

ANTIBACTERIAL ACTIVITY OF ISOLATED ENDOPHYTIC SEAWEED ASSOCIATED BACTERIA FROM SELECTED FIVE SEAWEEDS

Submitted in partial fulfilment of the requirements for the award of
Bachelor of Technology degree in Biotechnology

By

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DEPARTMENT OF BIOTECHNOLOGY

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SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY (DEEMED TO BE UNIVERSITY)

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Varsha Suresh (38230039)** who carried out the project entitled “**ANTIBACTERIAL ACTIVITY OF ISOLATED ENDOPHYTIC SEAWEED ASSOCIATED BACTERIA FROM SELECTED FIVE SEAWEEDS**” under our supervision from January 2022 to April 2022.

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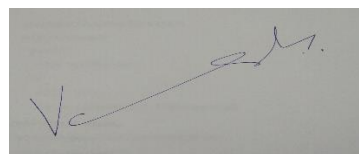
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DECLARATION

I, **Varsha suresh (38230039)** hereby declare that the Project Report entitled **“ANTIBACTERIAL ACTIVITY OF ISOLATED ENDOPHYTIC SEAWEED ASSOCIATED BACTERIA FROM SELECTED FIVE SEAWEEDS”** done by us under the guidance of **Dr. Ramesh Kumar. V** is submitted in partial fulfilment of the requirements for the award of Bachelor of Technology degree in Biotechnology.

DATE:



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SIGNATURE OF THE CANDIDATE

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I am pleased to acknowledge my sincere thanks to Board of Management of SATHYABAMA for their kind encouragement in doing this project and for completing it successfully. I am grateful to them.

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(VARSHA SURESH)

ABSTRACT

In this present study we synthesis the seaweed associated bacteria and antibacterial activity by using *Ulva Intestinalis*, *Gracilaria corticata*, *Sargasam wightii*, *Turbinaria conoides*, *Kappaphycus alvarezii*. Above-mentioned fresh seaweeds were collected from the Rameswaram, Southeast coast of India. We have surface sterilized the seaweeds and carried out two methods: Endophytic and soaking method. In endophytic method the seaweeds have been grinded with distilled water whereas in soaking method the seaweeds have been soaked with sterilized distilled water for 3 hours. The grinded and soaked seaweed distilled water is used for serial dilution. Media plate was prepared with nutrient agar, 50% of distilled water and 50% sea water. Spread plate method was carried out from that specified colonies were taken for future subculturing such as streak plate and nutrient slant. The seaweed associated bacteria was inoculated in nutrient broth and centrifuged. With 5 different human pathogens such as *Escherichia coli*, *Klebsiella pneumoniae*, *Enterococcus faecium*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* well diffusion method was carried out to find out the zone of inhibition. The synthesised certain seaweed associated bacteria have shown antibacterial activity comparatively more than other used seaweeds. The seaweed shown best zone of inhibition was taken for the DNA sequencing.

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LIST OF SYMBOLS AND ABBREVIATIONS

i.	<i>Ulva intestinalis</i>	:	<i>U. intestinalis</i>
ii.	<i>Gracilaria corticata</i>	:	<i>G. corticata</i>
iii.	<i>Sargassum wightii</i>	:	<i>S. wightii</i>
iv.	<i>Turbinaria conoides</i>	:	<i>T. conoides</i>
v.	<i>Kappaphycus alvarezii</i>	:	<i>K. alvarezii</i>
vi.	PCR	:	Polymerase Chain Reaction
vii.	µg	:	Microgram
viii.	µl	:	Microlitre
ix.	ml	:	Millilitre
x.	DNTP's	:	Deoxyribose nucleotide triphosphate

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1. INTRODUCTION

Seaweed protection against the different environmental stresses is taken care of by the metabolites. Components that are found from the Seaweed shows antibacterial, antifungal, antiprotozoal and antiviral activities. An unconventional drug which has the ability to control new diseases or multi-resistant strains of pathogenic microorganisms. Potent antimicrobial activity that belongs to the phlorotannin's, pigments lectins alkaloids polysaccharide fatty acid, and halogenated compounds are isolated from the green-blue and red algae this shows the antimicrobial activities and their promising applications (Prasanna et al.,2016a)

Total number of isolated cultivable isolates used were 148 in which 50% were influenced by γ -Proteobacteria and Firmicutes, wherein 53 of them showed consistent antibacterial activity against clinically significant pathogens. Against multidrug-resistant pathogens, such as methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecalis* the heterotrophs *Bacillus amyloliquefaciens* MTCC 12716 and *Shewanella algae* MTCC 12715 isolated from a red marine macroalga *Hypnea valentiae* exhibited the potential of anti-infective properties. Antioxidative properties was displayed in the extracts of *B. amyloliquefaciens* were and the activities showed considerable positive correlation ($r^2 > 0.8$, $P < 0.05$) with the inhibitory activities against pro-inflammatory cyclooxygenases, angiotensin converting enzyme-I, and 5-lipoxygenase, and hydroxymethylglutaryl coenzyme A reductase and dipeptidyl peptidase-4. In this review the approach of nuclear magnetic resonance-based fingerprinting to analyse the characteristic signals in the solvent extracts and to correspond them with the pharmaceutical properties were also underlined (Kizhakkekalam & Kajal Chakraborty.,2019).

Phylogenetic analysis of the 16S rRNA sequences shows that the microbial communities are remarkably different within the three forms studied and contains of mosaic sets of common and form-specific bacterial lineages. In spite of their high genetic similarity the divergence in bacterial composition is substantial between the TYP and LON forms. In three other laminarialean species isn't associated with the depth or locality of the algae settlements because the symbiont distribution within the *S. japonica* forms. They observe that phenotype differences between genetically

similar forms is also caused by host-symbiont interactions which will be a typical feature of evolution in algae and other eukaryote organisms. Bacterial symbionts could function the sensitive markers to match genetically similar algae forms and also as possible growth-promoting inductors to increase algae productivity (Balakirev et al., 2012)

The effect of process parameters food to microorganism's ratio (F/M) and hydraulic retention time (HRT) on the process performance, evaluated by settleability, microalgae incorporation to biomass and nutrient removal are deliberated. An innovative wastewater treatment that exploits the symbiosis between bacterial activated sludge and microalgae is seen because of the algal incorporation into the biomass process. Here the F/M were found to be necessary for stability when algae are incorporated into the biomass whereas the HRT had a notable influence in the growth rate of algae. But the complete nitrogen removal of the treatment was affected by these parameters. At high and low F/M values the stable flocs with incorporated algae and supernatants with low free-swimming algae concentrations were obtained (Medina Neis, 2007)

Seaweed construct metabolites which help to protect different environmental stresses. Antibacterial, antiprotozoal, antifungal and antiviral properties are shown by these compounds. New diseases or multi resistant strains of pathogenic microorganisms are controlled by macroalgae which is been cultured in huge volumes and shows an attractive source of potential compounds convenient for unconventional drugs. Halogenated compounds, fatty acids, phlorotannin, pigments, lectins, polysaccharides, alkaloids and terpenoids which belongs to antimicrobial activity are shown from the isolated from brown, green and red algae (María et al., 2016)

Scientific knowledge on primary, secondary metabolites and also the health-related functional properties of existing seaweeds. Polysaccharides, pigments, polyphenols, peptides and fatty acids of seaweed bioactive has been proved to possess different necessary biological properties which could potentially help in the nutraceutical and food development. These bioactive are deliberated for functional properties such as antibacterial, anticancer, antidiabetic, antioxidant, antiviral, antitumor and anticoagulant properties in in-vitro and in-vivo model system (Abirami et al., 2019).

Genus *Sargassum* is a group of edible brown seaweeds which shows high source of bioactive compound other than showing food source properties. The sulphated polysaccharides have attained more attention than compared to the other isolated bioactive components this is due to their proven health benefits (Asanka et al., 2018).

Marine macroalgae associated bacteria were analysed in order to showcase the anticancer, antagonistic and antibacterial property, Here the bacterial strains were isolated from 14 epiphytic and 6 endophytic. In which 3 strains exhibit antagonistic activity against *Staphylococcus aureus* and 1 strain showed activity against *Escherichia coli*. The bacterial extracts have the potential to inhibit the growth of *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli* from the antibacterial potential of seaweed-associated bacteria. Further the proliferation of SK-MEL-28 cell line in a dose dependent manner is inhibited (Jegan 2020).

Compounds like polysaccharides, polyphenols, pigments, terpenes, and many other elements show many bioactivities counting anticancer activity against the different kinds of cancer. From the seaweed, the obtained bioactive compound has been signified to cause apoptosis in cancer cells and trigger cell cycle arrest with low cytotoxicity against normal cells. This study shows that the bioactive compounds derived from seaweeds have the ability to shed light on anticancer activity (Omniya et al.,2020).

From red seaweeds the porphyran and carrageenan are derived. The latter is familiar from *Chondrus*, *Euchema* and *Gigartina* species, while the former is a characteristic polysaccharide of *Porphyra* in *Rhodophyceae*. With potential chemotherapeutic agents and by increasing immunity and targeting key apoptotic molecules, two polysaccharides have been found having anti-cancer property. This study shows the anticancer chemopreventive agents and the conceivable mechanism of porphyrin and carrageenan (Zhiwei et al.,2019).

Seaweeds may play a vital role in the prevention and treatment of chronic diseases. Seaweeds are a novel source of compounds with potential to be exploited in human health applications, as they are rich in unique bioactive compounds not found in terrestrial food sources, such as different proteins (lectins, phycobiliproteins, peptides, and amino acids), polyphenols, and polysaccharides. Antiviral, anticancer, and

anticoagulant qualities are claimed, as well as the capacity to alter gut health and obesity and diabetes risk factors. Despite the fact that the bulk of studies have been conducted in cell and animal models, there is evidence that seaweed and seaweed components have a positive influence on markers of human health and disease state. This is the first review to critically analyse this human research, with the goal of highlighting knowledge gaps that will aid in the planning and implementation of future investigations (Emma et al.,2014)

Minerals, vitamins, soluble dietary fibres, and flavonoids found in seaweeds are thought to protect against lifestyle-related disorders. Seaweed is widely consumed in East Asian countries, such as Japan. As a result, eating seaweeds may help Japanese people live longer by preventing lifestyle-related ailments. Two large Japanese cohort studies recently found a link between seaweed consumption and a lower risk of cardiovascular disease. Seaweeds, on the other hand, contain iodine and heavy metals such as arsenic species, both of which are thought to be harmful to one's health. The link between seaweed consumption and cancer and cardiovascular disease mortality and incidence, as well as risk variables like blood pressure and serum lipids. The negative impacts of iodine and arsenic species in seaweeds is also derived. Seaweeds are not extensively consumed in Western countries, dietary diversification and a growth in the number of immigrants from East Asia may lead to an increase in seaweed consumption (Utako et al.,2020)

When employed as food additives, seaweed-derived bioactive compounds can have a wide range of nutritional and therapeutic effects. Bioactive chemicals produced by red, brown, and green seaweeds exhibit biological activities such as cancer prevention, blood pressure lowering, antioxidant, slimming, and other properties that are closely related to the properties necessary for functional food products. The present state of knowledge on the health advantages of seaweed-derived bioactive compounds as dietary additives is reviewed in this chapter. These unique qualities are envisaged to be combined into functional food products to improve human health (Yimin.,2018)

Bioactive substances with a variety of chemical structures play an important role in illness prevention and physiological function maintenance. Several types of biomasses are being exploited for the identification of bioactive metabolites and

techno-functional biomolecules that are ideal for later uses in the cosmetic, food, and pharmaceutical areas due to a surge in industry demand for new bio-sourced molecules. Macroalgae are gaining prominence among the numerous biomasses accessible due to its potential nutraceutical and health benefits. Specific diterpenes, pigments, bioactive peptides, and polysaccharides give these health benefits. Numerous studies have found that native algal polysaccharides, as well as their derivatives, such as oligosaccharides and (bio)chemically modified polysaccharides, have useful biological functions. Only a few of these, however, can be commercialised and open up new markets for active compounds, extracts, or additives (Faiez et al.,2020)

2. REVIEW OF LITERATURE

The seaweed is taken into account has the as an upscale source of structurally different bioactive metabolites for the invention of novel functional food-based pharmacophores or the drugs. But the , clinical efficacy, mechanisms of action, and interaction with conventional foods, toxicity have not explored completely (Kannan et al.,2020). The secondary metabolites had been explored by using the pharmaceutical industries a good way to generate new capsules to treat numerous illnesses. Drug discovery for disregarded sicknesses has been unnoticed via the huge pharma, in particular due to the fact they affect human beings, maximum of them dwelling in developing international locations (Miriam et al.,2019).

Antimicrobial effect of various secondary metabolites which are isolated and purified from the different marine organisms and the clear structural idea of every secondary metabolites have been studied for helping the silico designing of the novel and potent antimicrobial compounds. The compound here has been isolated and purified by various purification techniques, and their structure also characterized and even the antibacterial activities have been reported by using broth microdilution as well as disc diffusion assays (Bamunuarachchi et al.,2021)

Bioactive compounds such as steroids, flavonoids triterpenoids, alkaloids, saponins, tannins that have antimicrobial potential were found from brown seaweed. Bacteria that attack aquatic organisms such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* bacteria and *S. aureus* activities has been inhibited by the brown seaweed extract. Endophytic microbes associated with seaweed such as bacteria and fungi are thought to be able to produce the same bioactive compounds. And many studies showed that the antibacterial activity of endophytic microbes can inhibit pathogenic bacteria and also the extract of brown seaweed could inhibit the pathogens ranged moderate to strong category (Fittrie et al., 2021).

Marine environment the antimicrobial molecules has categorized according to their main phylum and sources evolved from the marine environment. In particular strains of the *Bacillus* genus, Firmicutes phylum is the most revised group of bacteria producers of substances with antimicrobial activity, this is because of the difficult

cultivation of typical Actinobacteria from a marine sediment, whose members are the main producers of antimicrobial substances in land environments. From bacillus produced Actinomycetes and macrolactins a great diversity of chemical structures has been identified, such as fijimicyns and lynamycin (Paolo & Adriano 2020)

Totally 234 bacterial strains were remoted from 7 seaweed species in the Gulf of Mannar at the southeast riverside of India. The strains having constant antimicrobial activity had been tagged for more studies, and this constituted 9.8% of the lively strains isolated. Phylogenetic analysis using 16s rDNA sequencing with the prop of classical biochemical identification indicated the lives of two presiding phyla, Firmicutes and Proteobacteria. Antimicrobial activity evaluation blended with the outgrowths of amplifying genes decoding for polyketide synthetase and for non-ribosomal peptide synthetase substantiated that seaweed- related bacteria had a large- scale antimicrobial activity (Thilakan et al.,2015)

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DESCRIPTION OF SELECTED SEAWEEEDS

1.2.1 *Ulva intestinalis*

TAXONOMICAL CLASSIFICATION

Class - Ulvophyceae

Order - Ulvales

Family - Ulvaceae

Genus - Enteromorpha

Phylum - Chlorophyta

General information

Ulva intestinalis is a green alga in the family *Ulvaceae*, known by the common name's sea lettuce, gutweed and grass kelp. Until they were reclassified by genetic work completed in the early 2000s, the tubular members of the sea lettuce genus *Ulva* were placed in the genus *Enteromorpha*.

Geographical distribution

It typically grows attached to rocks, or free floating in protected areas. Its range extends from the Arctic Ocean, Bering Sea and Aleutian Islands, Alaska south to Mexico, and also includes Korea, Japan and Russia.

Applications

As suggested by its common name sea lettuce, *Ulva lactuca* is commonly used as a sea vegetable in salads. It is also served cooked, in soups, and along with meats and fish.

1.2.2 *Gracilaria corticata*

TAXONOMICAL CLASSIFICATION

Class - Florideophyceae

Order - Gracilariales

Family - Gracilariaceae

Genus – *Gracilaria*

Phylum - Rhodophyta

General information

Gracilaria is a genus of red algae (Rhodophyta) notable for its economic importance as an agarophyte, as well as its use as a food for humans and various species of shellfish. Various species within the genus are cultivated among Asia, South America, Africa and Oceania.

Geographical distribution

Gracilaria are found in warm waters throughout the world, though they also occur seasonally in temperate waters. It cannot tolerate temperatures below 10 °C (50 °F). *Gracilaria* are found in all oceans except the Arctic. Its centre of diversity is the Western Pacific, where it has been traditionally cultivated as a source of agar.

Applications

It has a gelatinous consistency and it is used in the cosmetic industry as a thickening agent. However, its most important use is as a gelatinous culture medium for the growth of microorganisms for scientific studies and medical examinations.

1.2.3 *Sargassum wightii*

TAXONOMICAL CLASSIFICATION

Class - Phaeophyceae

Order - Fucales

Family - Sargassaceae

Genus – *Sargassum*

Phylum - Ochrophyta

General information

The brown alga *Sargassum wightii* is one of the marine algal species rich in sulphated polysaccharides that possess a wide spectrum of biological properties, such as free radical scavenging and antioxidant effects.

Geographical distribution

Numerous species are distributed throughout the temperate and tropical oceans of the world, where they generally inhabit shallow water and coral reefs, and the genus is widely known for its planktonic (free-floating) species.

Applications

When *Sargassum* loses its buoyancy, it sinks to the seafloor, providing energy in the form of carbon to fishes and invertebrates in the deep sea, thus serving as a potentially important addition to deep-sea food web.

1.2.4 *Turbinaria conoides*

Class - Phaeophyceae

Order - Fucales

Family - Sargassaceae

Genus – *Turbinaria*

Phylum - Ochrophyta

General information

Turbinaria conoides is a tropical brown algae of the order Fucales native to coral reef ecosystems of the South Pacific. It can quickly colonize these ecosystems due in part to its method of dispersing by detaching older and more buoyant fronds that travel on surface currents, sometimes in large rafts of many individual thalli, or fronds. Some scientists are investigating whether the increase in density of seaweeds, and a decrease in living coral density, on coral reef ecosystems indicates a change in the health of the reef, focusing studies on this particular species of brown alga.

Geographical distribution

Turbinaria is mainly distributed in tropical coral reefs but is also found in the subtropics and temperate regions. Southeast Asia as well as the Indian Ocean contain the highest diversity of the genus. *Turbinaria conoides* is the species with the widest distribution within the genus, ranging from the West Atlantic, to the Indo-Pacific, and East Africa.

Applications

Turbinaria is utilized for its alginate extracts, which are used as thickening, gelling, and stabilizing agents in food and drinks, as well as in cosmetics and pharmaceutical products. The young thalli of *Turbinaria ornata* are consumed in Indonesia either fresh, salted, or with curry sauce. Dried *Turbinaria* can be ground and used as seasoning. In Samoa, it is used as a stir-fry ingredient. Brown algae are often used in animal feed as they can provide nutritional supplements and improve growth

1.2.5 *Kappaphycus alvarezii*

Class - Florideophyceae

Order - Gigartinales

Family - Solieriaceae

Genus – *Kappaphycus*

Phylum - Rhodophyta

General information

Kappaphycus alvarezii, the elkhorn sea moss, is a species of red algae. It is one of the most important commercial sources of carrageenans, a family of gel-forming, viscosifying polysaccharides. Farming methods affect the character of the carrageenan that can be extracted from the seaweed. This alga grows to two meters long and is green or yellow in colour. It is very fast-growing, known to double its biomass in 15 days.

Geographical distribution

Kappaphycus is a genus of red algae. Species are distributed in the waters of East Africa, Indonesia, Malaysia, Hainan Island, the Philippines, and Micronesia.

Applications

Kappaphycus Alvarezii used in a variety of commercial applications as gelling, thickening, and stabilizing agents, especially in food products such as frozen desserts,

chocolate milk, cottage cheese, whipped cream, instant products, yogurt, jellies, pet foods, and sauces. Aside from these functions, carrageenans are used in pharmaceutical formulations, cosmetics, and industrial applications such as mining.

3. AIM

To screen and analyse the study about the various seaweed associated bacteria for the antibacterial properties using endophytic and non-soaking method

Objectives

- ❖ Isolation of seaweeds associated microbes from Green, Brown and Red algae
- ❖ Extract the bioactive compounds from various seaweeds associated microbes
- ❖ To evaluate the antibacterial activity.
- ❖ Identification of potential bacterial by DNA sequencing

4. MATERILAS AND METHODS

4.1 SEAWEED COLLECTION

Seaweeds (*Ulva Intestinalis*, *Gracilaria Corticata*, *Sargasam Wightii*, *Turbinaria Conoides*, *Kappaphycus Alvarezii*) have been collected from Rameswaram coastal area. The collected seaweeds washed properly with the distilled water. Surface sterilization of seaweeds is done to prevent bacterial and fungal growth and to remove the debris found on the seaweeds.

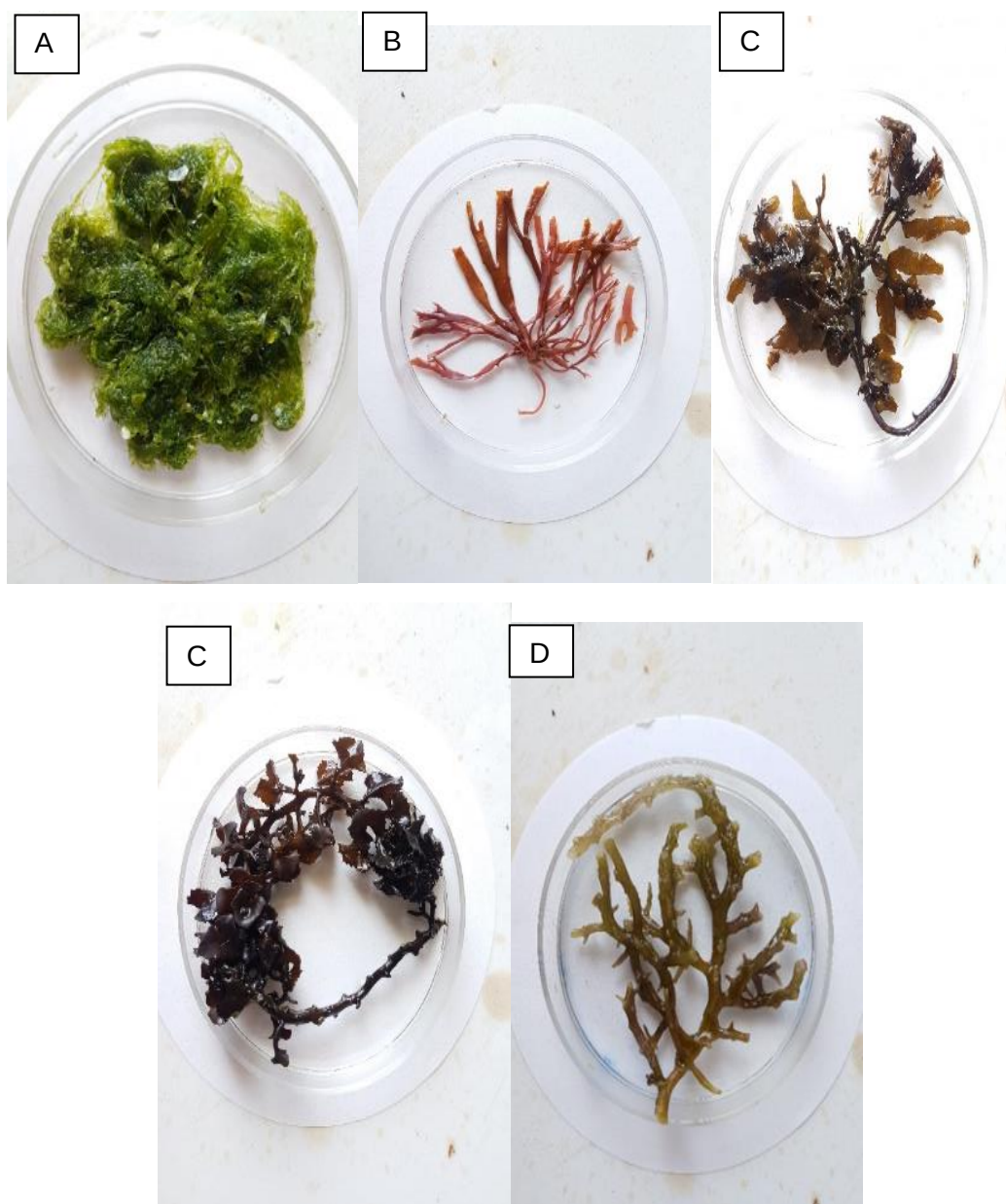


Figure 4.1: Collection of Marine Seaweeds :A. *Ulva Intestinalis*, B. *Gracilaria corticata*, C. *Sargasam wightii*, D. *Turbinaria conoides*, E. *Kappaphycus alvarezii*

4.2. GRINDING AND SOAKING METHOD

4.2.1 ENDOPHYTIC METHOD

After surface sterilization, grind the seaweeds with the help of the hand motor. Add 1ml of distilled water in the hand motor for grinding. The seaweeds are grinded into a fine substance and the grinded seaweeds have been taken to the 4-test tube serial dilution. Taken 1 part of a seaweed and add 9 parts of distilled water, Dilution tubes begin with 9-mL. 1-mL is added and mixed, then 1-mL is transferred to the next tube. The ending volume in the last tube would be 10-mL.

4.2.2 SOAKING METHOD

The seaweeds are soaked in the distilled water for 4 hours, the soaked water of 1ml is added to the 4-test tube serial dilution. Serial dilution is done by taking 1 part of a seaweed and add 9 parts of distilled water, Dilution tubes begin with 9-mL. 1-mL is added and mixed, then 1-mL is transferred to the next tube. The ending volume in the last tube would be 10-mL.



Figure 4.2: Collection of Marine Seaweeds (A) *Turbinaria conoides*, (B) *Sargasam wightii*, (C) *Gracilaria Corticata*, (D) *Ulva intestinalis*, (E) *Kappaphycus Alvarezii*

4.3 Media Preparation

11.2 grams of nutrient agar is taken from the 28.0 grams of nutrient agar for 1000ml box containing 0.5% of peptone, 0.3% beef extract, 1.5% agar, 0.5% NaCl, distilled water in the pH of 6.8 at 25 degrees Celsius for 20 plates. 200ml of distilled water and 200ml of sea water is added to the conical flask containing 11.2 grams of nutrient agar, the flask is packed with the cotton and the paper in the mouth of the flask and kept in the autoclave for 45mins. The autoclaved agar is prepared, pour nutrient agar into each autoclaved plate and leave plates on the sterile surface until the agar gets solidified. Replace the lid of each Petri dish and store the plates in a refrigerator. The colony produced is counted.

Table 4.1: Colony counting

SL.NO	SAMPLE IDENTIFICATION	No. Of colonies – non soaking method	No. Of colonies- soaking method
1	U1	302	569
2	U2	167	368
3	U3	106	310
4	U4	63	77
5	G1	SPREAD	SPREAD
6	G2	322	SPREAD
7	G3	247	SPREAD
8	G4	13	SPREAD
9	S1	301	1067
10	S2	195	724
11	S3	83	387
12	S4	45	201
13	T1	SPREAD	1080
14	T2	855	432
15	T3	536	91
16	T4	375	68
17	K1	SPREAD	988
18	K2	233	365
19	K3	145	131
20	K4	65	93

U – *Ulva Intestinalis*, G - *Gracilaria corticata*, S – *Sargasam wightii*, T – *Turbina conodias*, K –*Kappaphycus alvarezii*

4.4 Spread plate method

The spread plate technique is spreading a small number of bacteria suspended in a solution on a plate with a sterile spreader with a smooth glass surface (L - rod). To allow the agar to absorb the bacteria more rapidly, the plate must be dry and at room temperature. Add the serial diluted solution with the 200 µl pipette in the respective media for spread plate method and mark them with seaweed identification name. After 24hours check the plates for the bacteria growth.

4.5 Sub culture

4.5.1 Streak plate method

The process of streaking is used to isolate a pure strain from a single species of microbe, most commonly bacteria. The organism can then be recognised, examined, or tested by taking samples from the resultant colonies and growing a microbiological culture on a fresh plate. From the spread plate specific bacteria colonies are marked and taken for streak plate method. The selected bacteria colony is taken from the plate with a streak loop which is heated before to avoid contamination. Open the plate near a flame for a solid media and let the loop cool near the flame for about 10 to 15 seconds. Pick up an isolated colony far from any other colonies with the loop. Streak the loop in a zig zag pattern on one quadrant of the plate. Completely re-flame the loop and let it cool near the flame. Drag the loop through on portion of the streaked quadrant and continue a zig zag pattern into the next quadrant. Repeat steps for other 3 remaining quadrants. Let the loop cool after flaming between the streaks. After 24 hours the bacteria growth will be seen.



Figure 4.3: Endophytic Method – Streaked Plates

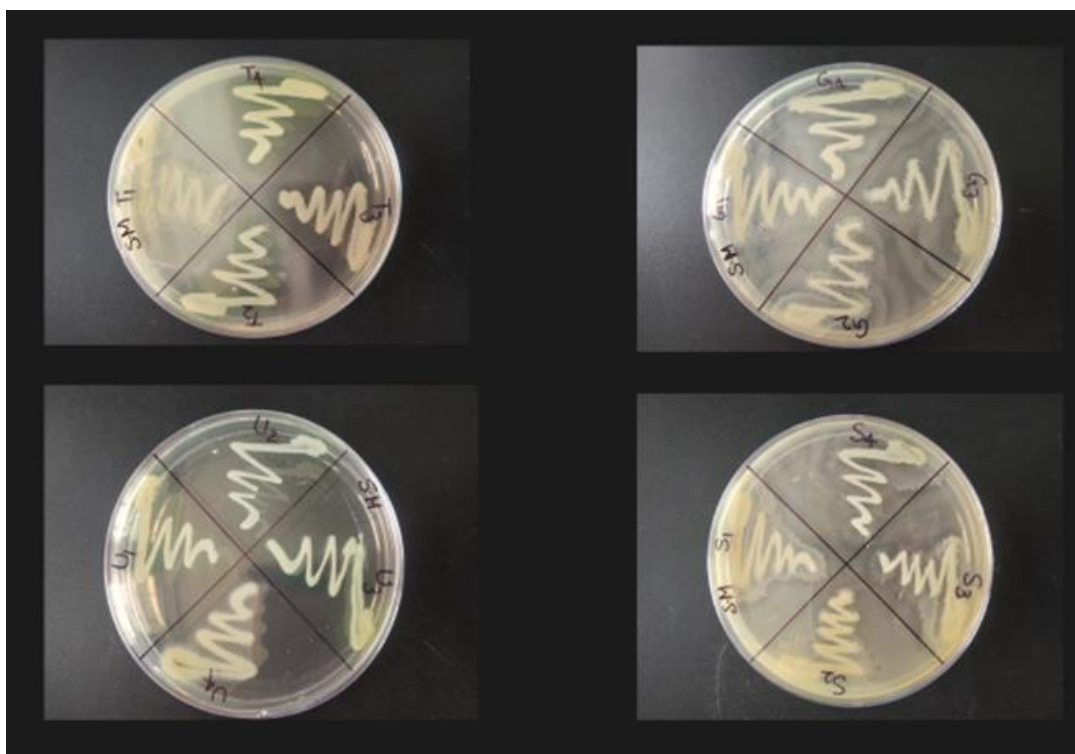


Figure 4.4: Soaking Method – Streak Plates

4.5.2 Nutrient slant preparation

Agar slants are used for storing pure cultures for a long term. There is less amount risk of contamination or of losing the culture due to the medium drying out because the small volume of air inside the tube and narrow opening combine to limit water loss and exposure to outside air, including dust and other particles. Slants are made by making agar in a beaker, dividing it into tubes, sterilising the capped tubes, then cooling them at an angle to create a slanted surface. Calculate the amount of agar you'll need. We get a slanted surface with good surface area and a 1 inch deep "butt" with 7 ml per 16 x 125 mm tube. Multiply the required number of tubes by 7 ml and add 10% for waste. Fill a rack with the desired number of tubes and an equal number of screw caps. In a beaker, weigh out and mix the desired amount of agar. To avoid leaking, the beaker should be nearly double the volume of agar. Place the beaker in the microwave with a stir bar and heat until the agar begins to froth. Keep an eye on the agar to make sure it doesn't boil over. If the agar isn't fully dissolved, heat it again. Use a sterilized loop to distribute the desired volume into each tube while stirring to keep the agar blended. Place a cap on each tube but don't tighten it. The tubes will be able to vent in this manner. Remove the rack safely and sterilise on a liquid cycle (cycle 2 for our autoclave), then tilt the tubes to allow the agar to cure. The seaweed associated bacteria is taken from the streak plate is inoculated in the slant test tube.

4.6 Bacteria inoculation and centrifuge method

The seaweed associated bacteria cultivated in the nutrient slant is taken with the help of the sterilized loop and added to the autoclaved nutrient broth test tubes. The bacteria inoculated test tube is kept in the beaker. The tubes are kept in 24 hours shaker machine for fast bacteria growth. From the shaker the test tubes are taken. The inoculated bacteria is taken and added to the eppendorf for centrifuge method. A centrifuge is a device that uses centrifugal force to separate various components of a fluid. This is done by spinning the fluid at required high speed within a container, for separating fluids of different densities of solids and fluids. 1.5ml of inoculated bacteria from the test tube is added to the eppendorf and kept in the centrifuge machine at 6000 rpm for 10 mins. The supernant and pellet is separated.

4.7 Zone of inhibitory activity with optimized bacterial isolates

The antibacterial activity of plants or microbial extracts is commonly assessed using the agar well diffusion method. The nutrient agar plate surface is inoculated by spreading a volume of microbial inoculum over the entire agar surface, similar to the approach employed in the disk-diffusion method. The human microbial pathogen used for spreading is 1. *Escherichia coli*, 2. *Klebsiella pneumoniae*, 3. *Enterococcus faecium*, 4. *Staphylococcus aureus*, 5. *Pseudomonas aeruginosa*

The above-mentioned bacteria are inoculated in the nutrient broth. 1.3 grams of nutrient broth is added to the 100 ml of distilled water and kept for 18 hours for the growth. Then, using a sterile cork borer or tip, a hole with a diameter of 6 to 8 mm is punched aseptically, and a volume (20–100 L) of the seaweed associated bacteria inoculated solution at the necessary concentration is put into the well. The agar plates are then incubated at the appropriate conditions for the test microorganism. The antibacterial ingredient diffuses across the agar medium and prevents the bacteria strain from growing.

4.8 DNA Sequencing

From the zone of diffusion, the highest diffused seaweed associated bacteria is taken for producing pure culture and provided for DNA sequencing. PCR product of the 16S gene is obtained, sequenced, and aligned against bacterial DNA data base, then the bacterium can be identified.

5. RESULTS AND DISCUSSION

5.1. Antibacterial activities

The zone of inhibition process as mentioned in the above paragraph was left around to check the antibacterial activity from the seaweed associated bacteria. From the 5 seaweeds taken for experimenting antibacterial activities, shown different responses with respect to the human pathogens used during both endophytic as well as soaking method. The endophytic method doesn't show reliable inhibition than compared to the soaking method. In soaking method since the used seaweeds have been soaked for more than 3 hours the seaweed associated bacteria have shown good antibacterial zone of inhibition.

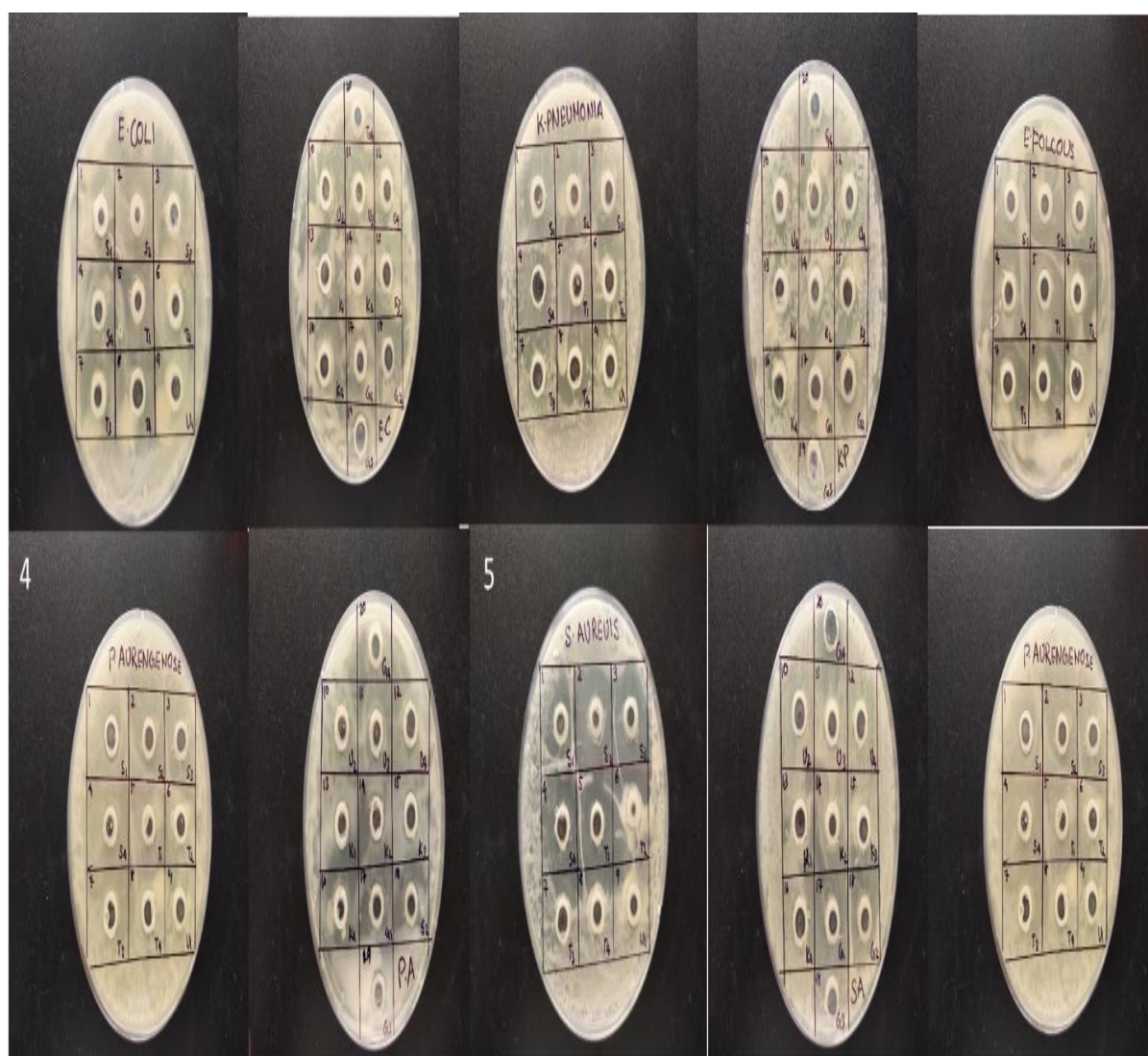


Figure 4.5: Antibacterial activities zone of inhibition: (1) *Escherichia coli*, (2) *Klebsiella pneumoniae*, (3) *Enterococcus faecium*, (4) *Staphylococcus aureus*, (5) *Pseudomonas aeruginosa*

Comparatively with another human pathogen in *Staphylococcus aureus* Sargasam wightii, *Turbinaria conoides* zone was formed relatively high. *Staphylococcus* has shown decent zone in all the four walls *Turbinaria* shows zone in one wall. *Turbinaria* has sent to further processing (i.e.) DNA Sequencing.

5.2. Molecular Identification by 16S rDNA Sequencing

Following Mohandass et al (2012) approach, the method of genomic DNA was extracted from bacterial isolates. The bacterial consensus universal forward and

reverse 16S rDNA primers 27F and 1492R were used to amplify the 16S rRNA gene in a final volume of 25 µl (Lane, 1991). 1x PCR buffer (Sigma, United States), 2.5 mM MgCl₂, 200 mM dNTPs, 1U of Taq DNA polymerase, 25 picomol of each forward and reverse oligonucleotide primer, and roughly 20 ng of genomic DNA were used in the reaction mixture. An initial denaturation at 94°C for 3 minutes was followed by 35 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute. After that, there was a last extension stage of 72°C for 5. minute. The samples were kept at 4 degrees Celsius until they were analysed. The PCR products were sequenced at the Bioserve Biotechnologies India pvt Ltd, Unit D4 to D7, 1st floor, Moula-ali industrial area, Hyderabad 500040. The sequences is sent to the Gen Bank, where they will be giving accession numbers and future processing.

Sequestration:

>CRO-1140-SampleA-10F

GGGCAAATACCGGATAATATATTTGAACCGCATGGTTGCAATAGTGAAAGACGG
TTTTGC

TGTCACCTTATAGATGGATCCGCGCCGCATTAGCTAGTTGGTAAGGTAACGGCTT
ACCAAG

GCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAAGTGA
CACGGTCC

AGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCT
GACGGAGC

AACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAACTCTGTTATTAGGGAA
GAACAA

ATGTGTAAGTAACTATGCACGTCTTGACGGTACCTAATCAGAAAGCCACGGCTA
ACTACG

TGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGG
CGTAAAG

CGCGCGTAGGCGGTTTTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGG
AGGGTCA

TTGGAAACTGGAAAAC TTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGC
GGTGAA

ATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTA
ACTGACG

CTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACCCCTGGGTAAAA

6.SUMMARY AND CONCLUSION

Seaweeds (*Ulva intestinalis*, *Gracilaria corticata*, *Sargasam wightii*, *Turbinaria conoides*, *Kappaphycus alvarezii*) have been collected from Rameswaram coastal area. The collected live seaweeds were used to find the antibacterial activity. Two methods have been followed i.e. endophytic method and soaking method, endophytic method doesn't showed much antibacterial activity compared to the soaking methods. Serial dilution has carried and out for both the methods and each test tube 100 µl was added to the 20 nutrient agar plates for spread plate method, from each plate 1 specific bacterial colony was taken for sub culturing method – streak plate. 4 colonies from 4 plates of one seaweed were taken and streaked in one plate. So total 5 streak plates were ready for 5 taken seaweeds. The seaweed associated bacteria was cultivated in the 20-nutrient broth, then taken for centrifuging method. 1.5 ml from each nutrient broth was taken and added to the eppendorf. At 10000rpm the centrifuged method was carried out. The human microbial pathogen1. *Escherichia coli*, 2. *Klebsiella pneumoniae*, 3. *Enterococcus faecium*, 4. *Staphylococcus aureus*, 5. *Pseudomonas aeruginosa* was inoculated for 18 hours and used to spread the nutrient agar plate to check the antibacterial activity. Each microbial pathogen was streaked for 2 plates for 20 zone of inhibition. 200 zone of inhibition with 5 human pathogens were made for looking into the antibacterial activity, 100 zone of endophytic method haven't showed proper activity. In another method i.e. soaking method, seaweed associated bacteria of *Sargasam wightii*, *Turbinaria conoides* have shown good zone of inhibition at the human pathogen *Staphylococcus aureus* plate, which was comparatively higher than the other seaweeds used. *Sargasam wightii*, *Turbinaria conoides*, among them *Turbinaria* has sent to future analysis - DNA sequencing

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