 5. *Escherichia coli*.

Table 3 (B) Antibacterial activity of isolated endophytic bacterial strains VITDSJ2 and VITDSJ3 with DCM extracts

Strain	Zones of inhibition of DCM extracts (in mm)				
	1	2	3	4	5
VITDSJ2	–	–	–	11	13
VITDSJ3	–	–	–	11	–

Notes: The same as Table 3 (A).

Table 4 Antibacterial activity of algal extracts with hexane against five human pathogens

Human bacterial pathogens	Zone of inhibition of hexane extracts of algae (in mm)	Zone of inhibition of methanolic extracts of algae (in mm)
1	14	12
2	15	13
3	No zone of inhibition	11
4	16	12
5	13	No zone of inhibition

Notes: The same as Table 3 (A).

Table 5 Colony characteristics of VITDSJ2 and VITDSJ3

Character	VITDSJ2	VITDSJ3
Size	0.1 mm in diameter	0.1 mm in diameter
Shape	Circular	Circular
Colour	Orange	White
Margin	Regular	Regular
Opacity	Opaque	Opaque
Consistency	Butyrous	Butyrous
Elevation	Flat	Flat
Gram nature	Gram negative	Gram positive
Morphology	Rods	Rods

3.5 Identification of Bioactive Compounds Through GC-MS

GC-MS analysis of the culture and algal extracts revealed peaks as represented in (Fig.6 (A) and Fig.6 (B)) of the macroalgae *Ulva reticulata* showed the presence of the bioactive compound hentriacontane.

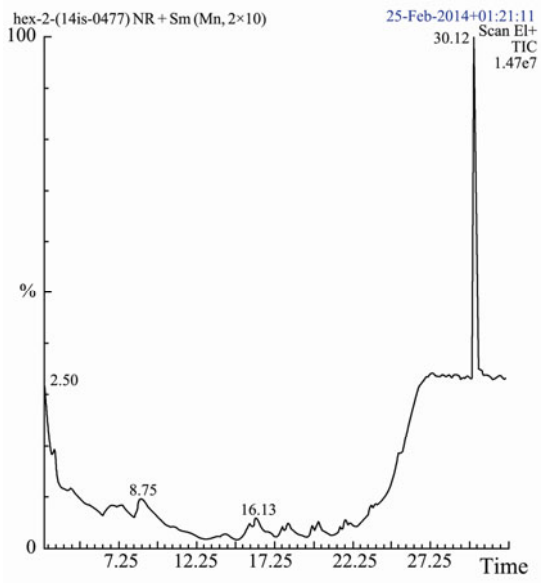

Fig.6 (A) GC-MS analysis of hexane extracts of VITDSJ2.

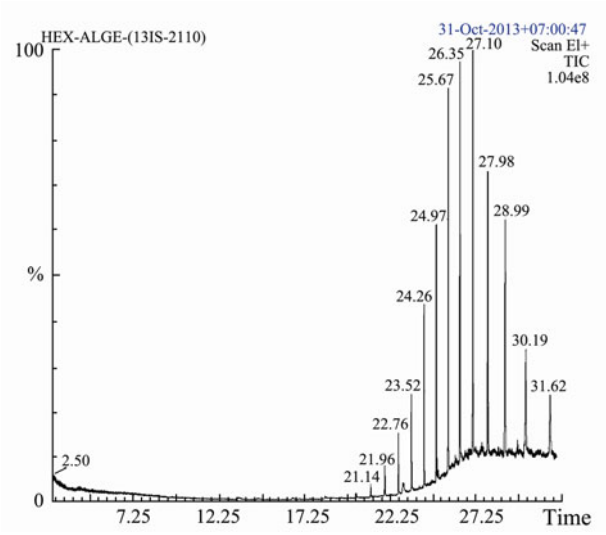

Fig.6 (B) GC-MS analysis of hexane extracts of *Ulva reticulata*.

3.6 Strain Identification

3.6.1 Morphological and biochemical characterization

The morphological characterization of the culture isolates VITDSJ2 and VITDSJ3 includes Gram's staining and study of colony characteristics. The gram nature of VITDSJ2 and VITDSJ3 was found to be gram negative and gram positive rods respectively. The colony characteristics of VITDSJ2 and VITDSJ3 are described in (Fig.7).The biochemical characterization of the effective isolate VITDSJ2 showed negative results for Indole, citrate and VP and positive for Methyl Red which indicates the ability of organism to oxidize glucose with production and stabilization of acid end products. TSI results showed acid butt (yellow colouration) and alkaline slant (red colouration) without gas production, indicating that the organism preferentially degrades glucose prior to the other sugars.

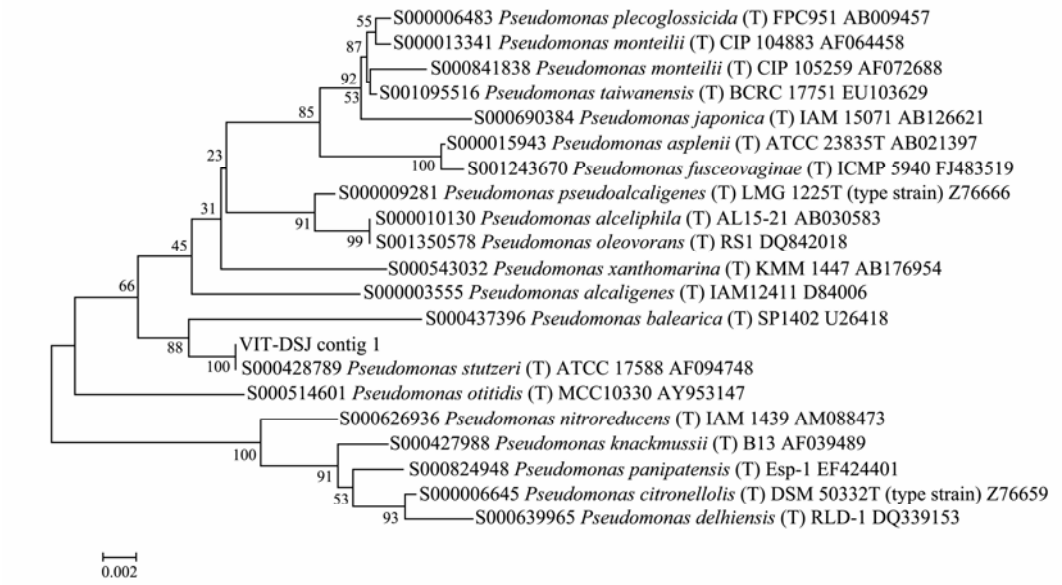

Fig.7 Phylogenetic tree showing the effective isolate VITDSJ2.

3.6.2 Molecular characterization

The 16S rRNA sequencing results showed that the effective culture isolate has 99% similarity to *Pseudomonas stutzeri.* Further phylogenetic tree was constructed and sequence was submitted to GenBank under the accession number-KJ437485.

4 Discussion

Seaweeds are ubiquitous in the marine environment; there are several seaweeds reported to possess antimicrobial activity (Nagaraj *et al*., 2014). In the present study for the isolation of endophytic bacteria, the green algae *Ulva reticulata* was selected since there have been many reports on the bacterial diversity associated with macroalgae (Mercado *et al*., 2012; Chakraborty, 2010; Hollants *et al*., 2013). There has been an expanding tendency of the genus Ulva and a species called as *U. laetevirens* has been reported by Yunxiang *et al*., (2014) off the northeast coast of the United States. Also Al-Saif *et al*., (2014) reported palmitic acid as the major component of the total fatty acids in *U. reticulata,* and stated that these fats and fatty acids from marine algae may play an important role in the formation of many other bioactive secondary metabolites which exhibit their inherent antibacterial activity. Hence, in our study we have focussed on the isolation of endophytic bacteria using Zobell marine agar (Inbaneson and Ravikumar, 2011) and three isolates were obtained, viz, VITDSJ1, VITDSJ2 and VITDSJ3, which were further screened for its potential antimicrobial activity (Strobel, 2003). Among the three isolates VITDSJ2 showed better antimicrobial activity against human pathogens which was reported by performing agar diffusion assay. Liu *et al*., (2009) proved that EPS from the endophytic bacterium *Paenibacillus polymyxa* EJS-3 to be a new source of natural antioxidants having therapeutic and nutritional values.

Previous studies showed that several compounds of endophytic bacterial origin have pharmaceutical potential like antibacterial, antifungal, and anticancerous activities (British Mycological Society Symposium Society Proceedings 2001; Strobel, 2003; Roy *et al*., 2010). Shweta *et al*., reported for the first time the production of camptothecine by endophytic bacteria isolated from *Miquelia dentate Bedd* . Some of the novel compounds capable of showing antibacterial activity include bromosphaerone, pestalone (Zhang *et al*., 2005), ilatol and iso-obtusol (Vairappan *et al*., 2001). The effective strain VITDSJ2 hence obtained was characterized by various phenotypic and genotypic methods and it was identified by 16S rRNA sequencing (Poonguzhali *et al*., 2009). Hence, it was found that the antimicrobial activity- showing bacteria are close neighbours of *Pseudomonas* genus. The bioactive compound in the hexane extracts of *Pseudomonas stutzeri* was found to be phenol, 3, 5-bis (1, 1-dimethylethyl) by GC-MS analysis. A previous report by Yogeswari (2012) has reported the presence of the compound phenol, 3, 5-bis (1, 1-dimethylethyl) in the crude extracts of fungi *Monochaetia kansensis*. This compound has also been analysed by GC-MS in the ethanolic extracts of Malaysian mango kernel and has been reported to be responsible for the antibacterial property against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa* (Abdullah *et al*., 2011). This is the first report of endophytic bacteria from marine macroalgae *Ulva reticulata* which is capable of showing antimicrobial activity. From the present study it could be concluded that the endophytic bacteria isolated from *Ulva reticulata* are capable of producing bioactive compounds which could effectively inhibit *Staphylococcus aureus* and *Escherichia coli* which did not show any zone for ampicillin. This is a novel report of endophytic bacteria from *Ulva reticulata.* This study also proves the fact that the endophytic bacteria of *Ulva reticulata* are solely responsible for various bioactive compounds which are present in it.

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