STUDIES ON BIOACTIVE MOLECULES OF ENDOPHYTIC BACTERIA OF THE SEAWEEDS

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by

ANSHAVI DATTARAJ DESHPRABHU

21P039004

Under the Supervision of

DR. CHANDA V BERDE

School of Earth, Ocean and Atmospheric Sciences Master's

in Marine Microbiology

GOA UNIVERSITY

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Examined by :

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DECLARATION

I hereby declare that the data presented in this Dissertation entitled, **"STUDIES ON BIOACTIVE MOLECULES OF ENDOPHYTIC BACTERIA OF THE SEAWEEDS"** is based on the results of investigations carried out by me in the M.Sc. in Marine Microbiology at the School of Earth, Ocean and Atmospheric Sciences, Goa University under the Supervision of Dr. Chanda Berde and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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Anshavi Deshprabhu
21P039004
M.Sc. Marine Microbiology
School of Earth, Ocean and Atmospheric Sciences, Goa University,
Taleigao Plateau, Goa

Date:

Place: Goa University

CERTIFICATE

This is to certify that the dissertation **"STUDIES ON BIOACTIVE MOLECULES OF ENDOPHYTIC BACTERIA OF THE SEAWEEDS"** is a bonafide work carried out by Ms Anshavi Dattaraj Deshprabhu under my supervision in partial fulfilment of the requirements for the award of the degree Master's in the Discipline M.Sc. Marine Microbiology at the School of Earth, Ocean and Atmospheric Sciences, Goa University.

Chanda

DR. CHANDA V BERDE

M.Sc Marine Microbiology

Date: 28/04/2023

Dean Senior Professor C. U. Rivonker

M.Sc. Marine Microbiology

School of Earth, Ocean and Atmospheric Sciences, Goa University.

Date:

Place: Goa University

School Stamp

Dr. C. U. Rivonker
Senior Professor and Dean,
School of Earth, Ocean
& Atmospheric Sciences,
Goa University.
Goa - 403 206.

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DR. CHANDA V BERDE

M.Sc Marine Microbiology

Date:

Dean Senior Professor C. U. Rivonker

M.Sc. Marine Microbiology

School of Earth, Ocean and Atmospheric Sciences, Goa University.

Date:

Place: Goa University

School Stamp

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ABBREVIATIONS

Gms	Grams
°C	Degree Celcius
R.T.	Room Temperature
Min	Minutes

Hrs	Hours
NA	Nutrient Agar
NB	Nutrient Broth
ZMA	Zobell Marine Agar
MEA	Malt Extract Agar
MEB	Malt Extract Broth
D/W	Distilled Water
ml	Millilitres
µg	Microgram
Spp	Species
g/L	Grams per litre
µg/ml	Micrograms per micro litre
%	Percentage
NaCl	Sodium Chloride
rpm	Revolutions per minute
ppt	Precipitate
No.	Number

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CHAPTER I

INTRODUCTION

Seaweeds are benthic macroscopic algae that grow on rocky shorelines and in marine and shallow coastal waters (Dhargalkar *et al*., 2004). Seaweeds are wonderful aquatic plants that have been commonly known as the "Medical Food of the Twenty-First Century." They have photosynthetic pigments and they use this pigments and the nutrients in seawater to photosynthesize and make food (Dhargalkar *et al*., 2004). Seaweeds are often found along the coastline between the highest and lowest tides, in addition to in the subtidal zone up to a depth where there is 0.01% of accessible light for photosynthetic activity. Seaweed distribution and variety are influenced by a multitude of environmental factors including plant pigments, light exposure, depth, temperature, tides and cost characteristics (Dhargalkar *et al*., 2004). Seaweeds resemble high vascular plants in terms of shape, but they differ greatly from them in structural and functional component.

The entire seaweeds body, which comprises of the hold fast, stipe and blade is known as the thallus and lacks true roots, stem and leaves. Although the holdfast resembles the root of the higher plants, its purpose is attachment, rather than food uptake. Although the stipe resembles a higher plants stem, it serves primarily to support the blade during photosynthesis and to absorb nutrients from the surroundings seawater. The blade may have different shapes (smooth, perforated, segmented) and imitates the leaves of higher plants. Its crucial function include photosynthesis and food absorption (Dhargalkar *et al*., 2004).

One of the many and diverse ecosystems is the seaweed, which is crucial to the marine environment. It primarily contributes to global primary production and offers a diversity of creatures with food and shelter. The surface of seaweeds provides bacteria with sheltered and nutrient-rich conditions for development (Ren *et al*., 2022). Seaweed therefore harbours a rich diversity of related microbes compared to other multicellular organisms. Antibacterial bacteria are believed to defend seaweeds from infections and the surface colonisation of competing species. Epiphytic bacterial communities have been observed to be particularly important for the morphological development of seaweeds. Certain bacterial species have bactericidal ability against particular diseases and host specificity; these specificities include intricate metabolic interactions between bacteria and seaweed (Egan *et al*., 2013).

Seaweeds can be classified in to three broad groups:

1)Green (Chlorophyta) Pigments: Chlorophyll a&b Reserved food: Starch Examples: Ulva, Acetabularia etc	
2) Brown (Phaeophyta) Pigments: Chlorophyll a & c, Fucoxanthin Reserved food: Mannital, Laminaria Examples: Padina, Sargassum etc.	
3) Red (Rhodophyta) Pigments: Chlorophyll a, d, carotene, phycoerythrin, phycocyanin Reserved food: Floridean starch Examples: Gracilaria, Gelidium etc.	 (Google image)

Fig. 1: Three broad groups of Seaweeds

Green algae (Chlorophyta)

They are found in marine and fresh habitats. Green algae contain photosynthetic pigments such as chlorophyll a and b in a special cell structure known as chromatophores. The size and form of the chloroplast might vary. It lacks a chloroplast, endoplasmic reticulum and has a double membrane envelope. Pyrenoids can be found in many different forms in chloroplasts, which are the primary sites of starch formation. Green algae's pyrenoids are variously thought of as large reserves of protein and as unique cell organelles (Fig.1) (Dhargalkar *et al*., 2004).

Brown algae (Phaeophyta)

They are found in marine habitats. The colour of the brown algae ranges from olive-yellow to deep brown. The colour is caused by the carotenoid pigment and fucoxanthin. The amount of fucoxanthin in different species of brown algae varies. The majority of the brown algae in the littoral zone are high in xanthophylls and fucoxanthin. Fucoxanthin-rich algae have much higher rate of photosynthesis in blue light then fucoxanthin-deficient algae. The other photosynthetic pigments of brown algae are Chlorophyll a and c, Beta-carotene and xanthophylls (Dhargalkar *et al*., 2004)

Red algae (Rhodophyta)

They are found in marine habitats, except for a few species. Red phycoerythrin and blue phycocyanin, two water soluble pigments, are also responsible for Rhodophyta's colour. Chlorophyll a and b, carotene and other colours are also present. Phycoerythrin pigment is found in greater abundance in deeper water and freely illuminated forms, which also have a higher phycoerythrin to chlorophyll ratio. The red algae appears to perform more photosynthesis in low light than brown and green algae (Dhargalkar *et al*., 2004)

Green seaweeds are also small, with a size range similar to red seaweeds (Kamleshbhai *et al*, 2022). Brown seaweeds are frequently enormous, ranging from gigantic kelp, which may develop up to 20m long, to thick leathery seaweeds 2-24m long and smaller species 30-60 cm long. Red seaweeds tend to be smaller in size, measuring anywhere from a few centimetres to roughly a metre in length; nevertheless, red seaweeds are not always red; they can be purple or even brownish red (Cunha *et al*, 2016).

On their surface and in their tissues, seaweed's microbial communities are home to a large and diverse collection of creatures, including archaea, bacteria, fungus, microalgae, protozoa, and viruses. (Lachnit *et al*., 2009). These microorganisms frequently carry out a variety of tasks connected to host development

and growth or stress defence, but they can also have negative impacts, such illness (Loos *et al.*, 2019). Some bacteria can also help seaweed survive by assisting the host in dealing with stress induced by sudden or severe environmental changes (Rosenberg *et al.*, 2010). Thus, seaweed and its associated microbial communities create a 'holobiont' (the host and its symbiont), which is a single biological entity with highly specialised symbiotic interactions required for both the host and the symbiont to function). As a result, holobionts may operate as a natural selection unit and provide a unit to target for the development of seaweed ecological and industrial uses (Rosenberg *et al.*, 2010).

In addition to living on the sea surface, these creatures also settle on marine animals and plants and develop distinctive relationships with their hosts (Lafi *et al*., 2005; Webster *et al*., 2008; Singh and Reddy 2014). In exchange for using nutrients (such as carbon) produced by their host, associated microorganisms secrete certain biologically active chemicals known as "bioactives" that shield their host from potentially damaging outside forces (Armstrong *et al*., 2001; Jiang *et al*., 2001; Jamal *et al*., 2006; Lane and Kubanek 2008). Seaweeds are a component of extremely productive ecosystems and serve as the habitat for many microorganisms that produce bioactive chemicals. Associated microorganisms can produce bioactive chemicals that have a wide range of biological actions, including antibacterial, antisettlement, antiprotozoan, antiparasitic, and anticancer properties (Ren *et al.*, 2022).

The abundance of marine endophytic bacteria found in macroalgae (seaweeds), which have a wide variety of bioactive chemicals such flavonoids, terpenoids, alkaloids, quinones, sterols, tannins, and polysaccharides, is drawing the attention of biochemical researchers (Erbabley *et al*., 2020). Isolation of bacteria associated with seaweeds (marine macroalgae) has attracted interest in recent years in a quest for novel bioactive compounds, due to the increasing demand for new therapeutic drugs from natural products in order to combat pathogens having multidrug resistance (Hagaggi *et al.*, 2022).

Endophytic seaweed–bacterial relationships

Bacteria not only live epiphytically on algal surfaces, but also within the thallus or cells. Algal thalli can be damaged by seaweed grazers or epiphytic bacteria that can break down algal cell walls, opening an entry point for pathogenic and opportunistic bacteria (Craigie *et al*., 1992; Correa *et al*., 1994; Craigie *et al*., 1996; Wang *et al*., 2008). In the event that these latter bacteria are able to penetrate the algal tissue and aid in the continued breakdown of the host, thallus rupture may eventually result (Goecke *et al*., 2010). In addition to these pathogenic relationships, nonharmful endophytic bacteria that are connected with seaweed are also described. More than 20 species of red and brown macroalgae have been found to have algal galls, which are aberrant tissue growths of seaweeds (reviewed in Apt). Endophytic bacteria in the red seaweed Prionitis cause galls by overproducing the phytohormone indole-3-acetic acid (IAA), which also provides a favorable microhabitat for the growth of the bacteria themselves (Ashen *et al.*, 2008) .Despite the fact that these endophytic bacteria have been linked to detoxification, nitrogen fixation, and photosynthetic activities (Chisholm *et al*., 2013), the true physiological makeup of these endobiotic siphonous seaweed-bacterial symbioses is still unknown. Secondary metabolites are biologically active substances that some endophytes create and exude. Seaweeds are a rich source of bioactive substances, which offer significant health benefits. Phenolic chemicals, polysaccharides, Polyunsaturated Fatty Acids (PUFAs), proteins, vitamins, and minerals are a few of the bioactive components found in seaweed. According to studies by (Debbarama *et al*., 2016), these chemicals exhibit biological activities and have the potential to be used as medications to treat degenerative disorders such as cancer, tumours, thrombosis, diabetes, inflammation, and others. Secondary metabolites, which some endophytes make and exude, are physiologically active substances that can assist the host plant in combating off bacterial, fungal, and other pest diseases. Endophytic organisms plays an important role in discovery of novel bioactive secondary metabolites. Bioactive secondary metabolites of endophytic organisms serves as a vital source for antimicrobial, antifungal, anti-insect and many more properties. Secondary metabolites derived from marine organisms are organic compounds with low molecular mass. In contrast to primary metabolites, secondary metabolites are not directly engaged in development, growth, or reproduction of the organisms (Netzker *et al.*, 2015). The marine environment is a rich source of secondary metabolites, which can be used to develop specialized product. Proteins, polysaccharides, and lipids are the primary algal metabolites, while phenolic compounds, halogenated compounds, sterols, terpenes, short peptides, and other bioactive substances are the secondary metabolites produced in algae tissues (Rosa *et al.*, 2019).

Mosquito larvicidal activity of seaweeds extracts

Mosquitos play a significant role in the spread of dengue, malaria, yellow fever, filariasis, and other diseases that are among the world's most serious health issues. The current return of these diseases is caused by an increase in the number of breeding sites in today's throwaway society. To manage the everincreasing population of vectors, synthetic insecticides such as organochlorine, organophosphorus, carbamates, pyrethrins, and pyrethroids are routinely utilised. Overuse of these chemical insecticides is not safer for the environment, and nontarget organisms have developed resistance. As a result, new tactics free of such concerns are required for current times in order to construct environmentally safe, biodegradable, cost effective indigenous approaches comprised of lawful scientific and technological weaponry with more powerful combatable features against such vectors. Natural materials are often favoured due to their lower toxicity to target species and inherent biodegradability. Plant products have long been utilised by human populations in various parts of the world to combat vectors and insect species. Many researchers have discovered that phytochemicals derived from plants can operate as larvicides and insect growth regulators, as well as have deterrent properties. Marine halophytes, which include seaweeds, mangroves, and seagrass, are a specialised group of plants adapted to extreme saline conditions. In the study, the species of mosquito used are *Culex quinquefasciatus* which is known to cause Lymphatic filariasis, which is transmitted through the bite of infected mosquito. (Syed Ali *et al.*, 2013).

The following is the list of marine algae across the Goa coast, based on fresh collections. Of the 145 specimens red algae comprise of 64 species, green algae comprise of 41 species and brown algae comprise of 40 species, 70 species represent new records for the Goa coast. Seaweeds grow along the Goa Coast in the intertidal zone and beyond the tidal area, where water splashes during high tide (spray zone), and below it (in the subtidal zone, on submerged reefs and rock boulders). Their growth is affected by the season and wave action. Goa is located on India's Central West Coast, between 14º 49' - 15º 52' N and 73º 38' - 74º 24' E. The state has a coastline of around 120 km. It is divided into sections by estuaries, beaches, rocky coasts, cliffs, bays, and creeks. There are seven estuaries and three bays, as well as sandy and rocky beaches. The climate is mild (atmospheric temperature 20-35°C) and humid (humidity 60-90%), with about 3000 mm8 of annual rainfall (Almeida *et al.*, 2014)

1. ***Erythrocladia irregularis*** Rosenvinge
2. ***Porphyra crispata***Kjellman
3. ***Porphyra suborbiculata***Kjellman
4. ***Porphyra vietnamensis***T. Tanaka & Pham.-Hoang Ho
5. ***Acrochaetiumrobustum***Børgesen
6. ***Ahnfeltia plicata***(Hudson) Fries
7. ***Gelidium micropterum*** Kützing
8. ***Gelidium pusillum*** (Stackhouse) LeJolis
9. ***Pterocladia capillacea***(Gmelin) Bornet
10. ***Gracilaria corticata***(J. Agardh) J. Agardh
11. ***Gracilaria foliifera*** (Forsskål) Børgesen
12. ***Gracilaria verrucosa*** (Hudson) Papenfuss
13. ***Grateloupia filicina***(Lamouroux) C. Agardh
14. ***Grateloupia filicina*** f. ***horrida***(Kützing) Børgesen
15. ***Grateloupia lithophila*** Børgesen
16. ***Peyssonnelia obscura***Webber -van Bosse var. ***bombayensis*** Børgesen
17. ***Hildenbrandia rubra*** (Sommerfelt) Meneghini
18. ***Amphiroa anceps***(Lamarck) Decaisne
19. ***Amphiroa fragilissima***(Linnaeus) Lamouroux

20. ***Amphiroa rigida*** Lamouroux
21. ***Cheilosporum spectabile*** Harvey ex Grunow
22. ***Corallina officinalis*** Linnaeus
23. ***Hydrolithon farinosum*** (Lamouroux) Penrose & Chamberlain
24. ***Hydrolithon reinboldii***(Weber-van Bosse & Foslie) Foslie
25. ***Jania rubens*** (Linnaeus) Lamouroux
26. ***Lithophyllum orbiculatum***(Foslie) Foslie
27. ***Mesophyllum erubescens***(Foslie) Lemoine
28. ***Catenella caespitosa***(Withering) L. Irvine
29. ***Chondracanthus acicularis***(Roth) Fredericq
30. ***Hypnea flagelliformis***Greville ex J. Ag.
31. ***Hypnea musciformis***(Wulfen) Lamouroux,
32. ***Hypnea spinella***(C. Agardh) Kützing
33. ***Hypnea valentiae***(Turner) Montagne
34. ***Ahnfeltiopsis pygmaea***(J. Agardh) P. C. Silva & De Cew
35. ***Champia compressa***Harvey
36. ***Champia parvula*** (C. Agardh) Harvey
37. ***Gastroclonium compressum*** (Hollenberg) Chang & B. Xia
38. ***Gelidiopsis variabilis***(J. Agardh) Schmitz
39. ***Antithamnion cruciatum*** (C. Agardh) Nägeli
40. ***Centroceras clavulatum*** (C. Agardh) Montagne
41. ***Ceramium cimbricum*** H. Petersen
42. ***Ceramium cruciatum*** Collins & Hervey
43. ***Gayliellaflaccida*** (Harvey ex Kützing) T. O. Cho & L. J. McIvor
44. ***Aglaothamnion tenuissimum*** (Bonnemaison) Feldmann-Mazoyer
45. ***Crouania attenuate***(C. Agardh) J. Agardh
46. ***Ptilothamnion speluncarum*** (F. S. Collins & Hervey) D. L. Ballantine & M. J. Wynne
47. ***Wrangelia argus***(Montagne) Montagne
48. ***Dasya ocellata*** (Grateloup) Harvey
49. ***Caloglossa leprieurii*** (Montagne) G. Martens
50. ***Caloglossa ogasawaraensis*** Okamura

51. ***Erythroglossum lusitanicum*** Ardré
52. ***Hypoglossum hypoglossoides***(Stackhouse) Collins & Hervey
53. ***Martensia fragilis*** Harvey
54. ***Acanthophora muscoides***(Linnaeus) Bory
55. ***Acanthophora spicifera***(Vahl) Børgesen
56. ***Bostrychia radicans***(Montagne) Montagne
57. ***Bostrychia tenella*** (Lamouroux) J. Agardh
58. ***Chondria armata*** (Kützing) Okamura
59. ***Chondria capillaris***(Hudson) Wynne
60. ***Herposiphonia secunda***(C. Agardh) Ambronn forma ***tenella*** (C. Agardh) Wynne
61. ***Laurencia obtusa*** (Hudson) J. V. Lamouroux
62. ***Neosiphonia ferulacea*** (Suhr ex J. Agardh) S. M. Guimarães & M. T. Fujii
63. ***Polysiphonia atlantica*** Kapraun & J. Norris
64. ***Polysiphonia denudata***(Dillwyn) Greville
65. ***Vaucheria longicaulis*** Hoppaugh
66. ***Feldmannia indica*** (Sonder) Womersley & Bailey
67. ***Feldmannia irregularis*** (Kützing) G. Hamel
68. ***Hinksia mitchelliae*** (Harvey) P. Silva
69. ***Ralfsia verrucosa*** (Areschoug) Areschoug
70. ***Sphacelaria rigidula*** Kützing
71. ***Canistrocarpus cervicornis***(Kützing) J. C. De Paula & O. De Clerck
72. ***Canistrocarpus crispatus*** (Lamouroux) Paula et Clerck
73. ***Canistrocarpus magneanus*** (Clerck etCoppejans) Paula et Clerck
74. ***Dictyopteris australis***(Sonders) Askenasy
75. ***Dictyota bartayresiana***Lamouroux
76. ***Dictyota ceylanica*** Kützing
77. ***Dictyota ciliolata***Kützing
78. ***Dictyota dichotoma***(Hudson) Lamouroux
79. ***Dictyota divaricata*** Lamouroux
80. ***Dictyota dumosa*** Børgesen

81. ***Padina antillarum*** (Kützing) Piccone
82. ***Padina australis*** Hauck
83. ***Padina boryana*** Thivy in Taylor
84. ***Padina gymnospora*** (Kützing) Sonder
85. ***Padina pavonica***(Linnaeus) Thivy in Taylor
86. ***Spatoglossum asperum*** J. Agardh
87. ***Spatoglossum variabile*** Figari & De Notaris
88. ***Stoechospermum polypodioides***(J. V. Lamouroux) J. Agardh
89. ***Chnoospora minima*** (Hering) Papenfuss
90. ***Colpomenia sinuosa***(Mertens ex Roth) Derbès & Solier
91. ***Iyengaria stellata*** Børgesen
92. ***Rosenvingea orientalis*** (J. Agardh) Børgesen
93. ***Sargassum cinctum*** J. Agardh
94. ***Sargassum cinereum***J. Agardh
95. ***Sargassum cinereum*** J. Agardh var. ***berberifolium***Børgesen
96. ***Sargassum crassifolium*** J. Agardh
97. ***Sargassum glaucescens*** J. Agardh
98. ***Sargassum ilicifolium***(Turner) C. Agardh 99. ***Sargassum plagiophyllum*** C. Agardh
100. ***Sargassum polycystum*** C. Agardh
101. ***Sargassum prismaticum*** Chauhan
102. ***Sargassum swartzii*** C. Agardh
103. ***Sargassum tenerrimum*** J. Agardh
104. ***Sargassum vulgare*** C. Agardh
105. ***Gayralia oxysperma*** (Kützing) Vinogradova
106. ***Ulva clathrata***(Roth) C. Agardh
107. ***Ulva compressa***Linnaeus
108. ***Ulva conglobata***Kjellman
109. ***Ulva flexuosa*** Wulfen
110. ***Ulva intestinalis***Linnaeus
111. ***Ulva lactuca*** Linnaeus
112. ***Ulva rigida*** C. Agardh

113. ***Ulva taeniata*** (Setchell) Setchell *et*Gardner
114. ***Chaetomorpha antennina*** (Bory) Kützing
115. ***Chaetomorpha linum***(O. F. Müller) Kützing
116. ***Chaetomorpha spiralis***Okamura
117. ***Cladophora bombayensis*** Børgesen
118. ***Cladophora coelothrix*** Kützing
119. ***Cladophora glomerata***(L.) Kützing
120. ***Cladophora herpestica*** (Montagne) Kützing
121. ***Cladophora lehmanniana***(Lindenberg) Kützing
122. ***Cladophora prehendens***Kraft & Millar
123. ***Cladophora prolifera***(Roth) Kützing
124. ***Cladophorarhizoclonioidea***van den Hoek & Womersley
125. ***Cladophora saracenica***Børgesen
126. ***Cladophora socialis*** Kützing
127. ***Cladophora vagabunda***(Linnaeus) van den Hoek
128. ***Rhizoclonium riparium***(Roth) Harvey
129. ***Rhizoclonium tortuosum*** (Dillwyn) Kützing
130. ***Boodlea composita***(Harvey) Brand
131. ***Cladophoroposis sundanensis***Reinbold
132. ***Phyllodictyon anastomosans*** (Harvey) G. T. Kraft & M. J. Wynne
133. ***Valoniopsis pachynema*** (Martens) Børgesen
134. ***Bryopis hypnoides*** Lamouroux
135. ***Bryopsis pennata***Lamouroux
136. ***Bryopsis plumosa*** (Hudson) C. Agardh
137. ***Caulerpa peltata***Lamouroux
138. ***Caulerpa racemosa***(Forsskål) J. Agardh
139. ***Caulerpa scalpelliformis*** (Brown ex Turner) C.Agardh
140. ***Caulerpa sertularioides***(Gmelin) Howe
141. ***Caulerpa sertularioides***(Gmelin) Howe forma ***brevipes*** (J. Agardh) Svedelius 142. ***Caulerpa sertularioides***(Gmelin) Howe forma ***longiseta*** (Bory) Svedelius
143. ***Caulerpa verticillata*** J. Agardh

144. ***Avrainvillea erecta***(Berkeley) A. Gepp & E. Gepp 145. ***Chlorodesmis hildebrandtii*** A.Gepp & E.Gepp

CHAPTER II

LITERATURE REVIEW

1.1 The seaweeds were collected from Cap Zebib (37° 16.2'N, 10° 3.6'E) on Tunisia's northern coast in the winter and summer of 2007. The collected seaweeds were then kept in bags, which had water from the same site (Ismail *et al*., 2016).

Seaweed samples were washed three times with autoclaved saline to remove white bacteria and loose bacteria, and then isolated as epiphytic bacteria (Burgess *et al*., 2003). Recover epiphytic organisms by vortexing 10 g of algae biomass in 90 ml of autoclaved seawater for 6 minutes. Bacteria were isolated using autoclaved saline and diluted back to 10^{-3}. Plate 100 µl of each dilution in triplicate on sea agar plates. Leave the plate at 20 °C for at least 7 days or until colonies form (Lemos *et al*., 2016). Select the colonies and inoculate on MA until cultured (J.G. *et al*., 2003).

18 bacterial type strains were used for checking the antimicrobial activity of all isolated epiphytes, which were: *Aeromonas salmonicida* LMG3780*, A. hydrophila* B3 , *Enterococcus faecalis* ATCC 29212, *Escherichia coli* O126-B16 (ATTC 14948), *E. coli* ATCC 25922, *E. coli* ATCC 8739, *Micrococcus* sp., *Pseudomonas cepacia , P. fluorescens* AH2, *P. aeruginosa* ATCC 27853, *Salmonella typhimurium* C52, *Staphylococcus aureus , S. aureus* ATCC 25923, *S. aureus* ATCC 6538, *Streptococcus* sp., (*Vibrio tapetis* CE CT4600, *V. anguillarum* ATCC12964T, *V. alginolyticus* ATCC 17749T. The yeast strain Candida albicans ATCC 10231 was also utilised in the experiment (Ismail *et al*., 2016).

Screening of isolates for antibiotics against human and fish diseases.

The 2 two methods were utilized in the experiment. First being the drop test which was performed on plates containing Trypto-Casein-Soy agar with 20 $g1^{-1}$ NaCl as specified by (James *et al*.,1996) and (Rao *et al*. 2005). Drops of an overnight culture's cell suspension were spotted onto agar plates containing a confluent lawn of the target strain (dried for 30 minutes at 30°C) and then incubated at 30°C. (Wiese *et al*.,2009) explained the overlay assay in detail with some modifications. Drops (10 ul) of an overnight culture of the isolates were placed onto TSA plates supplemented with 20 g l^{-1} NaCl and cultured for 24 h at 30°C being treated with a soft agar overlay comprising the desired strains [3 g l^{-1} Tryptone Soy Broth-(TSB-BIO RAD, 9 g l^{-1} agar (PRS, Panreac), 20 g l^{-1} NaCl, pH 7.2]. The target strain suspension contained ~107 cells ml1. After incubation (24 hours at 30°C), growth inhibition was assessed by measuring the zone of inhibition (mm).

For antibiotic sensitivity of isolated strains. The disc diffusion method (Barry & Thornsberry,1980) was used to investigate the sensitivity to various antibiotics on Muller Hinton agar (MH, BIO RAD) plates using the following antimicrobial compounds (BIO RAD, France), amounts are given per disc:

streptomycin (500 g), amoxicillin (25 g), tobramycin (10 g), nalidixic acid (30 g), oleandomycin (15 g), cefoxitin If a growth inhibition zone was seen around the disc, a strain was judged susceptible to an antimicrobial drug. The interpretation was done in accordance with the French Society of Microbiology's norms (Ismail *et al*., 2016).

1.2 The seaweeds, *Egregia menziesii* (Turner) A*reschoug* (Em), *Codium fragile* (Suringar) *Hariot* (Cf), *Sargassum muticum* (Yendo) *Fensholt* (Sm), *Endarachne binghamiae* (Petalonia binghamiaed) (J.), *Centroceras clavulatum* (Agardh) *Montagne* (Cc) and *Laurencia pacifica Kylin* (Lp) were caught at low tide on the coast near the Autonomous University of Baja California in Mexico. Collected samples were sent into frosted plastic bags (Villarreal-Gomez *et al*., 2010).

Sterile swab were used to wipe the algae surface before the bacterial extracts were inoculated into sea a gar and seawater (Difco) isolation plates. Leave the plates for 24-48 hours at 25 °C until colonies appear (Villarreal Gómez *et al*.,2010).

At a dosage of 10 mg ml^{-1} in DMSO, the algal and bacterial extracts were tested for antibacterial effectiveness against the pathogen bacteria Staphylococcus aureus (ATCC25923), *Klebsiella pneumoniae* (ATCC 13883), Proteus mirabilis (ATCC 35659), and Pseudomonas aeruginosa (ATCC 27853) (Villarreal Gómez *et al*,.2010).

1.3 The four species of seaweed, *Ceramium rubrum* (Rhodophyta), *Sargassum vulgare, Sargassum fusiforme, and Phaeophyta* (Phaeophyta), were collected from the Hurghada coast in Egypt's Red Coast, and analysis was performed to remove impurities. It was wash with water. Seaweed was sent to the laboratory in sterile plastic bags (Shafay *et al*., 2016).

The antibiotic susceptibility of the bacteria was determined using the Clinical and Laboratory Standards Institute's (CLSI, 2012) modified Kirby-Bauer disc diffusion method. Antibiotic concentrations were examined at the following levels: Ampicillin (AMP10µg), Amoxicillin (AX,25µg),

Amoxycillin/Clavulanic acid (AMC, 20/10µg), Piperacillin-tazobactam (TPZ, 100/10µg), Oxacillin (OX, 1µg), Ceftazidime (CAZ,30), Cefepime (FEP, 30µg), Ceftriaxone (CRO,30µg), Imipenem (IPM, 10µg), Meropenem (MEM,10µg), Cefoperazone/sulbactam (CES, 75/30µg), Aztreonam (ATM, 30µg), Gentamicin (CN,10µg), Amikacin (AK,30µg), Neomycin (N,30µg), Streptomycin (S,10µg), Tob ramycin

(TOB,10µg), Kanamycin (K30µg), Chloramphenicol (C,30µg), Colistin Sulfate (CT,10µg), Nalidixic acid (NA, 30µg), Ciprofloxacin (CIP, 5µg), Co-trimoxazole (SXT, 25µg), Tetracycline (TE, 30µg), Vancomycin (VA, 30µg). The antibiotic disks were then applied to the prepared plates and incubated at 37 °C for 18 h then, the diameter of the growth inhibition zones was measured (Shafay *et al*., 2016).

1.4 Seaweeds were obtained from a population of green algae (*Ulva lactuca*), brown algae (*Sargassum denticulatum, Hormophysa triquetra*) and red algae (*Hypnea cornuta*) and blue-green algae (*Sargassum denticulatum*) from Egypt (Musbah *et al*., 2019).

The antifungal activity was screened using the agar well diffusion method for the antifungal susceptibility test (Bauer *et al*., 1996). Candida species inoculates(108 cells/ml) were spread on Sabrouds Dextrose agar plates and left to dry at room temperature, wells were made on the surface of agar medium with 6 mm cork borer. Each well in plate was filled with 50 □m of algal crude extract using micropipette. The plates were incubated at 37 ºC from 24- 48 hrs, At the end of incubation the plates were observed for the zone of inhibition and the diameters of the zones were measured in mm (Karabay-Yavasoglu *et al*., 2007). All assays were carried out independently in triplicates and the mean result were calculated, also Fluconazole and dimethyl sulfoxide (DMSO) were used as positive and negative controls, respectively (Musbah *et al*., 2019).

1.5 Collected three species of seaweed, *Sargassum wightii, Turbinaria connoides, and Padina gymnocephalus*, from the Gulf of Mannar, Mandapam Coast, Tamil Nadu (India) (Minimol *et al*., 2019). A total of 25 g of dried seaweed was aseptically removed in phosphatebuffered saline for separation and serial dilutions were prepared. Then inoculate 1 ml of each dilution into a test tube containing 9 ml of E PS medium and0.2 g of KH2PO4; 1.5 grams K2HPO4; 0.2 gram MgS04 7H2O; 0.1 gram CaSO4 2H2O ; 2.0 mg of ferric chloride; 0.5 g of yeast extract and 20 g of sucrose / l. Samples were then plated on an equal volume of Tryptic Soy Agar (BD, Mumbai, India) and incubated at 37°C for 48 hours (Minimol *et al*., 2019) .

Exopolysaccharide (EPS) was isolated from bacteria using the method described by Berekaa and Ezzeldin (2018). The bacterial isolates were inoculated into the EPS culture medium and cultured at 37ºC in a shaker incubator (MaxQ 6000, Thermoscientific, USA) with 200 rpm rotation for 10 days. After

incubation, the cultures were centrifuged for 20 minutes at 9000 rpm (Centrifuge 5804R, Eppendorf, India). The supernatant was collected and blended in a 1:2 ratio with cold alcohol. Exopolysaccharide deposition of biomass was rinsed with distilled water and dried at 60^{o} C for 2-4 hours. The isolate with the highest EPS production was chosen for further investigation (Minimol *et al*., 2019).

1.6 Collected extracts of *Ulva, C. racemosa, Sargassum, Porphyra fanii, Gracilaria, Turbinaria decurrens, Turbinaria conoides,* and *Caulerpa toxifolia*, dissolved in DMSO grades, to generate graded sequences (Syed Ali *et al*., 2013).

In the present study, mosquito larvae of *Culex quinquefasciatus* at the late 3rd to 4th instar stage were used. 19 cleaned autoclaved plastic containers were filled with 100ml of RO (Reverse Osmosis) water and labelled. To the each container, 20 healthy larvae were introduced. 1container was kept as a control. In the other 18 containers 1ml of the supernatant was added. Mortality was recorded after 1hour, 24 hours and 48hours.

(Syed Ali *et al*., 2013) The WHO standard method was used to conduct the test for the larvicidal impact of seaweeds on mosquito larvae. 25 mosquito larvae of the early fourth instar were placed in a 250 mL enamel bowl comprising 199 mL of distilled water and 1 mL of extracts from plants (10-100 g). Each experiment was carried out in triplicate, with a control group present at the same time. Deaths were registered.

CHAPTER III

AIM AND OBJECTIVES

AIM

Isolation of endophytic bacteria from the seaweeds. Endophytes from seaweeds produce and secrete biologically active compounds that prevent bacteria, fungi and plant pests from growing in the host plant. Due to the rise in multi-drug resistant pathogens and other diseases, there is renewed interest in seaweeds endophytic bacteria as a rich source of natural products.

OBJECTIVES

1. Sample collection and isolation of bacterial endophytes of seaweeds.

2. Study of the antimicrobial peptides (Antimicrobial and Antifungal activity of culture supernatant).

3. Purification of proteins.

4. Study of exopolysaccharide production and its quantification.

5. Study of cytotoxicity of mosquito larvae.

6. Study of Protein estimation by Folin-Lowry's method.

CHAPTER IV

MATERIALS AND METHODS

1. Sample collection

The seaweeds were collected by hand at low tide from the coastal areas of Goa. The different sampling sites were Ashwem (N 15° 38' 37.6692", E 73° 43' 2.6508") (Sample 1), Miramar (15.4775° N, 73.8121° E) (Sample 2), Querim (15° 43' 1" north 73° 41' 27" east) (Sample 3 and Sample 4) and Anjuna (N 15° 34' 51.33", E 73° 44' 41.4384) (Sample 8) (Fig.2). The other seaweeds were collected from the seaweed cultivation project, Dona Paula. Collected samples was transferred in sterilized glass bottles. 8 samples were collected and transferred into bottles with seawater. Then endophytic bacteria were extracted from samples.

Fig. 2: Sampling sites for collection of seaweeds (Google images)

a) Surface sterilization of seaweed samples

For endophytic bacteria isolation, surface of the samples were clean to eliminate all associated bacteria. For surface sterilization first wash the samples with sterile saline then wash with 1% savlon for 3 minutes, then wash with 0.1% $HgCl_2$ for 2 minutes, lastly wash the samples with sterile distilled water 2-3 times. Surface clean samples were crushed in sterile mortar and pestle using sterile distilled water in aspectic condition. Endophytes were collected in sterile tubes and stored in freeze.

2. Isolation of endophytic bacteria

Seaweed extract were prepared and used for serial dilution. The sterilised seaweed samples were crushed in alcohol sterilised mortar & pestle. 5 ml of sterile saline are added & extract was collected in sterile test tubes. The extracted was then diluted serially upto 10^{-4} using sterile saline. Then 0.1 ml from 10^{-2},10^{-3},10^{4} were spread on ZoBell Marine Agar (Appendix 1) plates and incubated at room temperature for 24-48 hours (hrs). The plates were checked for viable counts. Colonies with distinct characteristics were selected. The bacterial colonies obtained were studied for cultural characteristics of isolates.

a) Growth and maintenance conditions

Endophyte bacterial isolates were sub-cultured using sterile wire loop on Nutrient Agar (Appendix1) slants for short term storage. The slants was incubated at room temperature for 24-48 hours and stored in refrigerator at 4°C. The purity of culture were checked by performing Gram staining and microscopic observations.

b) Colony characteristics of the selected colonies

a) **Size-** Measured by scale in millimetres.
b) **Shape-** Varies from round to irregular to filamentous and rhizoid.
c) **Colour-** Pigmentation of colonies (white, cream, yellow, etc).
d) **Elevation-** How high is the colony above the agar.
e) **Margin-** The colony was observed under the microscope for fine observation of the margin (raised/convex/flat/umbonate/crateriform).
f) **Opacity-** The colony was observed and tested for opacity viz. opaque, transluscent, transparent.
g) **Consistency-** The colony was picked and observed for consistency (consistent/mucoid/butyrous).

c) Gram Staining

Loopful of suspension was taken on clean grease slide. Suspension was then air dried and heat fixed through a Bunsen burner three times. Crystal Violet (primary stain) (Appendix 2) was added for 1 min. The slide was rinsed to remove unbound stain. Gram's iodine was added and kept for 1 min then decolorized for 30 sec with alcohol to remove the Crystal Violet stain. Then, Saffranine (secondary stain) was added for 1 min and water wash was given. Slide was air dried. Then one drop of oil was

placed on the slide and it was observed under oil immersion lens with phase contrast microscopy at 100x.

The isolated endophytic cultures were studied for colony and morphological characteristics by colony characteristics and Gram Staining.

3. Salt tolerance test of the cultures

Nutrient agar plates were prepared with sodium chloride (regular table salt) to with a salt concentration of 10% and 15%. Than all the selected isolates were spot inoculated on the nutrient agar plates with salt concentration of 10% and 15%. Next the plates were incubated at RT for 24 hours. The growth was checked after 24hours.

4. Production and extraction of Exopolysaccharide

The selected isolates were grown in Nutrient Broth (9ml) along with 1% of the Glucose (1ml). It was given 48 hours to grow. Then it was centrifuged at 5000rpm for 10 minutes. Supernatant was taken, and three times the volume of supernatant, chilled ethanol was added. It was further kept in deep freezer for 1 day. The eppendorfs were taken and preweighed. Precipitate formed was then again centrifuged and the precipitate was transferred in to preweighed sterile eppendorf tubes. The weight of the precipitate along with the eppendorf was noted.

5. Study of the antimicrobial peptide

a) Antibacterial activity of the culture supernatant

Host cultures used:

- **Gram positive bacteria- *Staphylococcus aureus*1, *Staphylococcus aureus* 2, *Bacillus* spp**

- **Gram negative bacteria-** ***Escherichia coli*** **1,** ***Escherichia coli*** **2,** ***Proteus*** **spp,** ***Salmonella paratyphi B,*** ***Klebsiella*** **spp,** ***Pseudomonas*** **spp .**

Th cultures were grown in Nutrient Broth and incubated for 4hours. From the NB 50ul of the culture was taken and spread plated on the labelled Nutrient Agar plates. Autoclaved F/12 Filter Paper disc with 6mm diameter were used. The disc was than dipped in the culture supernatant and placed on agar plates. The plates were than incubated at 28^0C for 24 hours. Further the diameter of the zones were measured. Agar cup method and Disk diffusion method was carried out .

b) Antifungal activity of the culture supernatant

Host cultures used: ***Aspergillus*** **spp,** ***Penicillium*** **spp** ***and Saccharomyces cerevisiae***

400ml of the Malt Extract Agar (Appendix 1) plates with 40mg Ampicillin using distilled water was prepared. The cultures *Penicillium* spp and *Aspergillus* spp were grown in sterile normal saline. While *Saccharomyces cerevisiae* were grown in Malt Extract Broth and incubated for 4 hours and then 50ul of the same was spread plated on the labelled Malt Extract Agar plate. Next the disks were dipped in culture supernatant and placed on agar previously inoculated with the test bacterium and incubated overnight at R.T. After incubation the zone of inhibition were measured and noted.

i) Agar cup method

The above test cultures where grown in Nutrient Broth and incubated for 4hours and then 50ul of the same was spread plated on the labelled Nutrient Agar plate. Wells were dug with the help of cork-borer. The wells were then filled with the 100µl supernatant and incubated overnight at R.T. After incubation the zone of inhibition were measured and noted. This method did not gave accurate results so Disk diffusion method was used. **ii) Disk diffusion method**

The above test cultures where grown in Nutrient Broth and incubated for 4hours and then 50ul of the same was spread plated on the labelled Nutrient Agar plate. Next, the disks were dipped in culture supernatant and placed on agar previously inoculated with the test bacterium and incubated overnight at R.T. After incubation the zone of inhibition were measured and noted.

6. Ammonium sulphate precipitation of antibacterial peptide

The cultures which gave positive results after antimicrobial screening were grown in Nutrient Broth at RT for 24-48 hours. The broth of each culture were centrifuged at 4000 rpm for 10 minutes. To the supernatant, 70% ammonium sulphate was added, dissolved properly and kept in refrigerator overnight. The supernatant were then centrifuged at 4000 rpm for 10 min and the pellet of precipitate were collected. This pellet were dissolved and stored in refrigerator at -20ºC for further studies. The cultures which gave positive results were again screened to check for zone of clearance.

7. Protein estimation by Folin- Lowry's method

After doing Ammonium sulfate precipitation, the best four samples were taken and subjected to protein estimatimation by Folin Lowry's method (Table.12) (Fig.30). The total protein content was determined by Folin Lowry's method .0.1 ml of sample were mixed with 5.0 ml alkaline $CuSO_4$ and kept for one min. Then 0.5 ml of Folin-Ciocalteau reagent was added and incubated at room temperature in a dark for 50 minutes. After incubation O.D. were taken at 600 nm. Then total protein were determined by the graph of concentration of protein. (Appendix 3)

8. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis analysis

1) Culture media

1ml of pre grown culture was taken and inoculated in sterile nutrient broth media and it was incubated at R.T. for 24-48 hrs. Cell material was harvested after 24 hrs by centrifugation for 10 minutes at 4000 rpm. Cell used for further protein extraction (Table.1).

2) Sample preparation

100 μl of pellet sample was mixed with 25 μl of SDS (Appendix 4) which was then boiled for 10 minutes in boiling water bath. Sample was taken from this boiled material and added to staining buffer then from the above sample 50 μl was taken and loaded in SDS-PAGE.

3) Preparation of slab gels

Thoroughly clean and dried glasses were assembled in the gel casting assembly. The two glass plates were sealed with the help of tygon tubing, clamp them and the whole assembly was placed in an upright position. Various components of resolving gel were mixed. The gel solution was poured into the mould in between the clamped glass plates ensuring that there are no air bubbles. Distilled water was overlaid on top as gently as possible and left for 30 minutes for gel to settle. When the gel was polymerized, the water layer was removed and rinsed with staking gel buffer. The staking gel components were mixed in the same way as described above for resolving gel. The staking gel was poured and immediately plastic comb was inserted in the staking gel ensuring that no air bubbles. The gel was allowed to polymerize for about 20 minutes, then the comb was without distorting or damaging the shapes of the wells. The reservoir buffer was poured in the lower and upper chambers.

Table 1: Addition table of solution casting for SDS-PAGE

	Separating gel(ml)	Staking gel(ml)
Gel concentration	10%	5%
Distilled Water	4.1	3.45

30% acrylamide	**3.3**	**0.83**
1.5M tris, pH 8.8,0.4% SDS	**2.5**	**0.63**
10% APS	**0.1**	**0.005**
TEMED	**0.004**	**0.005**

4) Electrophoresis of the sample

Around 50µl sample was loaded in the sample wells, also the molecular weight marker proteins was loaded in one of the wells. The current was switched ON and maintained at 80 volts for until the samples have travelled through the stacking gel. Then it was increased to 100 volts until the bromophenol blue dye reaches near the bottom of the gel slab this may require 3-4 hrs. After the electrophoresis was completed the current was switched OFF and the power supply was disconnected and the gel slab was carefully removed from between the glass plate. The gel was then destaining solution till the clear background of the gel was obtained.

5) Staining and Destaining of Gel

About 50 ml of staining solution was added to the tray containing gel. The gel was kept overnight in the staining solution for visualization of the bands. Next day the stain was removed and the gel was rinsed with distilled water till a considerable amount of stain reaches out from the gel. Again the destaining solution was added with constant shaking until distinct bands were observed.

9. Cytotoxicity of peptides against mosquito larvae

In this experiment, mosquito larvae of *Culex quinquefasciatus* at the late 3rd to 4th instar stage were used. 19 cleaned autoclaved plastic containers were filled with 100ml of RO (Reverse Osmosis) water and labelled. To the each container, 20 healthy larvae were introduced. One container was kept as a control. In the other 18 containers 1ml of the supernatant was added. Mortality was recorded after 1hour, 24 hours and 48hours. In between feed was given to the larvae in powdered form.

10. Antibiotic sensitivity of selected cultures

The 10 antibiotics, Ampicillin, Chloramphenicol, Erythromycin, Gentamicin, Kanamycin, Nalidixic acid, Rifampin, Streptomycin, Tetracycline, and Vancomycin (Hi media, India) (Appendix 5) were used in the experiment. The selected 18 cultures were grown in Nutrient Broth and incubated at RT for 24hours for growth to occur. Next the Nutrient Agar plates were prepared and 100ul of the culture was spread plated on it. The antibiotic discs were then placed on the plates. The plates were incubated at RT for 24 hours. The zone of inhibition were recorded and compared using standardise zone size interpretative chart.

CHAPTER V

RESULTS

1. Sample collection

Seaweed samples were collected from coastal area of Goa during low tide. Total 8 samples of seaweeds were collected in sterilized glass bottles (Fig 3-10) (Table.2). Total of 76 endophytic isolates were extracted from samples. All the samples were identified with identification chart (www.seaweeds.uib.no/key/, Seaweeds identification key).

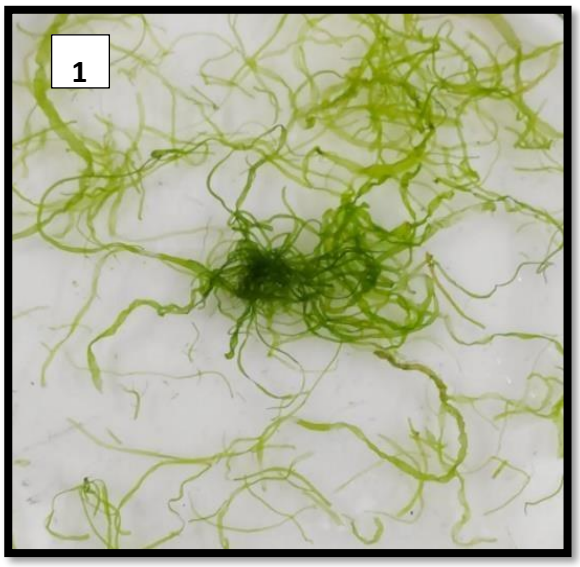


Fig. 3: ***Ulva intestinalis***

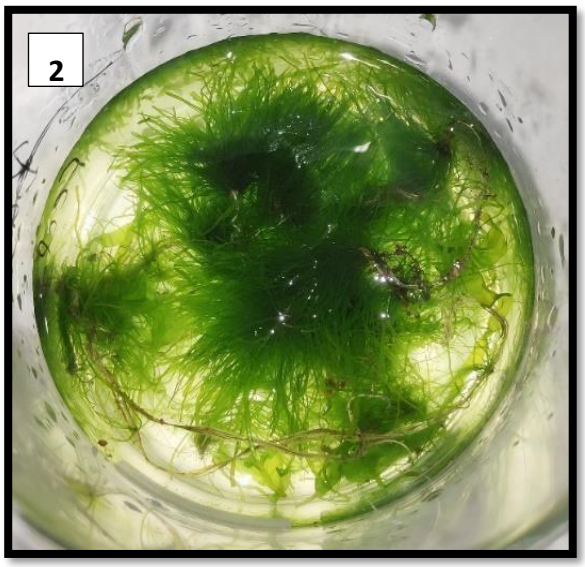


Fig.4: ***Ulva compressa***

Fig.5: ***Ulva lactuca***

Fig.6*: Sargassum polycystum*

Fig.7: Eucheuma denticulatum

Fig.8: Kappaphycus alvarezii

Fig.9: Kappaphycus striatus

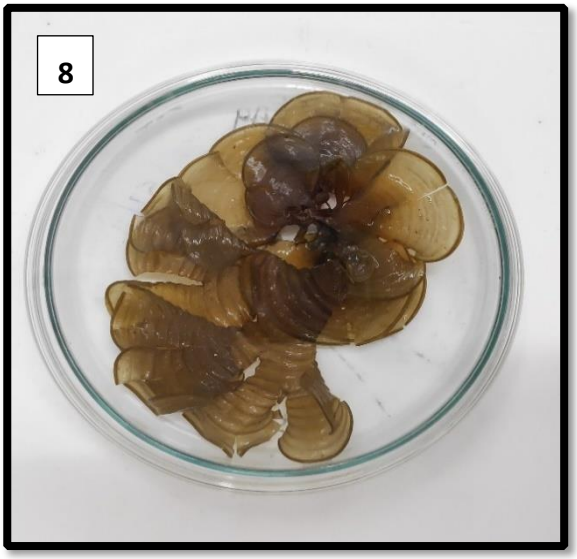


Fig.10: Padina antillarum

Table 2: Numbering of seaweed species

Serial No	Seaweed species name
1	***Ulva intestinalis***
2	***Ulva compressa***
3	***Ulva lactuca***
4	**Sargassum polycystum**
5	**Eucheuma denticulatum**
6	***Kappaphycus alvarezii***
7	***Kappaphycus striatus***
8	***Padina antillarum***

For associated bacteria the sample piece is cut and transferred in sterile tubes containing saline and shake the tubes. All loosely associated bacteria already remove in sampling glass bottle containing sea water. So only associated bacteria are mixed in saline. There was no growth observed around the seaweeds sample piece placed on control plate of Nutrient Agar. So clearly observed that the surface sterilization for seaweed endophytes sample procedure is successful (Fig.11).

Fig.11: Surface sterilized seaweed

2. Isolation of the endophytic bacteria

Seaweed endophytes isolated colonies were observed on Zobell Marine agar plates (Fig.12). Distinct colonies were pick, streak and maintained on the slants (Fig.13).

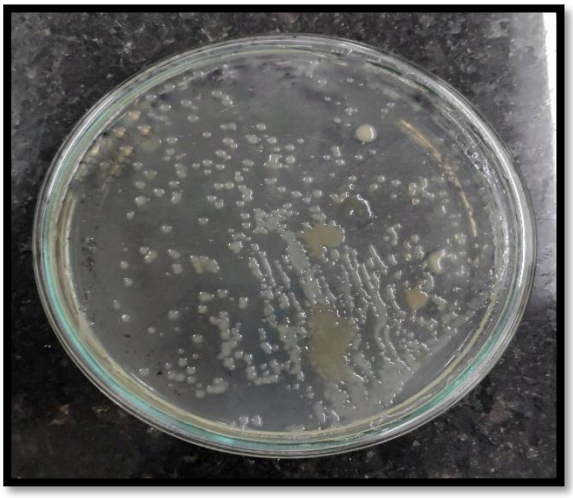


Fig.12: Isolated endophytic colonies

Fig.13: Slants of the isolated colonies

a) Colony characteristics of endophytic isolates

Organisms were isolated from seaweeds were spread plated on Zobell Marine Agar plates. Colony characteristics were determined for selected isolated colonies from seaweeds after 24- 48 hrs of incubation. Total **76 endophytic** isolates were studied (Table.3-10).

Table 3: Sample 1-Colony characteristics of isolated endophytic colonies of seaweeds after 24- 48 hours incubation at R.T.

Isolate	Size (mm)	Shape	Colour	Elevation	Margin	Opacity	Consistency
C1	<1	Circular	Translucent	Raised	Entire	Opaque	Butyrous
C2	1	Circular	White	Raised	Entire	Opaque	Butyrous
C3	<1	Circular	White	Raised	Entire	Opaque	Butyrous
C4	Pinpoint	Circular	Cream	Raised	Entire	Opaque	Butyrous
C5	<1	Circular	White	Raised	Entire	Opaque	Butyrous
C6	<1	Circular	White	Raised	Entire	Opaque	Butyrous
C7	1.5	Circular	White	Raised	Entire	Opaque	Butyrous
W1	<1	Circular	White	Raised	Entire	Opaque	Butyrous
W2	1	Circular	Translucent	Raised	Entire	Tranlucent	Butyrous
W3	<1	Circular	White	Raised	Entire	Opaque	Butyrous
W4	1	Circular	White	Raised	Entire	Opaque	Butyrous
W5	<1	Circular	White	Raised	Entire	Opaque	Butyrous
W6	1	Circular	Transparent	Raised	Entire	Transparent	Butyrous
W7	<1	Circular	White	Raised	Entire	Opaque	Butyrous
W8	<1	Circular	White	Raised	Entire	Opaque	Butyrous
W9	<1	Circular	White	Raised	Entire	Opaque	Butyrous

Table 4: Sample 2-Colony characteristics of isolated endophytic colonies of seaweeds after 24- 48 hours incubation at R.T

Isolate	Size (mm)	Shape	Colour	Elevation	Margin	Opacity	Consistency
1C	<1	Circular	Whitish Yellow	Raised	Circular	Opaque	Butyrous

2C	1	Circular	Whitish Yellow	Raised	Circular	Irregular	Butyrous
3C	1	Circular	Yellowish	Raised	Circular	Irregular	Butyrous
4C	<1	Irregular	White	Raised	Irregular	Irregular	Butyrous
5C	<1	Circular	Yellow	Raised	Circular	Irregular	Butyrous
6C	<1	Irregular	Whitish Yellow	Raised	Irregular	Irregular	Butyrous
7C	<1	Circular	Cream	Raised	Circular	Irregular	Butyrous
8C	1	Circular	Whitish Yellow	Raised	Circular	Irregular	Butyrous
1W	<1	Circular	White	Raised	Circular	Irregular	Butyrous
2W	<1	Circular	Whitish Yellow	Raised	Circular	Irregular	Butyrous
3W	1	Circular	Whitish Yellow	Raised	Circular	Irregular	Butyrous
4W	<1	Irregular	Whitish Yellow	Raised	Irregular	Irregular	Butyrous
5W	<1	Circular	White	Raised	Circular	Irregular	Butyrous
6W	1	Circular	White	Raised	Circular	Irregular	Butyrous
7W	1	Circular	Yellowish	Raised	Circular	Irregular	Butyrous
8W	<1	Circular	Yellowish	Raised	Circular	Irregular	Butyrous

Table 5: Sample 3-Colony characteristics of isolated endophytic colonies of seaweeds after 24- 48 hours incubation at R.T.

Isolate	Size (mm)	Shape	Colour	Elevation	Margin	Opacity	Consistency
1	<1	Circular	White	Raised	Entire	Opaque	Butyrous
2	<1	Circular	White	Raised	Entire	Opaque	Butyrous
3	<1	Circular	White	Raised	Entire	Opaque	Butyrous
4	<1	Circular	White	Raised	Entire	Opaque	Butyrous
5	<1	Circular	White	Raised	Entire	Opaque	Butyrous
6	<1	Circular	White	Raised	Entire	Opaque	Butyrous

Table 6: Sample 4-Colony characteristics of isolated endophytic colonies of seaweeds after 24- 48 hours incubation at R.T.

Isolate	Size (mm)	Shape	Colour	Elevation	Margin	Opacity	Consistency
i	1	Circular	White	Raised	Entire	Opaque	Butyrous

ii	<1	Circular	White	Raised	Entire	Opaque	Butyrous
iii	1	Circular	White	Raised	Entire	Opaque	Butyrous
iv	1	Circular	White	Raised	Entire	Opaque	Butyrous
v	1	Circular	White	Raised	Entire	Opaque	Butyrous
vi	1	Circular	White	Raised	Entire	Opaque	Butyrous

Table 7: Sample 5-Colony characteristics of isolated endophytic colonies of seaweeds after 24- 48 hours incubation at R.T.

Isolate	Size (mm)	Shape	Colour	Elevation	Margin	Opacity	Consistency
C1	3	Irregular	White	Raised	Undulate	Opaque	Butyrous
C2	<1	Circular	White	Raised	Undulate	Opaque	Butyrous
C3	2	Circular	White	Flat	Entire	Opaque	Butyrous
C4	<1	Circular	White	Flat	Entire	Transculent	Butyrous
C5	2	Circular	White	Raised	Entire	Opaque	Butyrous
C6	<1	Circular	White	Flat	Entire	Opaque	Butyrous
C7	2	Circular	White	Raised	Entire	Opaque	Butyrous
C8	3	Circular	White	Raised	Entire	Opaque	Butyrous
C9	2	Circular	White	Raised	Entire	Opaque	Butyrous
C10	1	Circular	White	Raised	Entire	Opaque	Butyrous

Table 8: Sample 6-Colony characteristics of isolated endophytic colonies of seaweeds after 24- 48 hours incubation at R.T

Isolate	Size (mm)	Shape	Colour	Elevation	Margin	Opacity	Consistency
C1	<1	Circular	White	Raised	Entire	Opaque	Butyrous
C2	<1	Circular	White	Raised	Entire	Opaque	Butyrous
C3	2	Circular	White	Raised	Entire	Opaque	Butyrous
C4	2	Circular	White	Raised	Entire	Opaque	Butyrous
C5	3	Circular	White	Raised	Entire	Opaque	Butyrous
C6	2	Circular	White	Raised	Entire	Opaque	Butyrous
C7	2	Irregular	White	Raised	Undulate	Opaque	Butyrous
C8	2	Circular	White	Raised	Entire	Opaque	Butyrous
C9	2	Circular	White	Raised	Entire	Opaque	Butyrous

C10	2	Circular	White	Raised	Entire	Opaque	Butyrous

Table 9: Sample 7-Colony characteristics of isolated endophytic colonies of seaweeds after 24- 48 hours incubation at R.T.

Isolate	Size (mm)	Shape	Colour	Elevation	Margin	Opacity	Consistency
C1	<1	Circular	White	Raised	Entire	Opaque	Butyrous
C2	2	Circular	White	Raised	Entire	Opaque	Butyrous
C3	1	Circular	White	Raised	Entire	Opaque	Butyrous
C4	1	Circular	White	Raised	Entire	Opaque	Butyrous
C5	2	Circular	White	Raised	Entire	Opaque	Butyrous
C6	1	Circular	White	Raised	Entire	Opaque	Butyrous
C7	1	Irregular	White	Raised	Entire	Opaque	Butyrous
C8	1	Circular	White	Raised	Entire	Opaque	Butyrous

Table 10: Sample 8-Colony characteristics of isolated endophytic colonies of seaweeds after 24- 48 hours incubation at R.T.

Isolate	Size (mm)	Shape	Colour	Elevation	Margin	Opacity	Consistency
1	<1	Circular	Whitish Cream	Raised	Entire	Opaque	Butyrous
2	1	Circular	Whitish Cream	Raised	Entire	Opaque	Butyrous
3	<1	Circular	Whitish Cream	Raised	Entire	Opaque	Butyrous
4	<1	Circular	White	Raised	Entire	Opaque	Butyrous

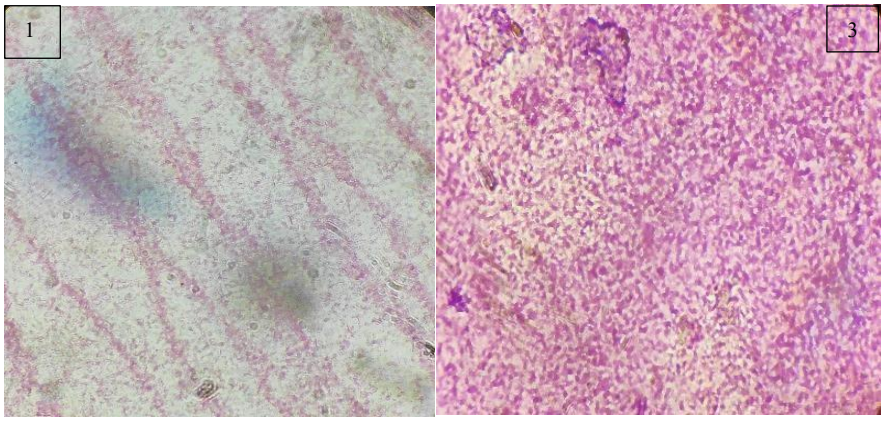
b) Gram staining

Gram staining of 18 different isolates were done. 8 samples were Gram negative, while 6 sample were Gram Positive (Table.11) (Fig 14).

Table 11: Results after performing Gram Staining of 18 different isolates.

No	Isolates	Gram nature	Shape
1	S1-W2	Gram negative	Cocci in clusters
2	S1-W4	-	-
3	S1-W6	Gram negative	Cocci in clusters

4	S2-1W	-	-
5	S2-2W	-	-
6	S2-6W	Gram negative	Rods
7	S2-4C	Gram negative	Rods
8	S2-5C	Gram positive	Cocci in cluster
9	S4-i	Gram positive	Cocci
10	S5-C2	Gram negative	Rod in chains
11	S5-C6	Gram positive	Cocci
12	S6-C5	-	-
13	S6-C7	Gram positive	Cocci
14	S6-C8	Gram positive	Cocci
15	S6-C9	Gram negative	Rods
16	S7-C10	Gram positive	Cocci
17	S7-C3	Gram negative	Cocci
18	S7-C5	Gram negative	Cocci in chains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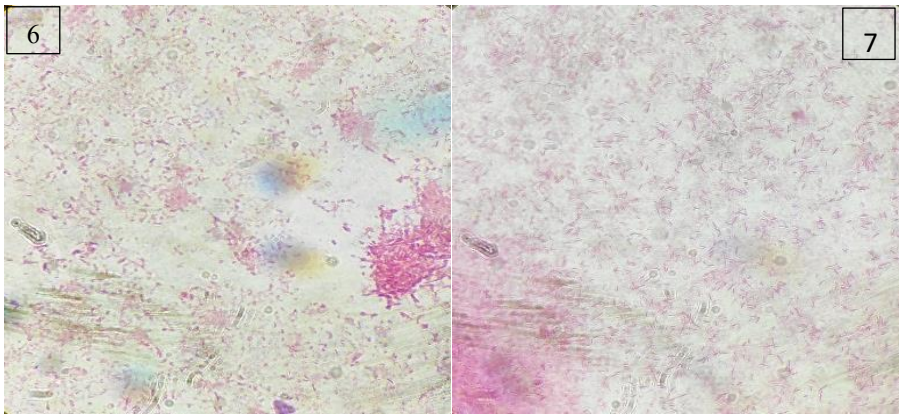
3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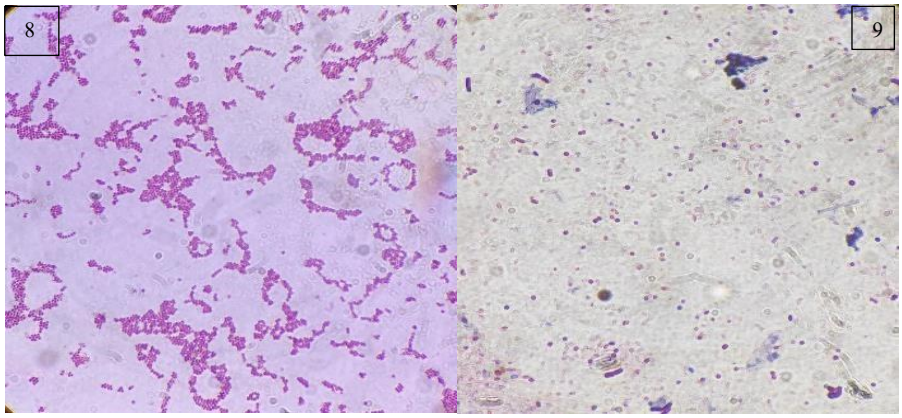
7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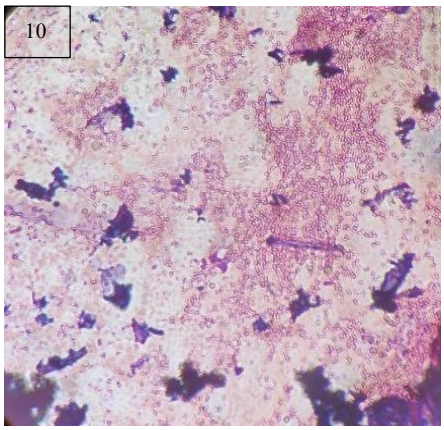
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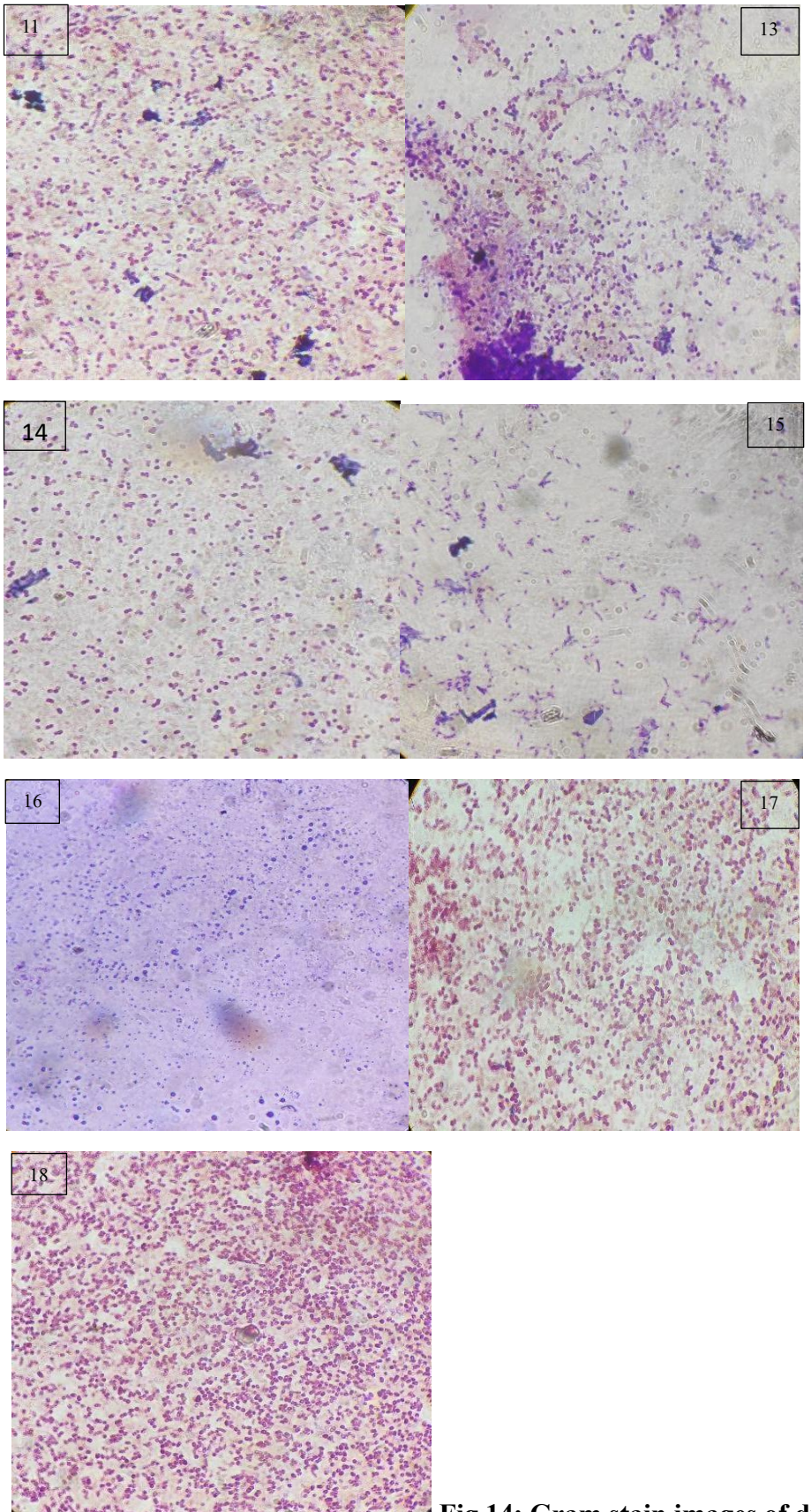

Fig.14: Gram stain images of different isolates

3. Salt Tolerance test of the cultures

The samples which were spot inoculated on plates containing 10% salt concentration had more growth as compared to the plates containing 15% salt concentration. For 10% salt concentration, out of 76 isolates spot inoculated, 48 isolates were positive, while 28 isolates were negative. For 15% salt concentration, out of 76 isolates spot inoculated, 6 isolates were positive, while 70 isolates were negative (Table.12) (Fig 15).

Table 12: Results obtained after performing salt concentration for 10% and 15 %

Isolate	Salt concentration 10%	Salt concentration 15%
Sample 2		
1C	+	+
3C	+	+
5C	+	+
7C	+	+
3W	+	+
4W	+	+
7W	+	-
8W	+	-
Sample 3		
1	+	-
2	+	-
3	+	-
4	+	-
5	+	-
6	+	-
Sample 4		
i	+	-
ii	-	-
iii	+	-
iv	+	-

v	+	-
vi	+	-
Sample 5		
C1	-	-
C2	+	-
C3	+	-
C4	+	-
C5	+	-
C6	+	-
C7	+	-
C8	+	-
C9	+	-
C10	+	-
Sample 6		
C1	+	-
C2	+	-
C3	+	-
C4	+	-
C5	+	-
C6	+	-
C7	+	-
C8	+	-
C9	+	-
C10	+	-
Sample 7		
C1	+	-
C2	+	-
C3	+	-
C4	+	-

C5	+	-
C6	+	-
C7	+	-
C8	+	-
Sample 8		
1	+	-
2	+	-

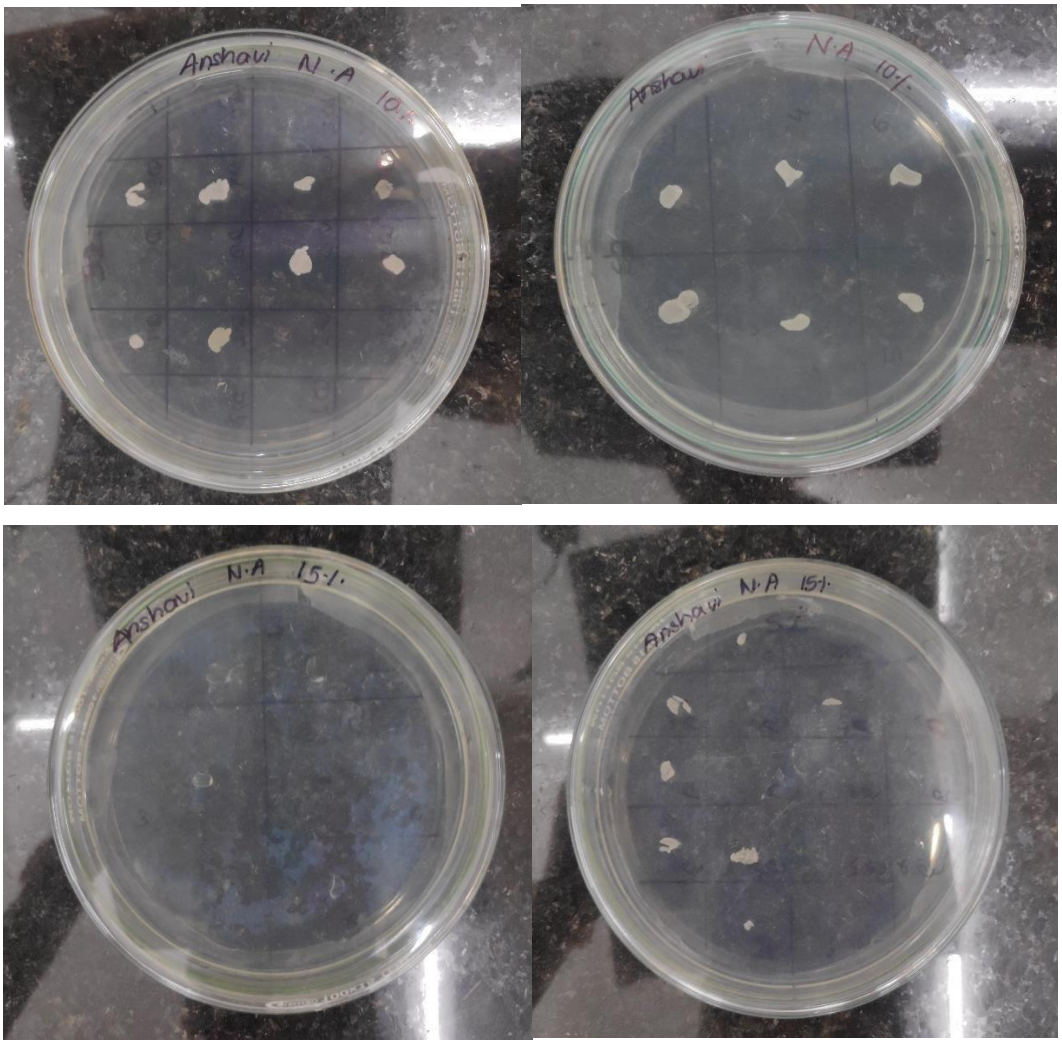

Fig.15: Images of Salt tolerance test

4. Production and extraction of of Exopolysaccharide

After performing the Exopolysaccharide production experiment the weight of the pellet of different isolates were obtained, which were than compared to each other. Isolate **S4-i** and isolate **S5-C6** gave maximum pellet (Table.13) (Fig 16 & 17).

Table 13: Results after performing Exopolysaccharide production experiment

No	Isolates	Weight of Eppendorf (gms)	Weight of Eppendorf + Dry weight of pellet (gms)	Dry weight of the pellet (gms)
1	S1-W2	1.150g	1.163g	0.013
2	S1-W4	1.165g	1.176g	0.011
3	S1-W6	1.180g	1.198g	0.018
4	S2-1W	1.163g	1.187g	0.024
5	S2-2W	1.156g	1.183g	0.027
6	S2-6W	1.164g	1.179g	0.015
7	S2-4C	1.158g	1.183g	0.025
8	S2-5C	1.160g	1.186g	0.026
9	S4-i	1.166g	1.208g	0.042
10	S5-C2	1.192g	1.228g	0.036
11	S5-C6	1.158g	1.201g	0.043
12	S5-C5	1.157g	1.189g	0.032
13	S6-C7	1.158g	1.183g	0.025
14	S6-C8	1.171g	1.197g	0.026
15	S6-C9	1.164g	1.184g	0.02
16	S7-C10	1.155g	1.174g	0.019

17	S7-C3	1.169g	1.183g	0.014
18	S7-C5	1.178g	1.195g	0.017

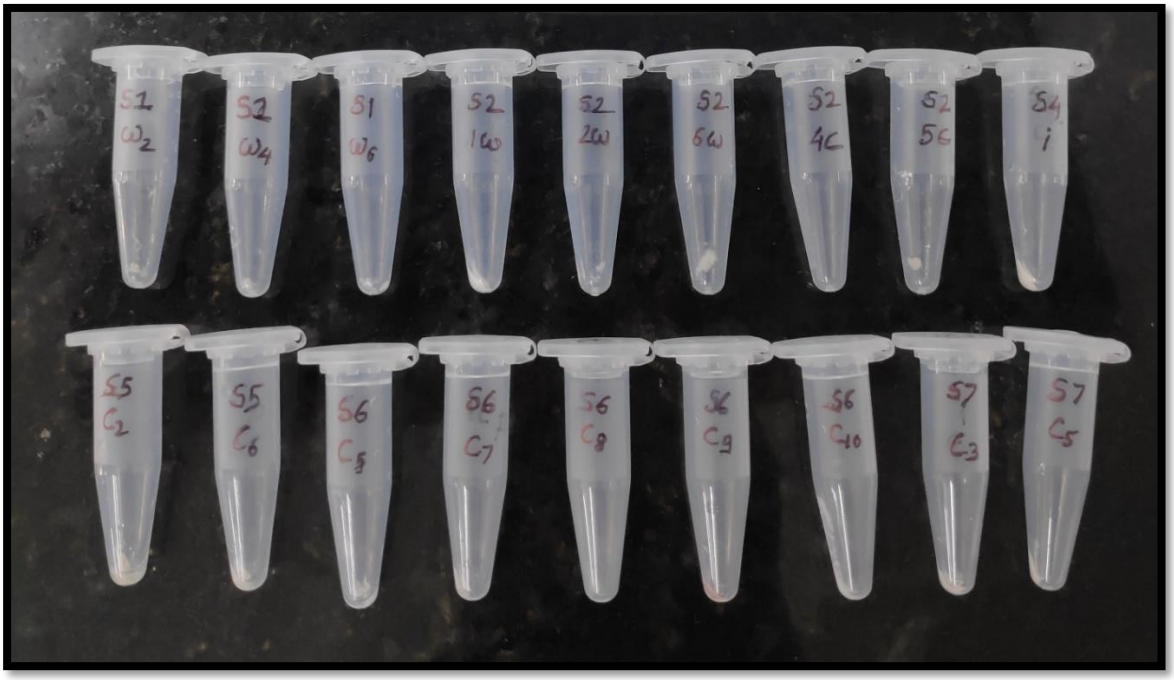

Fig.16: Eppendorfs containing pellet inside it

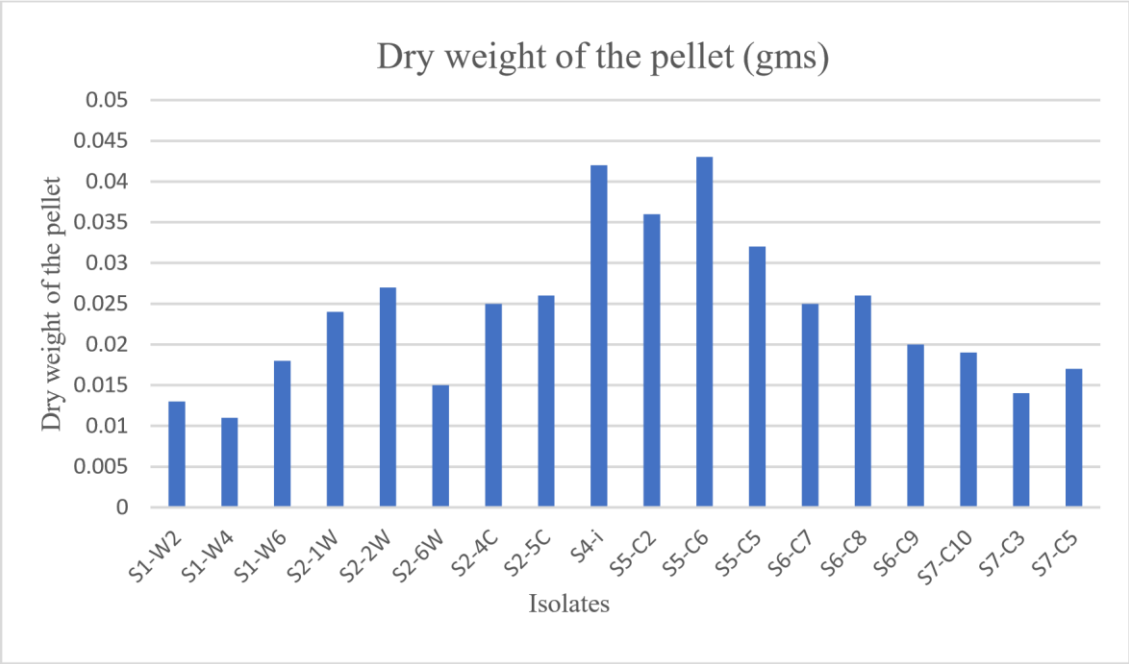

Fig.17: Graph of dry weight of the pellet

5. Study of the antimicrobial peptide

a) Antibacterial activity of the culture supernatant

All the 76 endophytic isolates were grown in Nutrient Broth at RT for 24 hours. The isolates were than tested across different test organisms and checked for zone of clearance. **Isolate No.12** showed antibacterial activity against 3 host cultures, *Escherichia coli* 1, *Staphylococcus aureus*1 and *Bacillus* spp**. Isolate No.14** showed the biggest zone of inhibition. Therefore, these isolates were selected for future studies (Table.14) (Fig 18, 19 & 20).

The isolates which gave positive results were than subjected to ammonium sulfate precipitation and than again screened and checked for zone of clearance.

Table 14: Results obtained after performing antibacterial screening

Isolate No	Test Microorganism	*E.Coli 1*	*S.aureus 1*	*E.Coli 2*	*S.aureus 2*	*Proteus spp*	*Bacillus*
	Sample 1				Zone of		
1	W2	-	+ 3mm	-	-		
2	W4	-	-	-	-		
3	W6	-	+ 1mm	-	-		
	Sample 2						
4	4C	+ 1mm	-	-			
5	5C	-	-	-	+ 1mm		
6	1W	-	-	-	-		
7	2W	-	+ 1mm	-	-		
8	6W	-	-	-	+ 2mm		
	Sample 4						
9	i	-	-	-	-		
	Sample 5						
10	C2	+ 4mm	-	-	-		
11	C6	+ 3mm	-	-	-		
	Sample 6						
12	C5	+ 9mm	+ 5mm	-	-		
13	C7	+11mm	+ 6mm	-	-		
14	C8	+12mm	+ 3mm	-	-		
15	C9		-	-	-		
16	C10	+ 5mm	-	-	-		
	Sample 7						
17	C3	-	-	-	-		
18	C5	-	-	-	-		

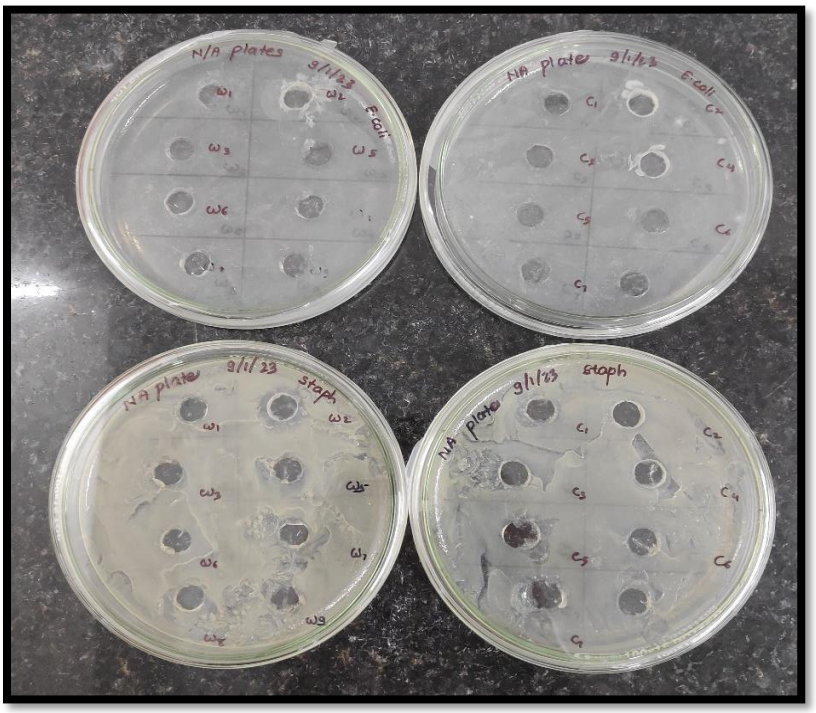

Fig.18: Antibacterial screening using Agar cup method

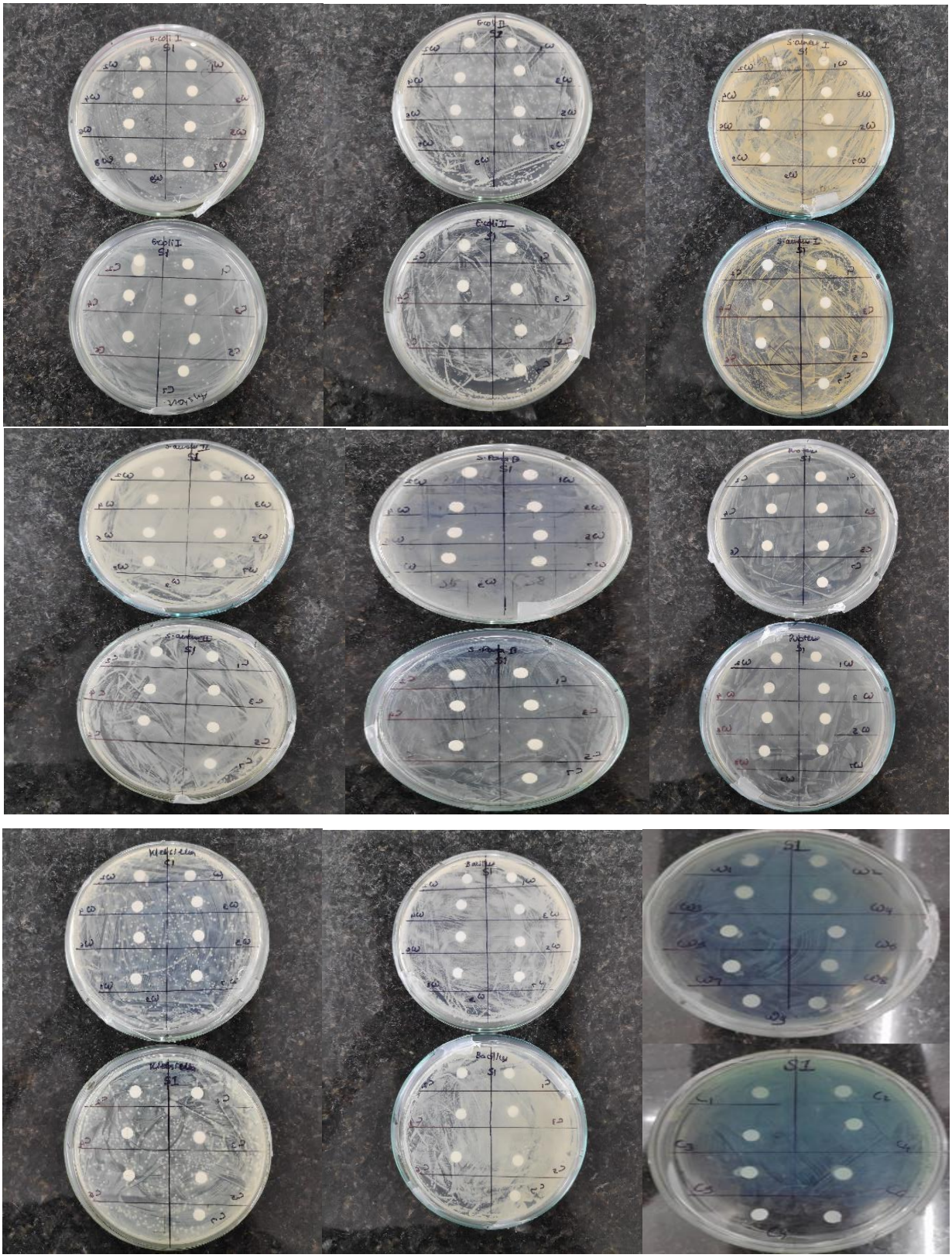

Fig.19: Antibacterial screening using Disc diffusion method

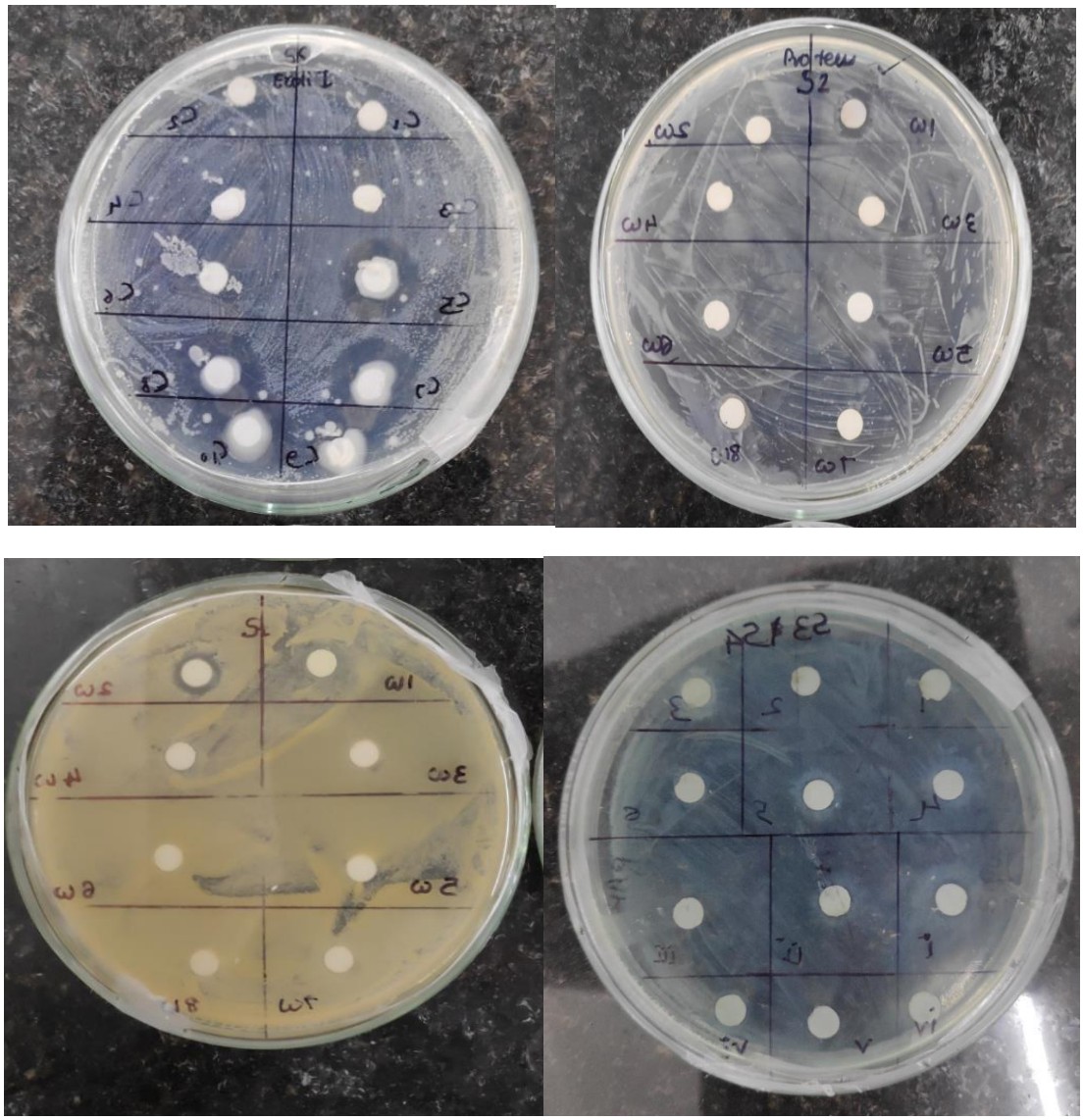

Fig.20: Samples showing positive results after antibacterial screening

b) Antifungal activity of the culture supernatant

All the 76 endophytic isolates were grown in Nutrient Broth at RT for 24 hours. The isolates were than tested across different test organisms and checked for the zone of clearance. **Isolate No.1** showed antifungal activity against 2 host cultures, *Apergillus and Penicillum*. **Isolate No.3** showed the biggest zone of inhibition (Table.15) (Fig.21 & 22).

Table 15: Results obtained after performing antifungal screening

Isolate No	Test Microorganism	*Aspergillus*	*Penicillium*	*S.cerevisiae*
	Sample 3		Zone of inhib	
1	1	**+ 3mm**	**+ 3mm**	-
	Sample 4			
2	i	-	**+ 3mm**	-
3	iv	**+ 4mm**	-	-
	Sample 5			
4	C3	-	**+ 2mm**	-

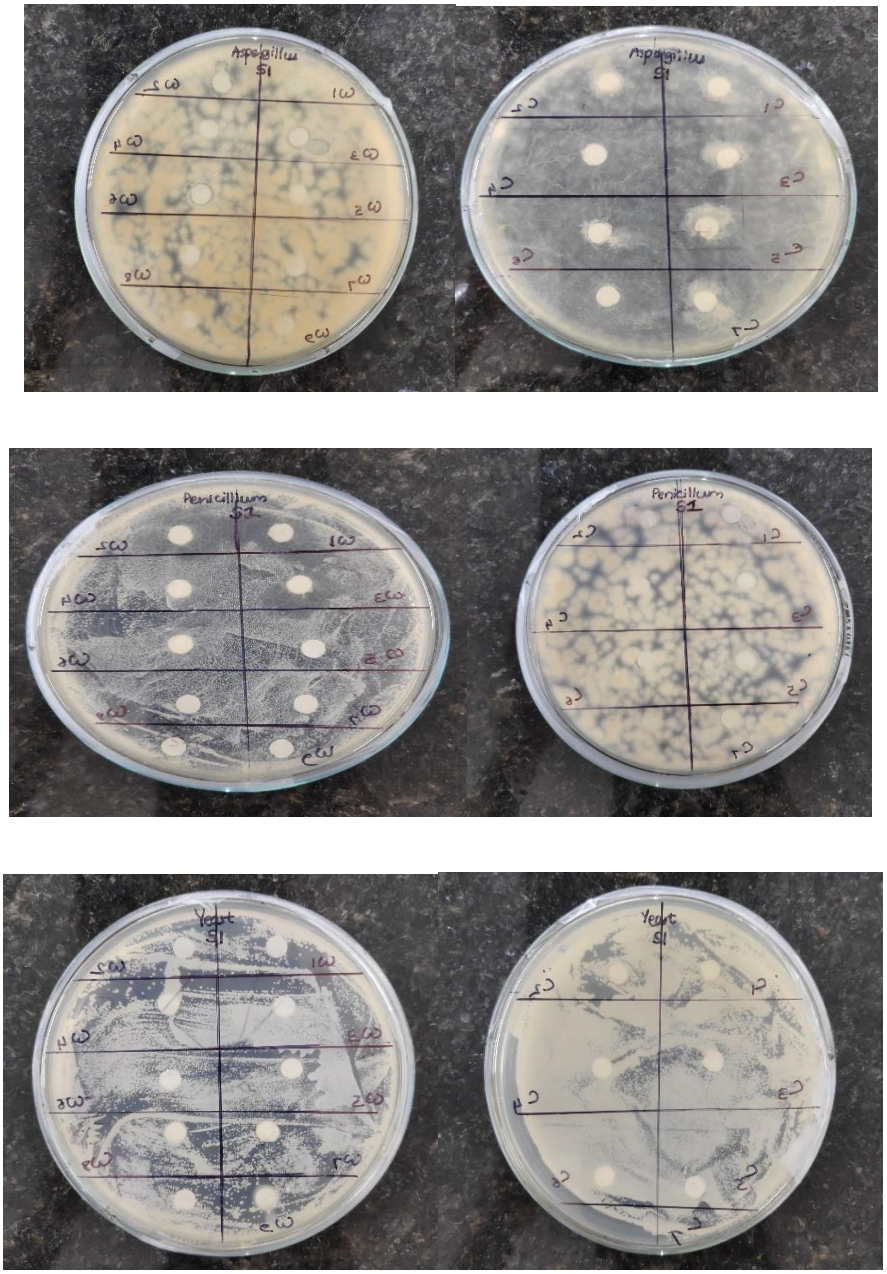

Fig.21: Antifungal screening using Disc diffusion method

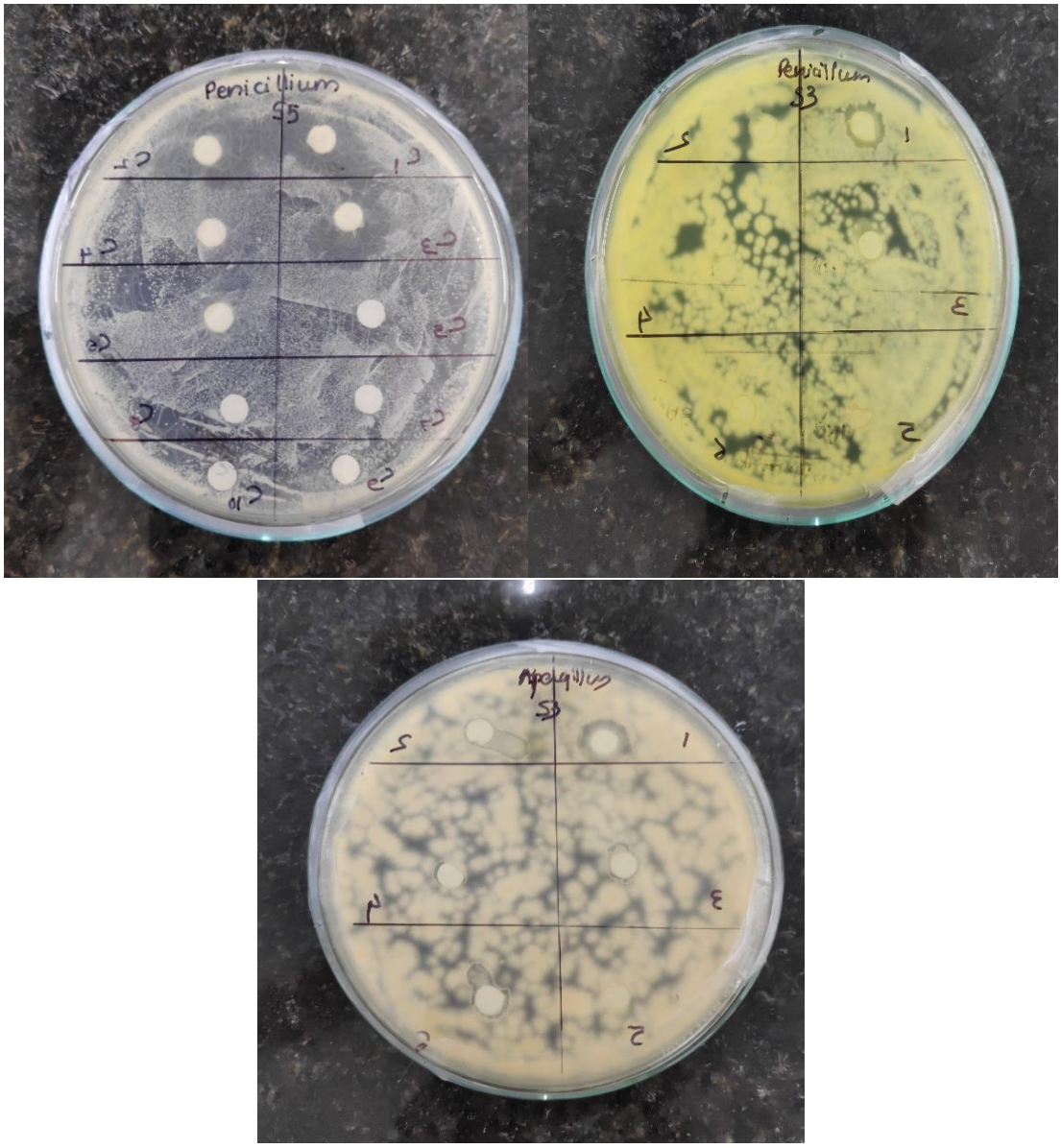

Fig.22: Samples showing positive results after antifungal screening

6. Ammonium sulphate precipitation of antibacterial peptide

The isolates which gave positive results after antimicrobial screening were than subjected to Ammonium Sulfate Precipitation and than again screened and checked for zone of clearance. **Isolate No.13** showed antimicrobial activity against 2 host cultures, *Escherichia coli* 1 *and Staphylococcus aureus*1. **Isolate No.15** showed the biggest zone of inhibition. Therefore, these isolates were selected for future studies (Table.16) (Fig 23).

Table 16: Results obtained after performing Ammonium Sulphate Precipitation

Isolate No		*E.Coli 1*	*S.aureus 1*	*E.Coli 2*	*S.aure spp s 2*	*Prote us spp*	*Bacillus us spp*	*S.para B*	*Klebsiella spp*	*Pseudo-Monas spp*
	Sample 1	Zone of inhibition (mm)								
1	W2	-	+ 1mm	-	-	-	-	-	-	-
2	W4	-	-	-	-	+ 2mm	-	-	-	-
3	W6	-	+ 2mm	-	-	-	-	-	-	-
	Sample 2									
4	4C	-	-	-		-		-	-	-
5	5C	-	-	-	+ 1mm		-	-	-	-
6	1W	-	-	-	-	+ 2mm	-	-	-	-
7	2W	-	+ 1mm	-	-	-	-	-	-	-
8	6W	-	-	-	+ 1mm	-	-	-	-	-
	Sample 4									
9	i	-	-	-	-	-	-	-	-	-
	Sample 5									
10	C2	-	-	-	-	-	-	-	-	-
11	C6	-	-	-	-	-	-	-	-	-
	Sample 6									
12	C5	-	+ 1mm	-	-	-	-	-	-	-
13	C7	+ 1mm	+ 2mm	-	-	-	-	-	-	-
14	C8	-	-	-	-	-	-	-	-	-
15	C9		-	-	-	-	-	-	+ 3mm	-
16	C10	-	-	-	-	-	-	-	-	-
	Sample 7									
17	C3	-	-	-	-	-	-	-	-	-
18	C5	-	-	-	-	-	-	-	-	-

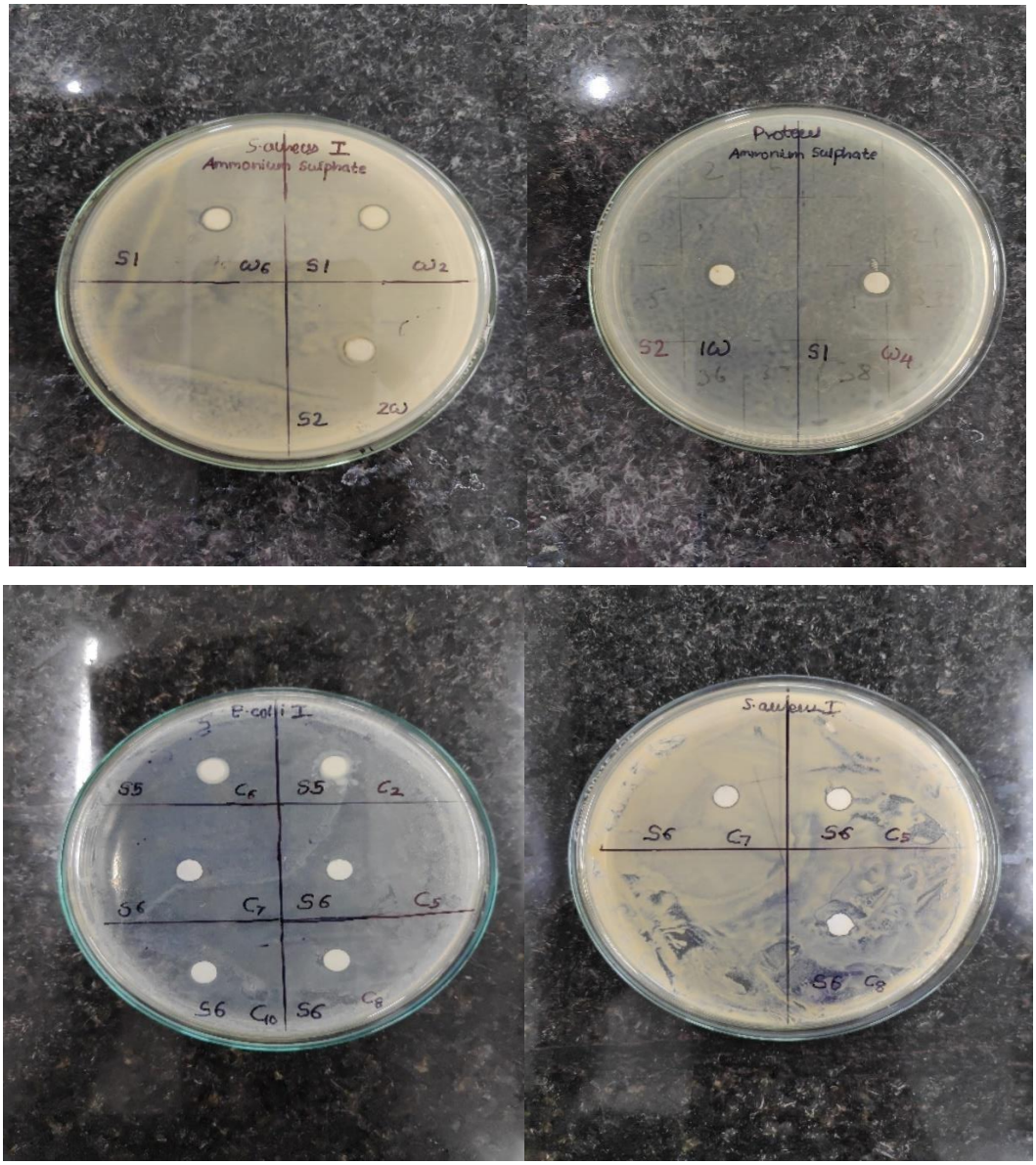

Fig.23: Results after performing Ammonium Sulphate Precipitation

8. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

The samples were analyzed by SDS-PAGE in which the protein profile was not clear on gel.

The molecular weight of protein could not be studied (Fig 24, 25 & 26).

The reason might be as follows :

- Time that the gel polymerizes is too long. So increase ammonium persulfate or TEMED or use fresh ammonium persulfate and new TEMED.
- The protein bands are not sufficiently resolved. Since insufficient electrophoresis has taken place, prolong the run. The gels pore size is not correct for the proteins that need to be separated. Use a gel with a different % acrylamide.
- The proteins are not fixed in the gel, so use a stain which will fix the proteins.

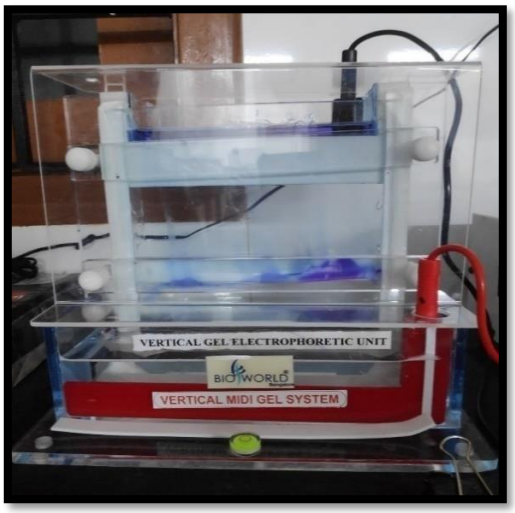

Fig.24: Electrophoresis unit **Fig.25: SDS-PAGE gel kept on Shaker**

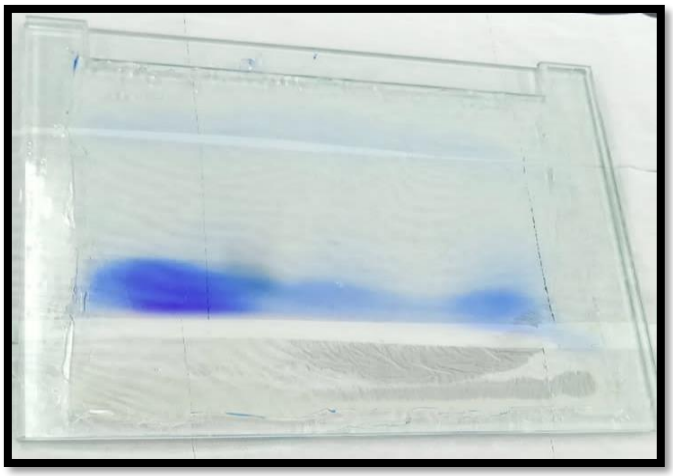

Fig.26: SDS-PAGE gel

9. Cytotoxicity of peptides against mosquito larvae

Total of 18 isolates were tested against the mosquito larvae. After 1 hour there was no effect on the mosquito larvae. For **Isolate No. 14,** after 24 hours, the percentage mortality was 60% while

the percentage mortality was 70% after 48 hours**.** For **Isolate No. 15,** after 24 hours, the percentage mortality was 55% while the percentage mortality was 65% after 48 hours. (Table.17) (Fig. 27 & 28).

Table 17: Results of cytotoxicity of mosquito larvae in Percentage Mortality

Isolate No	Isolates	Total larvae	After 1 hours	After 24 hours dead	% Mortality After 24 hours	After 48 hours dead	% Mortality After 48 hours
1	S1-W2	20	20	-		-	
2	S1-W4	20	20	-		-	
3	S1-W6	20	20	-		-	
4	S2-1W	20	20	-		-	
5	S2-2W	20	20	-		-	
6	S2-6W	20	20	-		-	
7	S2-4C	20	20	9 Dead	45%	10 Dead	50%
8	S2-5C	20	20	-		-	
9	S4-i	20	20	-		-	
10	S5-C2	20	20	-		-	
11	S5-C6	20	20	4 Dead	20%	6 Dead	30%
12	S6-C5	20	20	6 Dead	30%	8 Dead	40%
13	S6-C7	20	20	7 Dead	35%	8 Dead	40%
14	S6-C8	20	20	12 Dead	60%	14 Dead	70%
15	S6-C9	20	20	11 dead	55%	13 Dead	65%
16	S7-C10	20	20	-		-	
17	S7-C3	20	20	-		-	
18	S7-C5	20	20	-		-	
19	Control	20	20	6 Dead	30%	12 Dead	60%

Fig.27: Containers with mosquito larvae and control

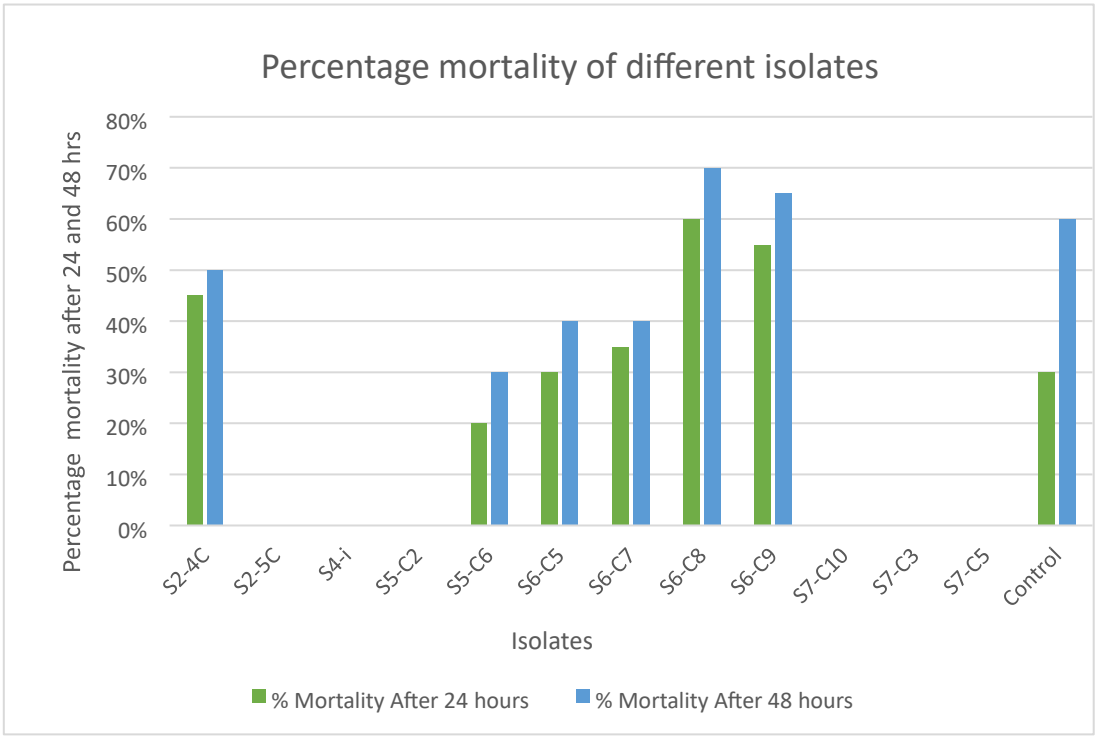

Fig.28: Graph of Percentage moratlity after 24 and 48 hrs

10.Antibiotic sensitivity of selected cultures

Total of 18 different isolates were tested against 10 different antibiotics. The zone of inhibition was measured and the results were interpretated based on the zone size interpretative chart. Most of the isolates were sensitive to the antibiotics (Table.18) (Fig. 29 & 30).

Table 18: Results after performing Antibiotic sensitivity test for 18 different isolates

No	Isolates	Zone of inhibition (mm)									
		1	2	3	4	5	6	7	8	9	10
1	S1-W2	-	-	-	-	-	28 NK	20 NK	23 S	22 S	20 S
2	S1-W4	13 R	24 S	20 MS	24 S	20 S	23 NK	18 NK	24 S	22 S	18 S
3	S1-W6	-	-	20 MS	-	-	-	-	-	-	-
4	S2-1W	13 R	15 MS	14 MS	23 S	22 S	17 NK	10 NK	20 S	21 S	19 S
5	S2-2W	-	-	-	30 S	24 S	20 NK	13 NK	25 S	21 S	-
6	S2-6W	-	-	13 R	23 S	21 S	13 NK	20 NK	12 MS	-	-
7	S2-4C	17 S	15 MS	-	21 S	23 S	17 NK	11 NK	21 S	22 S	-
8	S2-5C	16 MS	25 S	-	24 S	23 S	11 NK	20 NK	24 S	23 S	20 S
9	S4-i	-	21 S	28 S	26 S	20 S	22 NK	14 NK	23 S	21 S	13 S
10	S5-C2	15 MS	25 S	23 S	25 S	24 S	22 NK	18 NK	20 S	22 S	21 S
11	S5-C6	13 R	20 S	25 S	18 S	25 S	21 NK	26 NK	23 S	20 S	26 S
12	S6-C5	16 MS	25 S	26 S	23 S	21 S	22 NK	18 NK	11 R	21 S	20 S
13	S6-C7	17 MS	22 S	23 S	21 S	24 S	21 NK	18 NK	16 S	20 S	23 S
14	S6-C8	17 S	22 S	21 MS	22 S	16 MS	18 NK	13 NK	24 S	18 S	16 S
15	S6-C9	16 MS	24 S	22 MS	20 S	21 S	24 NK	23 NK	19 S	21 S	25 S
16	S7-C10	17 S	25 S	25 S	23 S	24 S	23 NK	18 NK	17 S	22 S	23 S

17	**S7-C3**	**15 MS**	**20 S**	**10 R**	**25 S**	**23 S**	**21 NK**	**20 NK**	**23 S**	**27 S**	**15 S**
18	**S7-C5**	**13 R**	**16 MS**	**25 S**	**23 S**	**18 S**	**14 NK**	**17 NK**	**21 S**	**22 S**	**13 S**

Key:

Sensitive (S), Moderately sensitive (MS), Resistant (R), Not Known (NK).

Ampicillin (1), Chloramphenicol(2), Erythromycin(3), Gentamicin(4), Kanamycin(5), Nalidixic acid (6), Rifampicin(7), Streptomycin(8), Tetracycline(9), and Vancomycin(10).

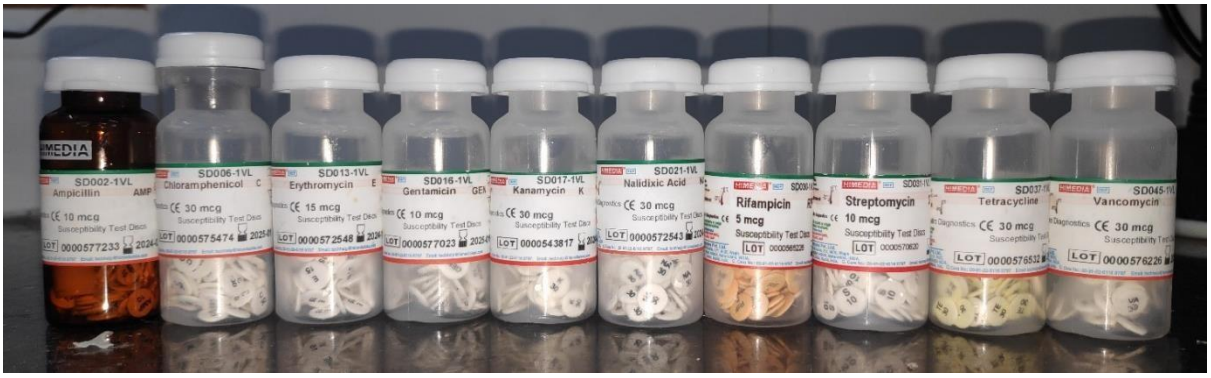

Fig.29: Different antibiotics used in the experiment

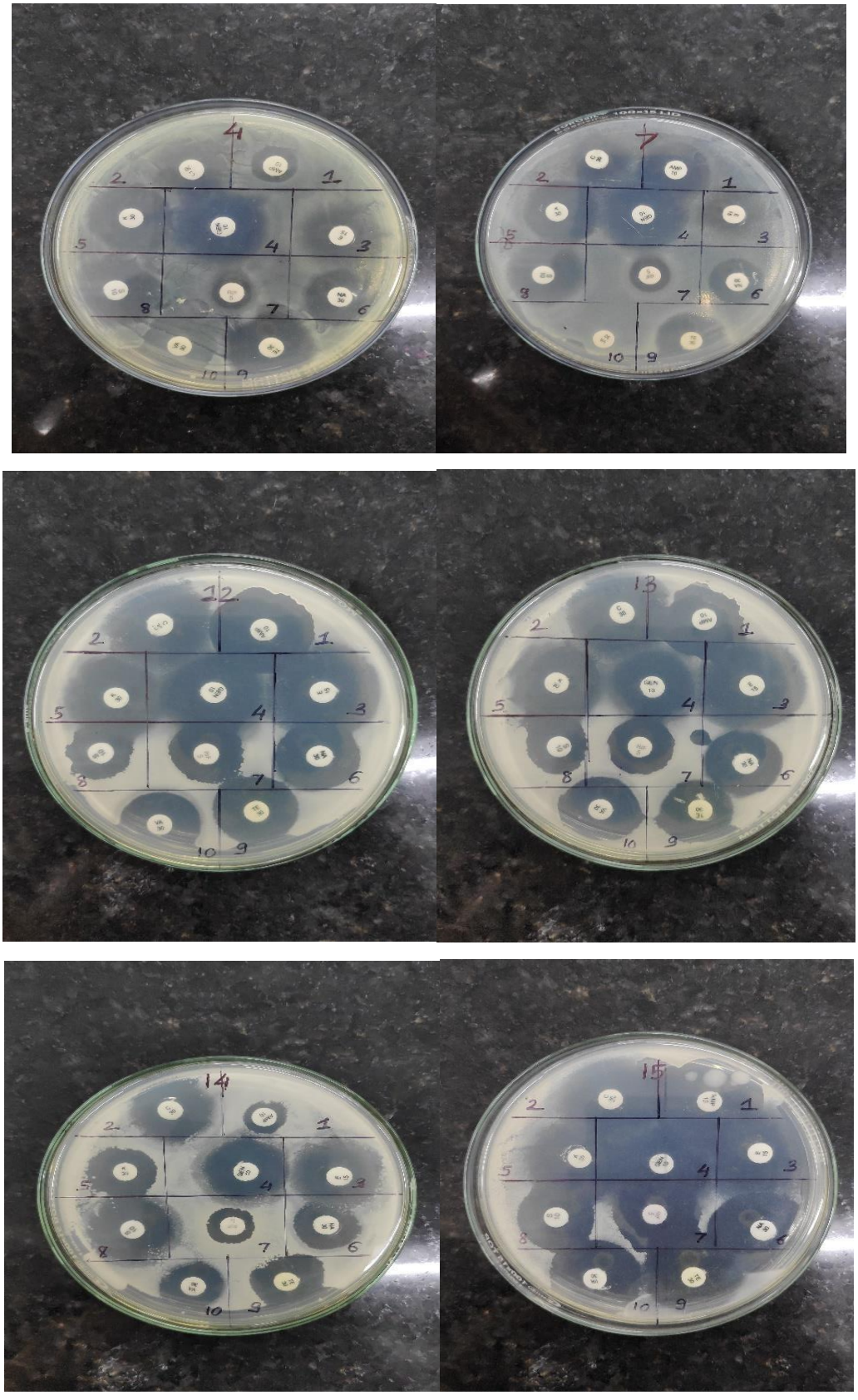

Fig.30: Plates showing results of antibiotic sensitivity test

CHAPTER VI

DISCUSSION

In this study, seaweed was collected by hand from the coast of Goa during low tide. A total of 8 algae samples were collected in a sterile glass jar. Different locations are Ashwem (*Ulva intestinalis*), Miramar (Ulva compacta), Querim (*Ulva lactuca* and *Sargassum polycystum*) and Anjuna (*Padina antillarum*). Other seaweeds (*Eucheuma denticulatum, Kappaphycus alvarezii, Kappaphycus striatus*) were collected from the Dona Paula seaweed cultivation project.

Ismail *et al*., (2016) collected seaweed from Cap Zebib (37° 16.2'N, 10° 3.6'E) on Tunisia's northern coast in the winter and summer of 2007. The collected seaweeds were than kept in bags, which had water from the same site.

Villarreal-Gomez *et al*., (2010) collected the seaweeds, *Egregia menziesii* (Turner) *Areschoug*

(Em), *Codium fragile* (Suringar) *Hariot* (Cf), *Sargassum muticum* (Yendo) *Fensholt* (Sm), *Endarachne binghamiae* (Petalonia binghamiaed) (J.), *Centroceras clavulatum* (Agardh) *Montagne* (Cc) and *Laurencia pacifica Kylin* (Lp) at low tide on the coast near the Autonomous University of Baja California in Mexico. Collected samples were sent into frosted plastic bags.

Shafay *et al*., (2016) collected four species of seaweed, *Ceramium rubrum* (Rhodophyta), *Sargassum vulgare, Sargassum fusiforme, and Phaeophyta* (Phaeophyta), from the Hurghada coast in Egypt's Red Coast, and analysis was performed to remove impurities. It was wash with water. Seaweed was sent to the laboratory in sterile plastic bags.

Musbah *et al*., (2019) Obtained seaweeds from a population of green algae (*Ulva lactuca*), brown algae (*Sargassum denticulatum, Hormophysa triquetra*) and red algae (*Hypnea cornuta*) and blue-green algae (*Sargassum denticulatum*) from Egypt.

Minimol *et al*., (2019) collected three species of seaweed, *Sargassum wightii, Turbinaria connoides, and Padina gymnocephalus*, from the Gulf of Mannar, Mandapam Coast, Tamil Nadu (India).

Syed Ali *et al*., (2013) collected extracts of *Ulva*, *C. racemosa*, *Sargassum, Porphyra fanii, Gracilaria, Turbinaria decurrens, Turbinaria conoides,* and *Caulerpa toxifolia*, dissolved in DMSO grades, to generate graded sequences.

For the isolation of seaweed related to this study, the sample (10 g) was suspended in sterile water (10 mL) and aseptically homogenized using a pestle and mortar in a laminar flow fume hood. The suspension was serially diluted in sterile seawater (9 mL) and the different dilutions were plated onto aseptically prepared isolation medium. The isolation medium used in this study is NA , ZMA. The incubation was done for 24-48 hrs at RT.

J.G. *et al*., (2003) Seaweed samples were washed three times with autoclaved saline to remove white bacteria and loose bacteria, and then isolated as epiphytic bacteria (Burgess *et al*., 2003). Recover epiphytic organisms by vortexing 10 g of algae biomass in 90 ml of autoclaved seawater for 6 minutes. Bacteria were isolated using autoclaved saline and diluted back to 10^3. Plate 100 µl of each dilution in triplicate on sea agar plates. Leave the plate at 20 °C for at least 7 days or until colonies form (Lemos *et al*., 2016). Ib., 1985). Select the colonies and inoculate on MA until cultured.

VillarrealGómez etal.,(2010) Sterile swab were used to wipe the algae surface before the bact erial extracts were inoculated into sea agar and seawater (Difco) isolation plates. Leave the pl ates for 24-48 hours at 25 °C until colonies appear.

Minimol *etal*., (2019) A total of 25 g of dried seaweed was aseptically removed in phosphate buffered saline for separation and serial dilutions were prepared. Then inoculate 1 ml of each dilution into a test tube containing 9 ml of EPS medium and 0.2 g of KH2PO4; 1.5grams K2 HPO4; 0.2 gram MgS04 7H2O; 0.1 gram CaSO4 2H2O; 2.0 mg of ferric chloride; 0.5 g of ye ast extract and 20 g of sucrose / l. Samples were then plated on an equal volume of Tryptic So y Agar (BD, Mumbai, India) and incubated at 37°C for 48 hours.

In the present study, the bacterial extracts were tested against the following bacteria: *Staphylococcus aureus*1, *Staphylococcus aureus* 2, *Bacillus* spp, *Escherichia coli* 1*, Escherichia coli* 2, *Proteus* spp*, Salmonella paratyphi B, Klebsiella* spp*, Pseudomonas* spp . Incubate for 24 hours at room temperature by measuring the zone of inhibition (mm) around t he disc with the test bacteria

Ismail *et al*., (2016) 18 bacterial type strains were used for checking the antimicrobial activity of all isolated epiphytes, which were: *Aeromonas salmonicida* LMG3780*, A. hydrophila* B3 , *Enterococcus faecalis* ATCC 29212, *Escherichia coli* O126-B16 (ATTC 14948), *E. coli* ATCC 25922, *E. coli* ATCC 8739, *Micrococcus* sp., *Pseudomonas cepacia , P. fluorescens* AH2, *P. aeruginosa* ATCC 27853, *Salmonella typhimurium* C52, *Staphylococcus aureus , S. aureus* ATCC 25923, *S. aureus* ATCC 6538, *Streptococcus* sp. (, *Vibrio tapetis* CECT4600, *V. anguillarum* ATCC12964T, *V. alginolyticus* ATCC 17749T. The yeast strain Candida albicans ATCC 10231 was also utilised in the experiment.

Screening of isolates for antibiotics against human and fish diseases.

The 2 two methods were utilized in the experiment. First being the drop test which was performed on plates containing Trypto-Casein-Soy agar with 20 $g1^{-1}$ NaCl as specified by (James *et al*.,1996) and (Rao *et al*. 2005). Drops of an overnight culture's cell suspension were spotted onto agar plates containing a confluent lawn of the target strain (dried for 30 minutes at 30°C) and then incubated at 30°C. (Wiese *et al*.,2009) explained the overlay assay in detail with some modifications. Drops (10 ul) of an overnight culture of the isolates were placed onto TSA

plates supplemented with 20 g l^{-1} NaCl and cultured for 24 h at 30°C being treated with a soft agar overlay with the desired strains [3 g l^{-1} Tryptone Soy Broth- (TSB-BIO RAD, 9 g l^{-1} agar (PRS, Panreac), 20 g l^{-1} NaCl, pH 7.2]. The target strain suspension contained ~107 cells ml1. After incubation (24 hours at 30°C), growth inhibition was assessed by measuring the zone of inhibition (mm).

Villarreal-Gómez *et al*., (2010) At a dosage of 10 mg ml^{-1} in DMSO, the algal and bacterial extracts were tested for antibacterial effectiveness against the pathogen bacteria Staphylococcus aureus (ATCC25923), Klebsiella pneumoniae (ATCC 13883), Proteus mirabilis (ATCC 35659), and Pseudomonas aeruginosa (ATCC 27853).

In the present study, the fungal isolates were tested against, *Aspergillus* spp*, Penicillium* spp *and Saccharomyces cerevisiae.* Growth inhibition was evaluated after incubation at RT for 24 hrs, by measuring the zone of inhibition in mm, around the disc with the test organism.

The antifungal activity was screened using the agar well diffusion method for the antifungal susceptibility test (Bauer *et al*., 1996). Candida species inoculates(108 cells/ml) were spread on SD agar plates and left to dry at room temperature, wells were made on the surface of agar medium with 6 mm cork borer. Each well in plate was filled with 50 □m of algal crude extract using micropipette. The plates were incubated at 37 ºC from 24- 48 hrs, At the end of incubation the plates were observed for the zone of inhibition and the diameters of the zones were measured in mm (Karabay-Yavasoglu *et al*., 2007). All assays were carried out independently in triplicates and the mean result were calculated, also Fluconazole and dimethyl sulfoxide (DMSO) were used as positive and negative controls, respectively (Musbah *et al*., 2019).

In the present study, for exopolysaccharide production, the selected isolates were grown in Nutrient Broth (9ml) along with 1% of the Glucose (1ml). It was given 48 hours to grow. Than it was centrifuged at 5000rpm for 10 minutes. Supernatant was taken, and three times the volume of supernatant, chilled ethanol was added. Precipitate formed was than again centrifuged and the precipitate was transferred in to preweighed sterile eppendorf tubes. The weight of the precipitate along with the eppendorf was noted.

Minimol *et al*., (2019) Exopolysaccharide (EPS) was isolated from bacteria using the method described by Berekaa and Ezzeldin (2018). The bacterial isolates were inoculated into the EPS

culture medium and cultured at 37ºC in a shaker incubator (MaxQ 6000, Thermoscientific, USA) with 200 rpm rotation for 10 days. After incubation, the cultures were centrifuged for 20 minutes at 9000 rpm (Centrifuge 5804R, Eppendorf, India). The supernatant was collected and blended in a 1:2 ratio with cold alcohol. Exopolysaccharide deposition of biomass was rinsed with distilled water and dried at 60º C for 2-4 hours. The isolate with the highest EPS production was chosen for further investigation.

In the present study, For antibiotic sensitivity of isolated strains, the sensitivity to various antibiotics were checked by the disc diffusion method. The various antibiotics used were, Ampicillin, Chloramphenicol, Erythromycin, Gentamicin, Kanamycin, Nalidixic acid, Rifampin, Streptomycin, Tetracycline, and Vancomycin. A isolate was considered susceptible to an antimicrobial compound if any growth inhibition zone was observed around the disc The interpretation was done using standardise zone size interpretative chart.

Ismail *et al*., (2016) For antibiotic sensitivity of isolated strains. The disc diffusion method (Barry & Thornsberry,1980) was used to investigate the sensitivity to various antibiotics on Muller Hinton agar (MH, BIO RAD) plates using the following antimicrobial compounds (BIO RAD, France), amounts are given per disc: streptomycin (500 g), amoxicillin (25 g), tobramycin (10 g), nalidixic acid (30 g), oleandomycin (15 g), cefoxitin If a growth inhibition zone was seen around the disc, a strain was judged susceptible to an antimicrobial drug. The interpretation was done in accordance with the French Society of Microbiology's norms (Soussy,2008).

The antibiotic susceptibility of the bacteria was determined using the Clinical and Laboratory Standards Institute's (CLSI, 2012) modified Kirby-Bauer disc diffusion method (Shafay *et al*., 2016). Antibiotic concentrations were examined at the following levels: Ampicillin (AMP, 10µg), Amoxicillin (AX,25µg),Amoxycillin/Clavulanicacid(AMC,20/10µg), Oxacillin (OX,1 µg),Piperacillintazobactam(TPZ,100/10µg), Ceftazidime (CAZ,30), Cefepime (FEP,30µg), C eftriaxone (CRO,30µg), Imipenem (IPM,10µg), Meropenem (MEM,10µg),Cefoperazone/sulb actam(CES,75/30µg), Aztreonam (ATM,30µg), Gentamicin (CN,10µg), Amikacin (AK,30µg), Neomycin (N,30µg), Streptomycin (S,10µg), Tobramycin (TOB,10µg), Kanamycin (K,30µ g), Chloramphenicol (C,30µg), ColistinSulfate (CT,10µg),Nalidixicacid(NA,30µg), Ciproflox acin (CIP,5µg), Co-trimoxazole (SXT, 25µg), Tetracycline (TE, 30µg), Vancomycin (VA, 30µg). The antibiotic disks were then applied to the prepared plates and incubated at 37 °C for 18 h then, the diameter of the growth inhibition zones was measured.

In the present study, mosquito larvae of *Culex quinquefasciatus* at the late 3rd to 4th instar stage were used. 19 cleaned autoclaved plastic containers were filled with 100ml of RO (Reverse Osmosis) water and labelled. To the each container, 20 healthy larvae were introduced. 1container was kept as a control. In the other 18 containers 1ml of the supernatant was added. Mortality was recorded after 1hour, 24 hours and 48hours.

Syed Ali *et al*.,(2013) used the WHO standard method was used to conduct the test for the larvicidal impact of seaweeds on mosquito larvae. 25 early fourth instar larvae of mosquitoes were deposited in a 250 mL enamel a container filled with 199 mL of distilled water and 1 mL of extracts from plants (10-100 g). Each experiment was carried out in triplicate, with a control group present at the same time. Deaths were registered

Future prospects

- Mass analysis of purified antibiotic peptide.
- Finding proper molecular weight of the peptide.
- Identification of cultures by biochemical tests and 16SrRNA.
- Study new species of bacteria.

CHAPTER VII

CONCLUSIONS

- Total 8 different species of seaweed samples were collected from the selected sampling sites.

- *Ulva intestinalis, Ulva compressa, Ulva lactuca*, Sargassum polycystum, Eucheuma denticulatum, *Kappaphycus alvarezii, Kappaphycus striatus and Padina antillarum* were identified.

- 76 endophytes were isolated from the seaweeds.

- Antimicrobial activity of gram negative bacteria is greater than the gram positive bacteria.

- **Isolate No.12** showed antibacterial activity against 3 organisms, *Escherichia coli* 1, *Staphylococcus aureus*1 and *Bacillus spp*. **Isolate No.14** showed the biggest zone of inhibition. Therefore, these isolates were selected for future studies.

- **Isolate No.1** showed antifungal activity against 2 organisms, *Apergillus and Penicillum*. **Isolate No.3** showed the biggest zone of inhibition.

- After Ammonium sulphate ppt, **Isolate No.13** showed antimicrobial activity against 2 organisms, *Escherichia coli* 1 *and Staphylococcus aureus*1. **Isolate No.15** showed the biggest zone of inhibition. Therefore, these isolates were selected for future studies.

CHAPTER VIII

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CHAPTER IX

APPENDIX

Appendix 1

Media composition 1. Nutrient agar :- HiMedia

Ingredients	Gms/litre
Peptone	5
Yeast extract	3
Sodium chloride	5
Agar	15
Distilled water	1000 ml
pH	6.8-7.2

2. Zobell Marine Agar :- HiMedia

Ingredients	Gms/litre
Peptone	5
Yeast extraxt	1
Ferric citrate	0.100
Sodium chloride	19.45
Magnesium chloride	8.80
Sodium sulphate	3.240
Calcium chloride	1.800
Potassium chloride	0.550
Sodium bicarbonate	0.160
Potassium bromide	0.080
Strontium chloride	0.034
Boric acid	0.022
Sodium silicate	0.004
Sodium fluorate	0.0024
Ammonium nitrate	0.0016
Disodium phosphate	0.008
Agar	15
Distilled water	1000 ml
pH	7.5-7.7

3. Malt Extract Agar :- HiMedia

Ingredients	Gms/litre
Malt extract	20.0 g
Glucose	20.0 g
Peptone	1.0 g
Agar	20.0 g
Distilled water	1 liter
pH	5.4

4.Saline :-

Ingredients	Gms/litre
Sodium chloride	0.85
Distilled water	100 ml

Appendix 2

Gram's staining 1. Crystal violet:-

Ingredients	Quantity
Ammonium oxalate	8 gm
Crystal violet	10 gm
Alcohol	100 ml
D/W	900 ml

2. Gram's iodine:-

Ingredients	Quantity
Iodine	1 gm
Potassium iodide	2 gm
D/W	300 ml

3. Decolorizer:-

Ingredients	Quantity
Ethanol	95 ml
D/W	5 ml

4. Basic fuschin:-

Ingredients	Quantity
Basic fuschin	1 gm
Alcohol	10 ml
D/W	100 ml

Appendix 3

Protein Estimation by Folin-Lowry's method

Standard : 1mg /ml BSA

Diluent : Distilled water

Reagents: Solution A: 2% sodium carbonate in 0.1 N NaOH

Solution B: 0.5% copper sulphate solution in 1% sodium potassium tartarate solution.

Alkaline copper sulphate solution: 50 ml solution A + 1 ml of solution B. Prepare it just before using.

Folin- ciocalteau reagent- 1 part Folin-Phenol [2 N]: 1 part water (1:1)

Table 19: Protein estimation of Unknown with OD at 660nm

Test tube No.	Conc. Ug/ml	Stock (ml)	Diluent (ml)	Alkaline CuS04		Fc reagent		OD at 660nm
Blank	-	-	1.0	5.0	**Mix And wait for 1 min**	0.5	**Incubate for 50 Min in dark**	
1	20	0.2	0.8	5.0		0.5		0.397
2	40	0.4	0.6	5.0		0.5		0.625
3	60	0.6	0.4	5.0		0.5		0.685
4	80	0.8	0.2	5.0		0.5		0.775
5	100	1.0	-	5.0		0.5		0.925
Uknown 1	-	0.1	-	5.0		0.5		0.776
Uknown 2	-	0.1	-	5.0		0.5		0.877
Uknown 3	-	0.1	-	5.0		0.5		0.929
Uknown 4	-	0.1	-	5.0		0.5		0.825

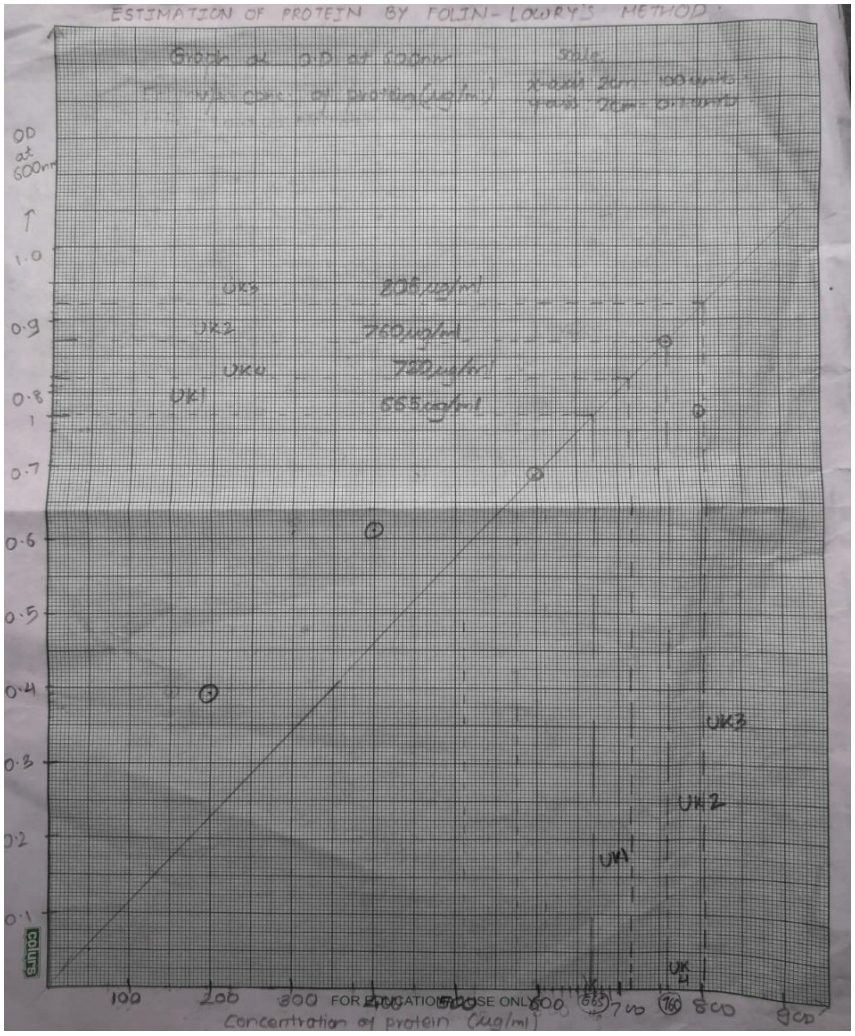

Fig. 30: Graph of the unknown sample

The concentration of unknown proteins estimated by Folin Lowrys Method was found to be:

1) S6-C5 (Isolate 12): Uknown 1 : 665ug/ml

2) S6-C8 (Isolate 14): Uknown 2 : 760ug/ml

3) S6-C7 (Isolate 13): Uknown 3 : 805ug/ml

4) S6-C9 (Isolate 15): Uknown 4 : 715ug/ml

Appendix 4

SDS-PAGE reagent composition

1. **30% acrylamide**: weigh 29g acrylamide, 1g N, N – methylene bis-acrylamide. Add 60 ml warmed deionized water and heat to 37 ℃. Add deionized water to make a final volume of 100ml; filter; Then we have 30% (w / v) acrylamide stock solution; Acrylamide and bisacrylamide were transformed slowly into acrylic acid and double acrylic acid during storage, so the pH of the solution should be no more than 7.0 and it should be placed in a brow bottle at 4 ℃.

2. **10% sodium dodecyl sulfate (SDS)**: weigh 10g SDS and 90ml deionized water; heat to 68 ℃ and add a few drops of concentrated hydrochloric acid until the pH becomes 7.2; then water to 100ml; after the whole processes, we have 10% (w/v) SDS.

3. **Stacking gel buffer (1mol / L Tris-HCl pH 6.8)**: dissolve 12.12g Tris in 80ml deionized water. Adjust the pH to 6.8 with concentrated hydrochloric acid; add deionized water to 100ml and store at 4℃.

4. **Resolving gel buffer** (1.5mol / L Tris-HCl pH 8.8): dissolve 18.16g Tris in 80ml deionized water; adjust the pH to 8.8 with concentrated hydrochloric acid; add deionized water to 100ml; store at 4 ℃.

5. **10% ammonium persulfate (AP)**: ammonium persulfate provides the free radical necessary for the catalysis of the Polymerization of Acrylamide and Bis-acrylamide; Use deionized water to prepare a small amount of 10% (w/v) solution and store at 4 ℃. Since ammonium persulfate will decompose slowly, it should be freshly prepared every other week.

6. **TEMED (N, N, N, N – tetramethylethylenediamine)**: by catalyzing ammounium persulfate to form free radicals, TEMED accelerated the polymerization of acrylamide and bis-acrylamide. Since TEMED only functions in a free base form, the polymerization reaction would be inhibited when the pH is low.

7. **Tris- glycine electrophoresis buffer**: weigh 15.1g Tris and 94g glycine; Dissolve in 900ml deionized water; then add 50ml 10% (w/v) SDS and deionized water to 1000ml. Dilute 5-fold when using. The final concentration would be: Tris, 25mmol/L; glycine, 250mmol/L; SDS, 0.1% and the pH of the buffer is 8.3.

Appendix 5

Antibiotics	Disc content	Diameter of zone of inhibition (mm)		
		Resistant (mm or less)	Intermediate (mm)	Sensitive (mm or more)
Neomycin	30 mcg	12	13-16	17
Gentamicin	10 mcg	12	13-14	15
Vancomycin	30 mcg	14	15-16	17
Ampicillin	10 mcg	13	14-16	17
Bacitracin	10 units	8	9-12	13
Erythromycin	15 mcg	13	14-22	23
Penicillin G	10 units	14	--	15
Streptomycin	10 mcg	11	12-14	15
Chloramphenicol	30 mcg	12	13-17	18

Name of antibiotics (dose)	Inhibitory zone diameter to nearest millimeter (mm)		
	Sensitive (S)	Moderately sensitive (MS)	Resistant (R)
Amoxicillin (30 μg/disk)	≥18	14–17	≤13
Cloxacillin (5 μg/disk)	≥25	22–24	≤21
Cephalothin (30 μg/disk)	≥18	15–17	≤14
Cephradine (25 μg/disk)	≥18	13–17	≤12
Cefuroxime (30 μg/disk)	≥23	15–22	≤14
Cefixime (5 μg/disk)	≥19	16–18	≤15
Kanamycin (30 μg/disk)	≥18	14–17	≤13
Streptomycin (10 μg/disk)	≥15	12–14	≤11
Neomycin (30 μg/disk)	≥17	13–16	≤12
Vancomycin (30 μg/disk)	≥12	10–11	≤9
Erythromycin (15 μg/disk)	≥23	14–22	≤13
Azithromycin (15 μg/disk)	≥18	14–17	≤13
Ciprofloxacin (15 μg/disk)	≥21	16–20	≤15
Levofloxacin (5 μg/disk)	≥17	14–16	≤13
Tetracycline (30 μg/disk)	≥15	12–14	≤11
Doxycycline (30 μg/disk)	≥14	11–13	≤10
Cotrimoxazole (25 μg/disk)	≥16	11–15	≤10
Chloramphenicol (30 μg/disk)	≥18	13–17	≤12