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A Promising Anti-Biofilm Activity of Seaweeds *(Fucus serratus).*

Manka Marycleopha [1], Elisa Kalugendo [2]
[1]Msc Microbiology, Department of Microbiology, Allagapha University, Karaikudi- 630003 Tamilnadu, India.
[2] M.Sc. Biochemistry, PG & Research Department of Biochemistry, Bharathidasan College of Arts and Science, Erode - 638116, Tamilnadu, India.

Abstract:-The aim of the present study was to screening of the eco-friendly anti-biofilm antimicrobial compound from marine seaweeds, characterization of the bioactive metabolites though FTIR.

Method
Confirmation of biofilm activity was evaluated using Congo red agar method where the organisms where streaked over Congo red agar method,

Results
The different parameters namely morphological, biochemical testes have been used for characterization and identification of marine biofilm bacteria

Conclusion
Anti- biofilm metabolites assess from the above results indicates the presence of active constituents in the extractions of natural plants of seaweeds which showed better antimicrobial activity against micro-fouling bacteria.

Keywords: *- Anti-Biofilm, Seaweeds, Antimicrobial Activity, Invitro-Biofilm Inhibitory Concentration And FTIR Analysis.*

I. INTRODUCTION

Biofilm is the aggregate of microbes are highly resistant to antibiotics, up to 1000 times more resistant. Biofilm are mass of microbes attached to the biotic or abiotic surface. Biofilm can be beneficial or harmful to human and animals. Most of the microbes which affect humans they form persistence of biofilm (Railkin AI., (2004). Most of the biofilm bacteria are resistant to antibiotics activities. Biofilm are mostly found in the moist environment where the sufficient of nutrients are available and attachment is available. Biofilm can be formed by a single bacteria species although they contain many species examples *fungi*, *protozoa*; *algae* etc. (Maki, 2002). Biofilm bacteria affect the other growth of other bacteria in the same biofilm (Caccamese,S.,*etal*,1985). Marine biological fouling, usually termed marine bio fouling, can be defined as the undesirable. Marine biological fouling, usually termed marine bio fouling, can be defined as the undesirable accumulation of microorganisms, plants and animals on artificial surfaces immersed in sea water (Abarzua S, Jakubowski S., 1995) .

Micro fouling is the initial step in the growth of befouling on hard substrata submerged in marine waters. (: Davy, A.R. *et al*, 2008). Despite the focus of modern microbiology research on pure culture plankton (free-swimming) bacteria. It is now widely recognized that most bacteria found in natural clinical and industrial setting persist in association with surface (Hay M E, Steinberg P D .,(1992). Furthermore, these microbial communities are often composed of multiple species that with each other and their environment. The termination of biofilm architecture, particularly the spatial arrangement of micro colonies (cluster of cells) related to one another has profound implication for the function of these complex communities (Oliver,J.D., (1982) .

Kingdom	Chromista
Phylum	Ochrophyta
Class	Pheaophyceae
Order	Fucales
Family	Fucaceae
Genus	Fucus

Table 1:- Showing classification of seaweeds

Fig 1:- *Fucus serratus*

II. MATERIALS AND METHODS

Valinokkam 5 m above sea level and located at 9.17° N 78.65° E. Ramanathapuram District (South of Rameswaram Island) in Tamilnadu. Because it is near the seashore, Valinokkam is famous for salt production.

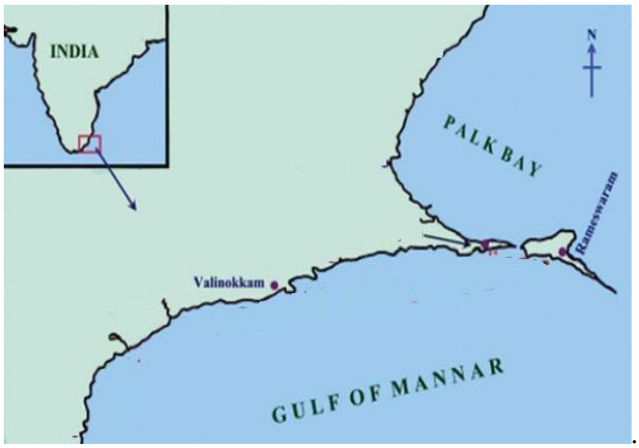

Fig 2:- **Valinokkam** Ramanathapuram District-India

B. Sample collection and extract preparation

Live and health samples of the seaweeds like Focus serrates were collected by hand picking during low tide from Valinokkam, Gulf of Mannar, and South India. These samples were thoroughly washed with seawater to remove all epiphytes, shells etc., and again washed with fresh water to remove the surface salts, sand particles if any and allowed to dry in the shady place for 3 to4 days. The collected samples were identified by using standard books and manuals. The dried samples were then placed on a blotting paper to remove the excess moisture before preparation of seaweeds sample was grounded to fine powder prior to solvent extraction.

C. Crude extract preparation

Seaweed were collected and thoroughly washed with sterile seawater to remove all epiphytes, *etc*., and again washed with fresh water to remove the surface salts, sand particles if any and allowed to dry in the shade for one week. The dried samples were then placed on blotting paper to remove the excess moisture before preparation of the extracts; the samples were ground to fine powder prior to solvent extraction. Solvent extracts were taken in 250ml conical flask add with the same volume of solvents like chloroform (w/v) were added to get the natural concentrations of the seaweed; and they were extracted by cold steep method at –10°C (Wright, 1998). The concentrated extract (about 100ml) was again filtered through a WHATMAN No. 1 filter paper fitted with a Buchner funnel using suction pressure. Finally, it was reduced to thick oily natured crude extract in a rotary vacuum evaporator at 40°C, collected in air–tight plastic vials and stored in the refrigerator for further activity studies.

D. Identification of the bacteria

For the characterization of bacterial strains, a loop full of bacterial culture was inoculated into sterile nutrient broth for total heterotrophic bacteria and was incubated overnight .The fresh overnight culture was subjected to microscopic, physiological test for the characterization and identification.

E. Characterization and identification

Different test was performed as per the keys given in Bergeys manual.

1. For gram positive cocci pigment production, catalase, and Glucose fermentation test.
2. For gram positive spores formers: MR-VP, citrate utilization, ONPG, Carbohydrate fermentation test.
3. For gram positive non spores formers, iodole, Catalase, Oxidase, Urease, carbohydrate fermentation test.
4. for gram negative rods: Catalase, Oxidase, IMVIC test, etc.

F. Confirmation of biofilm activities by Congo red agar method.

Confirmation of biofilm activity was evaluated using Congo red agar method where the organisms where streaked over Congo red agar. Congo red Agar was prepared and poured on Petri plate it was left to solidify. Then the isolated bacteria were streaked on the agar and were incubated for 37ºc for 24hr. After incubation the black color was observed and interpreted.

G. Antibacterial activity by disc diffusion method

Antimicrobial activity was evaluated using the agar disc method in Petri dishes using Zobell marine agar. Briefly 50-500µl of the extract was loaded on agar Zobell marine agar early stage biofilm bacterial isolates were spread on Zobell marine agar plates with sterile effusion and the plates were placed on incubator at 370C for 24 hr. After incubation clear zone around the disc was evidence of antimicrobial activity. Diameters of the zones of inhibition were measured in millimeter.

H. Invitrobiofilm inhibitory concentration (BIC) assay

The ant biofilm activity was carryout by using 96 well plates against pathogenic bacteria such as *Salmonella* sp., *Klebseilla* sp and *E.coli* and biofilm marine bacteria sp. The biofilm formation was measured by OD@490 value by using Elisa Reader.

I. Biofilm viability analysis through light microscopic method

The biofilm bacteria was inoculated in the broth and incubated for 24 hr, further, the seaweed extract was taken and spread on the glass slide and it was incubated for 24hr. After incubation the treated glass slide with methanol extract and untreated glass slide was immersed into the conical flask which contains broth with biofilm bacteria. Then it was incubated for another 24 hr. Then the slide was taken and washed slightly with distilled water and crystal violet was spread on the slide and left for 5 min. The dye was washed away and left to dry and then observed under light microscope.

J. FTIR analysis of bioactive extract

The FTIR spectra of crude chloroform extracts was recorded with subjected to FTIR (Instrument Model RXI) spectrometer. The scanning wavelength of infrared was at 4000–400 cm−1 at a resolution of 4 cm−1 and an interval of1.0 cm−1.

III. RESULTS AND DISCUSSION

A: Results

A. Isolation and identification of marine biofilm bacteria

The different parameters namely morphological, biochemical testes have been used for characterization and identification of marine biofilm bacteria. In the present investigation, were found to dominating in the marine biofilm which was isolated characterized by morphologically and chemically.

Organism	**R3Z4**	**R3A3**	**B2A6**	**B3A7**	**R4A15**	**B3A13**	**R3A9**
SHAPE	Cocci cluster	Cocci cluster	Cocci cluster	Cocci cluster	Cocci cluster	Cocci cluster	Cocci cluster
COLOR	VW	C	PO	W	W	C	C
VP	+	+	+	-	+	+	+
MR	+	+	_	+	-	+	+
I	-	+	+	+	+	-	+
C	+	-	+	+	+	+	+
ISI	+	-	-	+	-	+	+
CAT	+	-	+	_	+	+	+
OX	-	-	-	-	-	-	-

Key: YW-yellow white, C-Colorless, PO-pale orange, PY-pale yellow, W-white, _ Negative, + -Positive

Table 2:- Shows the biochemical characterization of biofilm bacteria

Organism	**B3A21**	**B3A1**	**B3A8**	**B3A12**	**R2A14**	**R2A18**	**R3A10**
SHAPE	Cocci cluster	Cocci cluster	cocci	cocci	cocci	cocci	cocci
COLOR	C	O	PY	O	O	PY	W
VP	+	+	+	+	+	_	_
MR	+	+	+	+	+	_	_
I	+	+	+	+	+	_	_
C	+	+	+	_	-	_	_
ISI	+	+	+	+	_	+	+
CAT	-	+	_	+	_	_	_
OX	+	_	_	_	_	_	-

Key: YW-yellow white, C-Colorless, PO-pale orange, PY-pale yellow, W-white, _ Negative, + -Positive

Table 3:- Shows the biochemical characterization of biofilm bacteria

B. Evaluation on antibacterial activity by disc diffusion method

Antimicrobial activity was evaluated using the sterile disc method. After incubation, the clear zone around a disc was evidence of antimicrobial activity. Diameters of the zones of inhibition were measured in millimeters.

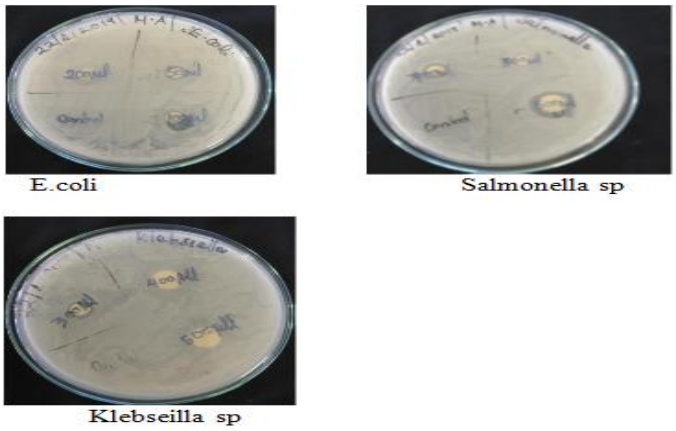

Figure3:-shows the antimicrobial activity of *Focus serratus* against pathogenic bacteri

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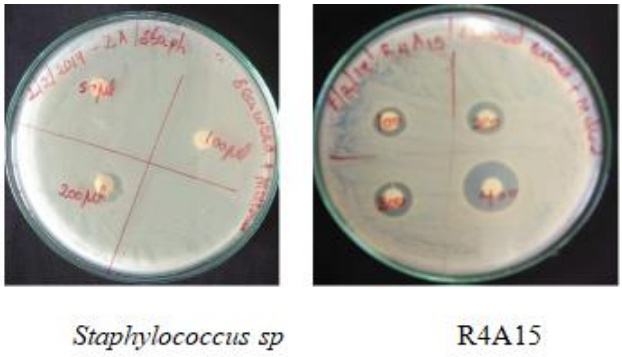

Figure 4:- Shows the antimicrobial activity of *Focus serratus* against marine biofilm bacteria.

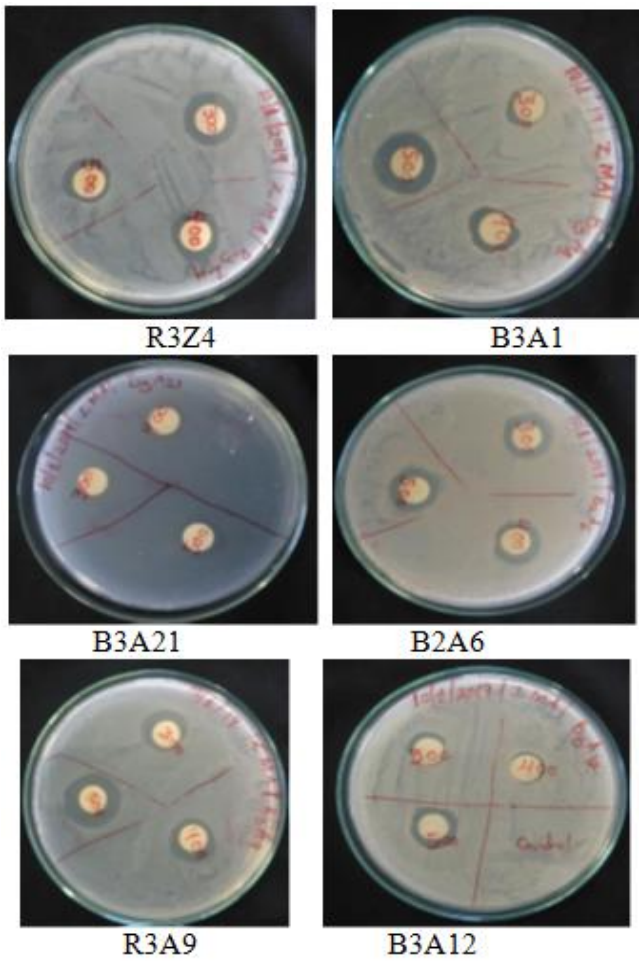

Figure5:- Shows the antibacterial activity of *Fucus serratus* against marine biofilm bacteria.

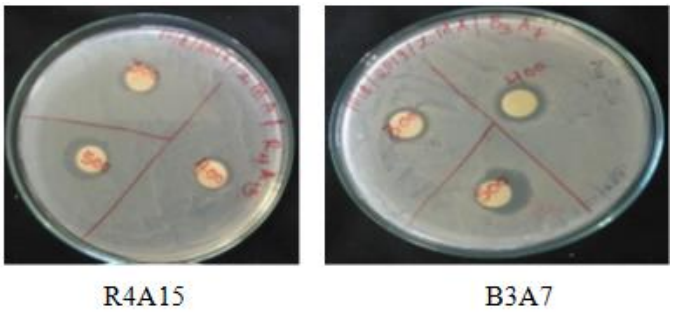

Figure 6:- shows the antibacterial activity of *Fucus serratus* against marine biofilm bacteria.

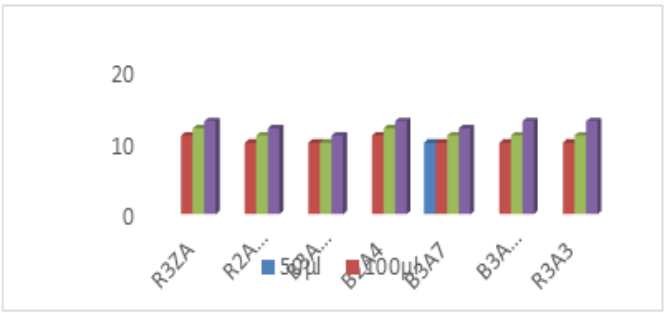

Figure7:- Shows the antimicrobial activity against micro fouling bacteria.

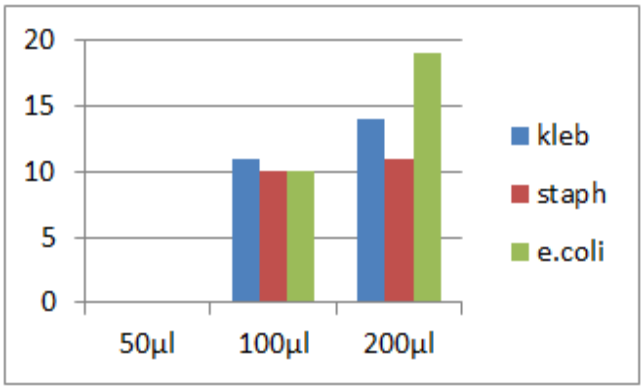

Figure 8:- Shows the antimicrobial activity against micro fouling bacteria.

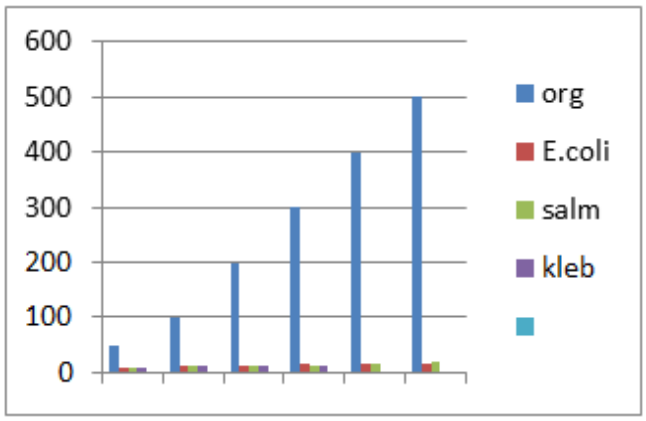

Figure 9:- Shows the antimicrobial activity against pathogenic bacteria

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E. Biofilm viability analysis through light microscopic

The chloroform extract of *Fucus serratus* showed effectively kill the viability of marine biofilm bacterial isolates

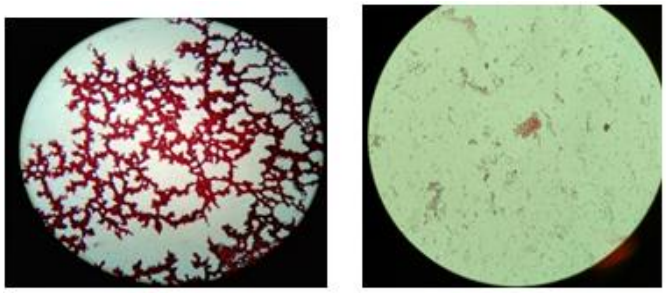

Figure 10:- *Shows the* biofilm viability analysis of *Fucus serratus* against marine biofilm bacteria B2A6

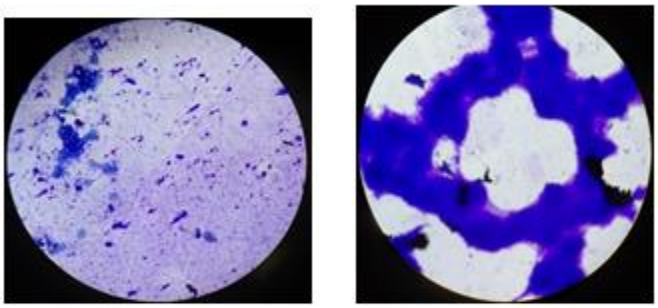

Figure 11:- Shows the biofilm viability of *Fucus serratus* against marine biofilm bacteria B2Z3

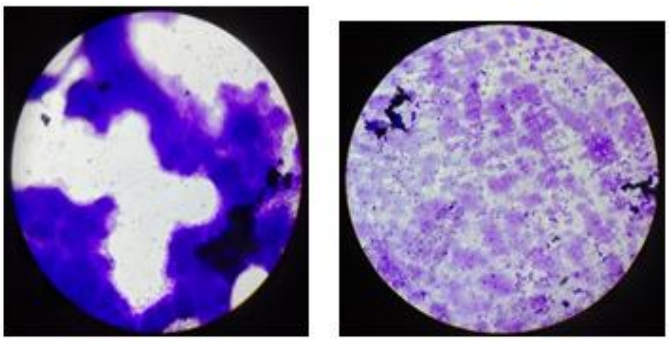

Figure 12:- shows the biofilm viability of *Fucus serratus* against marine biofilm bacteria B2Z7

F. FTIR characterization of seaweed extracts.

The FTIR spectra of chloroform extract of seaweeds was recorded with subjected to FTIR (Model RXI) spectrometer with scanning wavelength of IR was at 4000–550 cm^{-1}**(Figure14,a,b,c,d)**. The IR spectrum in the mid–infrared region was used for discriminating and identifying various function groups variation in spectral features of the IR band suggests that bind to hydroxyl, amino ,carbonyl and phosphoryl functionalities.

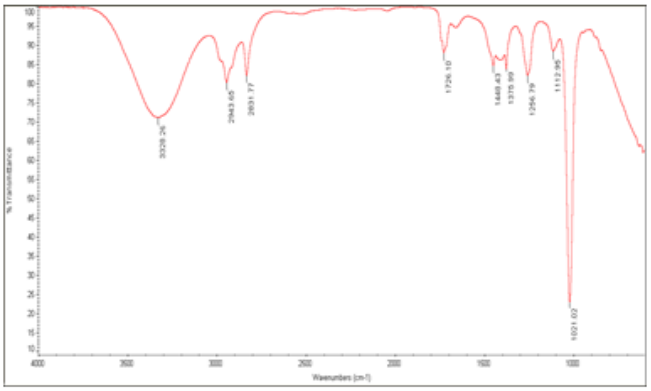

Figure 13:- Shows functional moieties and characterization through FTIR chloroform

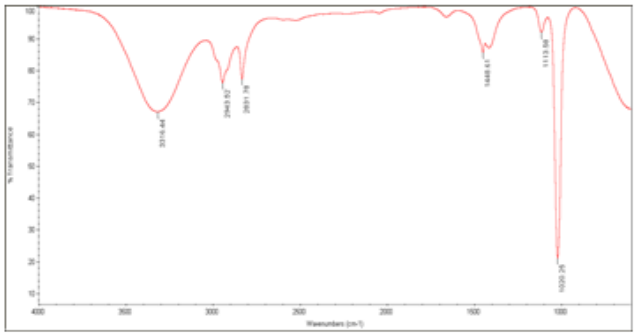

Figure 14:- Shows functional moieties and characterization through FTIR methanol extract.

S/NO	Frequency	Bond	Functional moiety
1	3316.44	Strong	Alcohol OH stretch
2	2943.52	Weak	=C-H Stretch
3	2831.78	Variable	C-H aldehydic
4	1448.61	Medium	CH_2 bend
5	1113.58	Strong	C-O-C stretch
6	1020.25	Strong	C-F

Table 4:- Shows the FTIR showing the chloroform extract from *Fucus serratus*

S/N	Frequency	Bond	Functional moiety
1	3328.26	Strong	Alcohol OH stretch
2	2943.65	Weak	-C-H stretch
3	2831.78	Weak	=C-H stretch
4	1726.10	Strong	C=O ester
5	1448.43	Weak	C=C aromatic
6	1375.99	Medium	CH_3 bend
7	1256.79	Strong	NO_3 stretch
8	1112.95	Strong	C-F
9	1020.25	Strong	C-F

Table 5:- Shows the FTIR showing the methanol extract from *Fucus serratus*

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G. Invitro biofilm inhibitory concentration (BIC) assay

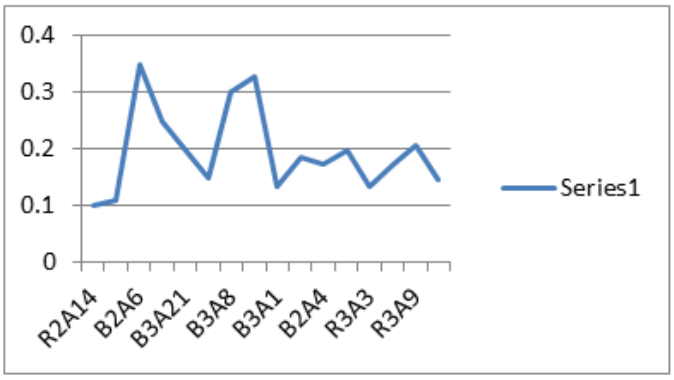

Figure 15:- Shows control biofilm growth

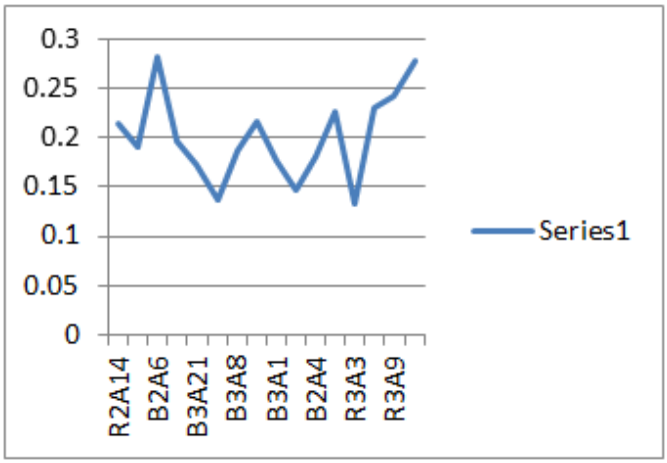

Figure 16: Shows biofilm growth with extract

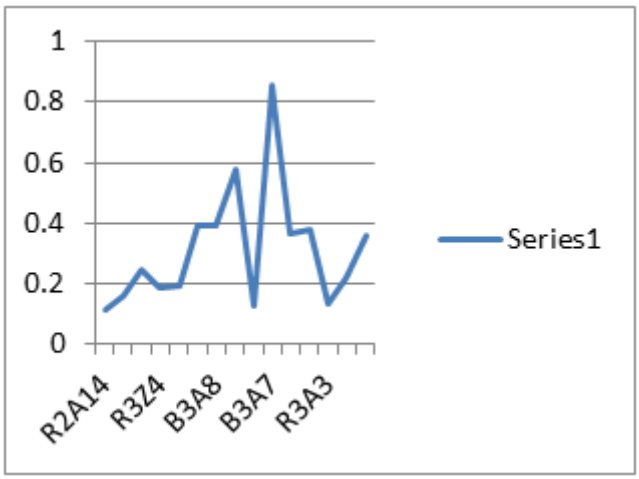

Figure 17:- Shows the control biofilm growth

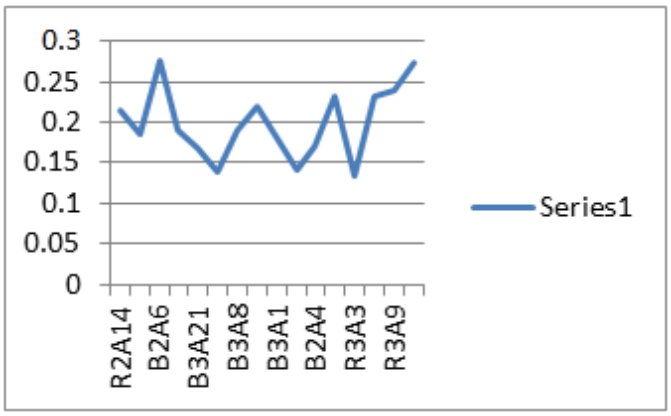

Figure 18:- Shows biofilm growth with extract

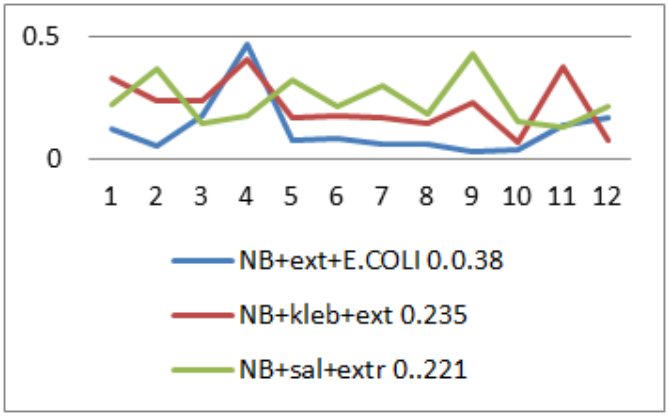

Figure 19:- Shows 0 hrs pathogenic bacteria with extract

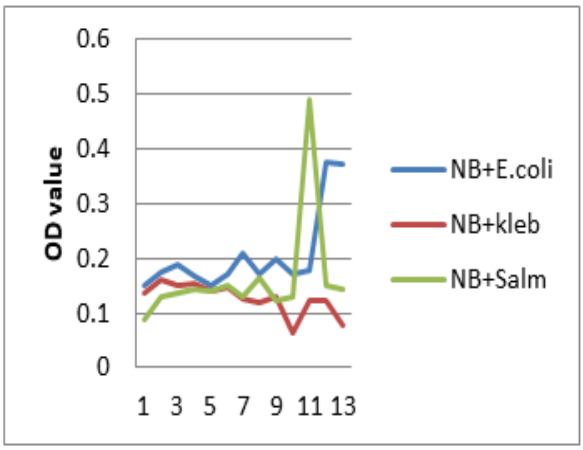

Figure 20:- Shows control pathogenic growth

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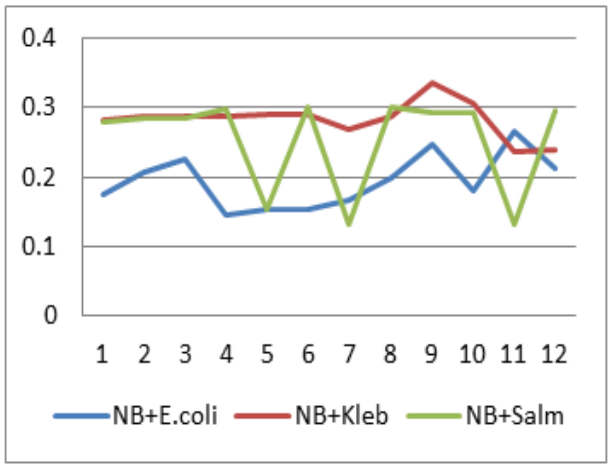

Figure 21:- Shows control pathogenic growth.

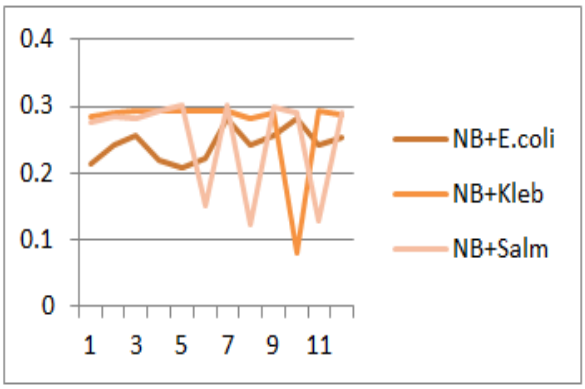

Figure 22:- Shows control pathogenic growth after 24hrs

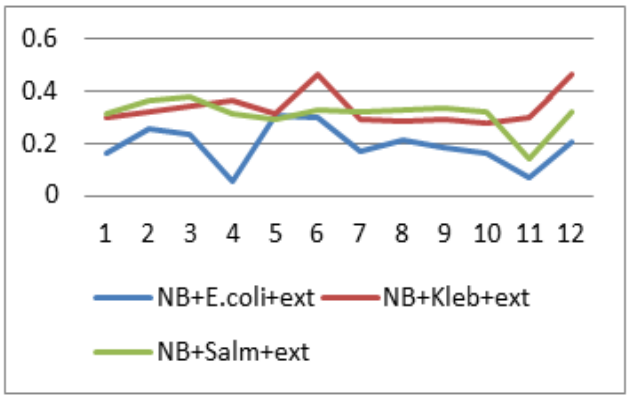

Figure 23:- Shows control pathogenic growth after 24hrs

B: Discussion

The algae extracted in methanol and chloroform was found to show considerable antibacterial activity, similar observation was earlier made by (Prem *et al*., (1997) , who reported that the hypobranchial glands of *Chicoreus virgineus* and egg capsules of *Rapanarapiformis* extracted with polar solvents like ethanol and methanol showed wide spectral antibacterial activities. (Sastry *et al*., 1994) showed successive extraction with benzene, chloroform and methanol. Similarly, (Marasneh *et al.*, 1995) has shown antibacterial activity in organic extracts of six species of marine algae against multi-antibiotic resistant bacteria. It has been reported that biofilm bacteria may be 150–3000 times more resistant to free chlorine than free floating bacteria this is due to excessive production of exo-polymers by biofilm bacteria. In the present study of seaweed, the tested biofilm marine bacteria, **B3A13, R3A9, B3A7, B3A1, R3Z8** was found to be more sensitive to chloroform seaweed extract. It is evidenced that all the chloroform extracts of seaweeds possess anti-biofilm metabolites. In the present study, chloroform extract of natural plants shows significant in the present study, chloroform extract of natural plants shows significant activity towards the marine biofilm bacteria viz. The marine biofilm bacterial characterization was given in the table 2 in the biofilm bacteria. The present study of chloroform extract of *Fucus serratus* shows significant antibacterial on well as antibiofilm activities were shown (**Fig .4, 5, 6, 7, 8).** For discriminating and identifying various function groups' variation in spectral features of the IR band suggests that bind to hydroxyl, amino, carbonyl and phosphoryl functionalities (**Figure8, 8.1**). In earlier reports, Caccamese *et al.* (1985) proved that the brown and red algal extract showed higher antibacterial activity against *Bacillus* Sp., and *E. coli*. Similarly, Padmini and 38 Sreenivasa Rao, (1991) [10] reported that red and brown algal extracts showed greater antibacterial activity than green algae. On contrary, green algae were found to be more effective against biofilm bacteria compared to brown algae (*Padina* sp.). The variation in the effects of algal extracts suggests that they are not simply functioning as broad spectrum toxins against marine biofilm bacteria; rather they appear to have specific activities against one or several biofilm bacteria. According to the previous reports, the results from the present study support that the micro-fouling bacteria are more susceptible to natural plant extract which was also supported from earlier works with different species of seaweeds (Del Val *et al.*, 2006), indicating that the more susceptibility of Gram-positive and Gram negative bacteria to the chloroform extract was due to the differences in their cell wall structure and their composition. Further in Gram-negative bacteria, the outer membrane acts as a barrier to many environmental substances including antibiotics and the presence of thick marine layer in the cell wall also prevents the entry of the inhibitors (Thomas KV, Brooks S., 2010) .The overall anti biofilm metabolites assess from the above results indicates the presence of active constituents in the extractions of natural plants of seaweeds which showed better antimicrobial activity against micro-fouling bacteria. Hence they can be considered as potential natural sources of bioactive metabolites act as leading anti-biofilm molecules for the investigation of natural anti-foulant preparations.

IV.CONCLUSION

The collection of seaweed *Fucus serratus* from vallinokkam Gulf of Mannar, Tamil Nadu Costal area and washed thoroughly with sea water and allowed to dry, the sample was then grinded and the powder was placed in the solvents extraction. Each 10g of seaweeds was taken in 250ml conical flask with the same volume solvents (w/v) like chloroform was added to get the natural concentrations of the seaweeds, by cold steep method. The biofilm bacteria were isolated and subjected to microscopic, physiological

and biochemical tests for the characterization and identification. Different tests were performed per the keys given in Bergey's manual .Antimicrobial activity was evaluated using the disc diffusion method in Petri dishes by using Zobel marine agar. Briefly, 50 to 500µl of each extract were spread on agar. After incubation clear zone around the disc was evidenced of antimicrobial activity. Diameters of the zones of inhibition were measured in millimeters; each test was made in duplicate, the extract was subjected to FTIR analysis for identification of the active functional groups.

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