

Original Research Article

Isolation of Secondary Metabolites from Marine Algal bacterial Population against Foot Ulcer Associated Pathogens

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A B S T R A C T

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Marine algae have been reported to possess a wide range of bio active properties (Baslow 1969). The seaweed *Gracilaria* is a group of marine algae belonging to the class *Florideophyceae* has come to attention for more pharmacological products and have received comparatively less bioassay attention. From this present the study endophytic bacteria were isolated from seaweeds along the East Coast of India, Palk Strait to find out the potential of antibacterial activity of Total Heterotrophic bacterial (THB). Based on the morphological characters, 36 different strains were isolated. The effect of bioactive compound from chosen Heterotrophic bacteria strains were assayed for antibacterial effect through Minimum inhibitory concentration and Minimum Bactericidal concentration and tested for the antimicrobial sensitivity against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa* Sp by cross streak assay. Of them, 12 strains were showed sensitivity against pathogenic bacteria. The isolated endosymbiotic strains which shown sensitivity against four pathogenic bacteria were subjected for the Minimum Inhibitory Concentration (MIC) assay by following standard methodology. It shows that the strain no ENG6 shown the MIC value of 250µg against *Staphylococcus aureus* and *Escherichia coli* the ENG30 showed MIC value of 1000µg against *Klebsiella pneumonia*, the ENG36 showed MIC value of 250µg against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The ENG6 showed MBC value of 250µg to two of the pathogenic bacteria against *Staphylococcus aureus* and *Escherichia coli*, ENG30 showed MBC value of 1000µg against *Klebsiella pneumonia*. The ENG36 showed MBC value of 250µg against *Pseudomonas aeruginosa*

Introduction

The ocean environment contains over 80% of world's plant and animal species and with more than 150,000 seaweeds found in the intertidal zones and tropical waters of

the oceans, it is a primary source of natural products. Seaweeds are rich and varied source of bioactive natural products and have been studied as potential biocidal and

pharmaceutical agents. In recent years, there are numerous reports of macro algae derived compounds that have a broad range of biological activities such as antibacterial, antifungal, antiviral, antineoplastic, antifouling, anti-inflammatory, antitumor, cytotoxic and antimitotic activities. Presently seaweeds constitute commercially important marine renewable resources which are providing valuable ideas for the development of new drugs against cancer, microbial infections and inflammations. The marine biotopes contain an unmatched metabolic and organism diversity. (*ESF Marine Board Feasibility Study Group Report* December 2001). The marine environment is a rich source of both biological and chemical diversity and has been the source of unique chemical compounds with the potential for industrial development as pharmaceuticals, cosmetics, nutritional supplements, molecular probes, fine chemicals and agro chemicals. The objective of the present study is to bring into limelight the potential activities of the crude extracts of these algae and to exploit these untapped resources in various ways for the benefit of the man kind. In recent years, a significant number of novel metabolites with potent pharmacological properties have been discovered from the marine organisms.

Seaweeds offer a wide range of therapeutic possibilities both internally and externally. Sea weeds are extensive profile source of secondary metabolites. More than 600 secondary metabolites have been isolated from marine algae. Although a majority of these (about 60%) are terpenes, but some fatty acids are also common (20%) with nitrogenous compounds. Many of these compounds are bioactive and have been extensively studied using bioassays and

pharmacological assays (Paul *et al.*, 1987). The production of inhibitory substances from seaweeds was noted as early as in 1917. Since then, numerous studies have been carried out to detect and extra antimicrobial compounds from marine algae of all three groups *viz.* Rhodophyceae, Phaeophyceae and Chlorophyceae. Seaweeds provide an excellent source of bioactive compounds such as arylterpenoids, dietary fiber, protein, essential fatty acids, vitamins and minerals). In the present investigation an attempt has been made here to find out the bioactivities of protein from sea weed, *Gracilaria edulis* (Boobathy *et al.*, 2010). The production of inhibitory substances from seaweeds was noted as early as in 1917. Since then, numerous studies have been carried out to detect and extra antimicrobial compounds from marine algae of all three groups *viz.* Rhodophyceae, Phaeophyceae and Chlorophyceae. Seaweeds provide an excellent source of bioactive compounds such as arylterpenoids, dietary fiber, protein, essential fatty acids, vitamins and minerals). In the present investigation an attempt has been made here to find out the bioactivities of protein from sea weed, *Gracilaria edulis* (Boobathy *et al.*, 2010).

Foot lesions in diabetic patients are a major medical, social and economic problem. Infectious agents are associated with amputation of the infected foot if not treated promptly. Infection with multidrug-resistant organisms may increase the duration of hospital stay and cost of management, as well as morbidity and mortality. People with diabetes develop foot ulcers because of neuropathy (sensory, motor, and autonomic deficits), ischaemia, or both. The initiating injury may be from acute mechanical or thermal trauma or from repetitively or

continuously applied mechanical stress. Patients with clinically significant limb ischaemia should be assessed by a vascular surgeon to determine the need for angioplasty, stenting, or femorodistal bypass. When infection complicates a foot ulcer, the combination can be limb or life-threatening. Infection is defined clinically, but wound cultures reveal the causative pathogens. Tissue specimens are strongly preferred to wound swabs for wound cultures. Antimicrobial therapy should be guided by culture results, and should aim to cure the infection, not to heal the wound.

Secondary metabolites produced by endophytes usually produce the enzymes necessary for the colonization of plant tissues. It was demonstrated that most endophytes are able to utilize, at least *in-vitro*, most plant cell components. Most of investigated endophytes utilize xylan and pectin, show lipolytic activity and produce non-specific peroxidase, chitinase and gluconase

The discovery, development and clinical use of antibiotics during the 20th century decreased substantially the morbidity and mortality from bacterial infections. Marine bacteria being a heterotrophy with simple cell multiplication process, which can be cultivated in large amounts inexpensively. This has prompted the present study, to assess the possible utilization of associated bacteria as resources, to meet the sufficient supply of desired metabolites. The present investigation was initiated to screen the antibacterial efficacy of marine algae associated bacterial population against the foot ulcer pathogens

Materials and Methods

Description of the Study Area

Thondi is situated in the Palk Strait region of Tamil Nadu. The study area lies in the latitude of 79° 44' 10" N and longitude of 79° 10' 45" E. *Gracillaria edulis* species of seaweeds were collected during month of (July 2013) for the isolation of endophytic THB strains.

Isolation of Endophytic Organisms

1 gm of fresh sea weeds species were aseptically weighed, washed thrice with sterilized distilled water and were plated with the Zobell marine agar medium. The bacterial species enumerated on the Zobell marine 2216e (Hi-media) agar medium were counted and the total number of bacterial counts was expressed as Colony Forming Unit (CFU).

Isolation of Foot ulcer associated pathogens

The fresh Pus swab samples were collected in a sterile container. The foot ulcer associated pathogens were isolated by using selective media. The chosen isolates were identified using standard staining and biochemical test. (Bergey's manual Holt et al., 1994).

Antibacterial sensitivity assay

The antagonistic activity was tested by following cross streak assay method using 36 different THB strains against foot ulcer associated pathogen via, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Single streak of the isolated strains was

done on sterile Muller Hinton Agar plates followed by overnight culture of foot ulcer associated pathogens were streaked at perpendicular to the original streak of isolates and incubated at $37\pm 2^{\circ}\text{C}$. 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.

For MIC determination 0.5 ml of various concentration of extracts (63, 125, 250, 500, 1000, 1500, 2000 μg) was prepared with Dimethyl sulphoxide (DMSO) and mixed with 0.5ml of nutrient broth. 50 μl of bacterial inoculums serves as positive control. Nutrient Broth alone served as negative control. Whole setups in duplicates were incubated at 37°C for 48 hours. The MIC was the lowest concentration of the extract that did not permit any visible growth after 24 hours of incubation and it was examined on the basis of turbidity.

To avoid the possibility of misinterpretations 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

About 36 bacterial strains were isolated as endosymbiont from the marine algae *Gracillaria. edulis* and all of them have been tested for the antimicrobial sensitivity against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* sp by cross streak assay. Of them, twelve strains were shown sensitivity against four pathogenic bacteria.

The isolated endosymbiotic strains which shown sensitivity against four pathogenic bacteria were subjected for the Minimum Inhibitory Concentration (MIC) assay by following standard methodology. It shows that the strain no ENG6 shown the MIC value of 250 μg against *Staphylococcus aureus* and *Escherichia coli* the ENG30 showed MIC value of 1000 μg against *Klebsiella pneumoniae*, the ENG36 showed MIC value of 250 μg against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The ENG6 showed MBC value of 250 μg to two of the pathogenic bacteria against *Staphylococcus aureus* and *Escherichia coli*, ENG30 showed MBC value of 1000 μg against *Klebsiella pneumoniae*. The ENG36 showed MBC value of 250 μg against *Pseudomonas aeruginosa*.

The world's oceans comprise the largest part of the biosphere and contain the most ancient and diverse forms of life. The endosymbiotic heterotrophic bacteria have been isolated from sea weed species. Foot lesions in diabetic patients are a major medical, social and economic problem. The presence of microorganisms in a wound, however, does not in itself define a clinical infection. It is important to

Table.1 Cross streak assay method against diabetic foot ulcers sample associated pathogens

Endophytic Bacterial Strain No	Foot ulcer bacterial pathogen			
	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>	<i>Klebsiella pneumoniae</i>	<i>Staphylococcus aureus</i>
ENG1	+	-	+	-
ENG 6	+	-	+	+
ENG 10	+	+	-	-
ENG 11	+	+	-	-
ENG 15	+	-	+	+
ENG 17	+	-	-	-
ENG 20	-	-	-	+
ENG 25	+	-	-	-
ENG 26	+	-	-	-
ENG 30	+	-	-	+
ENG 35	-	+	-	+
ENG 36	-	+	-	+

ENG – Endophytic Gracillaria;

[+] present of activity.;

[-] absent of activity.

Figure.1 The Picture Showing MBC For Against Diabetic Foot Ulcer Associated Pathogens

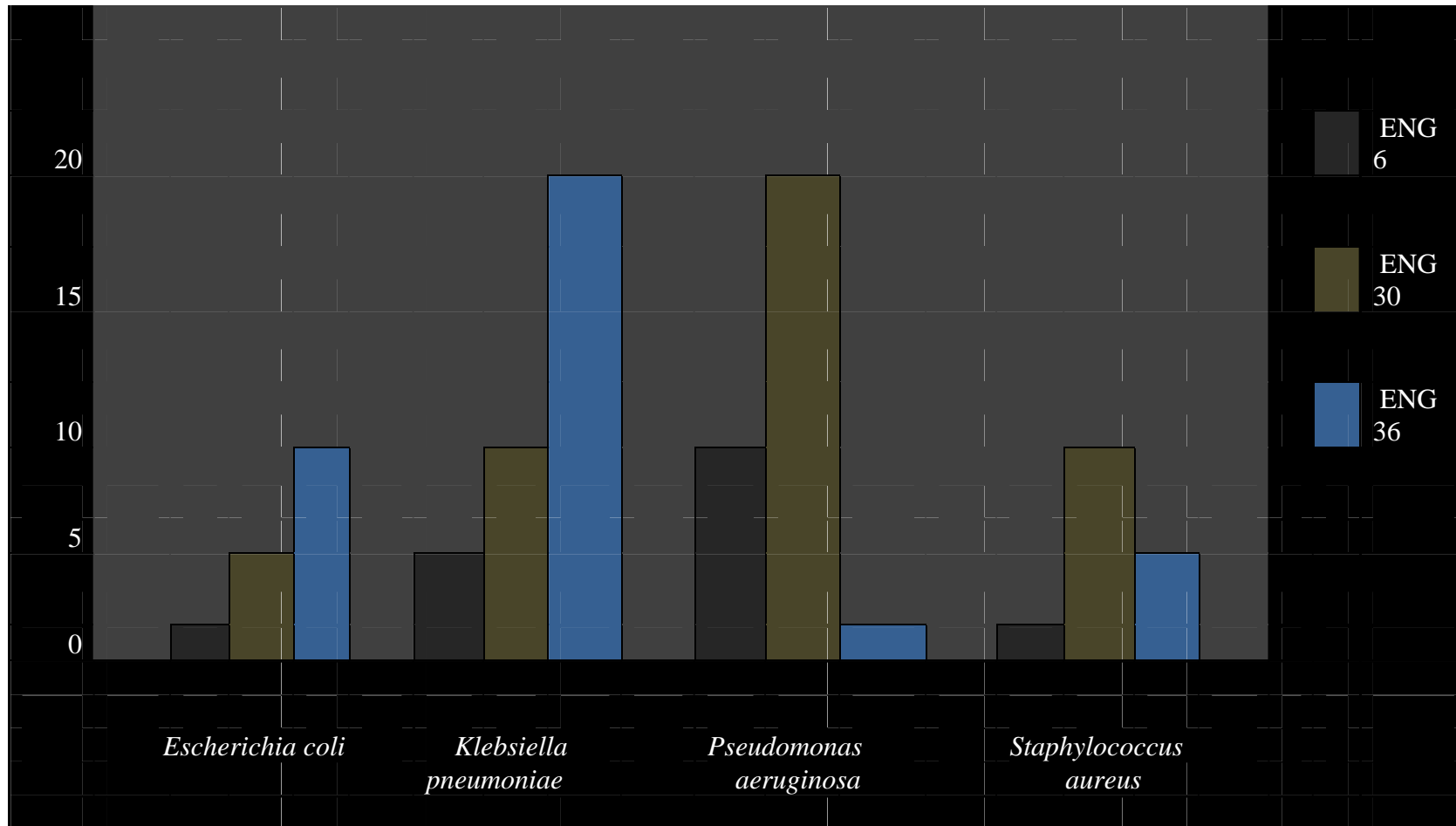


Table.2 Minimum inhibitory concentration test

Strain Number	2000µg	1000µg	500µg	250µg	125µg	63µg
<i>Escherichia coli</i>						
ENG 6	-	-	-	-	+	++
ENG 30	-	-	-	+	++	+++
ENG 36	-	-	+	+	++	+++
<i>Pseudomonas aeruginosa</i>						
ENG 6	-	-	+	+	++	+++
ENG 30	-	+	++	+++	++++	++++
ENG 36	-	-	-	-	+	+
<i>Klebsiella pneumoniae</i>						
ENG 6	-	-	-	+	+	+
ENG 30	-	-	+	++	+++	++++
ENG 36	-	+	++	+++	+++	++++
<i>Staphylococcus aureus</i>						
ENG 6	-	-	-	-	+	++
ENG 30	-	-	+	++	+++	++++
ENG 36	-	-	-	+	+	++

ENG – Endophytic *Gracilaria*; - : Absence of Growth; + : Presence of Growth
 ++ : Medium Growth; +++ : Large Growth; ++++ : Very Large Growth

recognize that there is a spectrum, or continuum, of disease (Williams *et al.*, 2004). Infectious agents are associated with amputation of the infected foot if not treated promptly. Reducing the initial bacterial pathogens in foot ulcers is of prime importance in an attempt to improve the Life of the person.

Hence the present study was undertaken to produce valuable secondary metabolites from the marine algae against foot ulcer associated pathogens. Based on the morphological characters, 36 strains were isolated and all of them have been tested for the antimicrobial sensitivity against *Staphylococcus aureus*, *Escherichia Coli*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa* by cross streak assay. Generally, the endophytic bacteria isolated from seaweeds showed maximum sensitivity against several human bacterial pathogens. Moreover, the bioactive compounds from endophytic bacteria showed maximum sensitivity with minimum concentration than the bioactive compounds from epiphytic bacteria of other biological origin. The earliest marine bio active compounds were isolated from endosymbionts of sponges. A wide range of chemical and functional diversity has been observed among bioactive compounds. Of the various chemical classes of compounds, polyketides, alkaloids, fatty acids, peptides and terpenes are the most abundant ones. Majority of them show antimicrobial, antitumor and anticancer properties. Tritetracontane, Nonadecane, Docazane, Indolirine, Trifluoroacetic acid, n-octadecyl ester are highly abundant and have been shown to be multifunctional. Triterpenes attract attention because of their biological activities of endosymbionts. Hence, steps have been undertaken to find out the reason for the maximum activity of

endophytic bacteria from seaweeds and complete structural elucidation of the potential metabolites will also be focused in future

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