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Research Article

ANTIMICROBIAL POTENTIAL OF THE BACTERIA ASSOCIATED WITH *SARGASSUM WIGHTII* AGAINST PLANT FUNGAL PATHOGENS

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ABSTRACT

This study focuses on the isolation and characterization of the antifungal bacteria associated with the marine algae *Sargassum wightii* from two study sites (coast of Vizhinjam, site1 and Varkala, site2,Thiruvananthapuram, Kerala).The isolates were characterized by morphology, Gram Staining and Biochemical tests. Ethyl acetate extracts of bacterial supernatant were screened for antibacterial activity. From the eleven isolates, eight strains revealed antibacterial potential against the plant pathogens *Rizoctonia solani, Fusarium monoliforme* and *Claviceps purpurae*. The two isolates showing maximum activity from each site were identified upto species level by 16S rRNA profiling. The one with maximum activity was identified as *Pseudomonas stutzeri* (site1) and *Staphylococcus vitulinus* (site2). The HPTLC assay was carried out for the most active isolate. Spot of the EPS developed on HPTLC was analyzed by GC-MS analysis. *S.vitulinus* can produce Pyrrolo[1,2-a pyrazine-1,4 dione hexahydro-3-(phenylmethyl)-compound,2(Acetoxymethyl)-3-(methoxycarbonyl)biphenyl compound and *P.stutzeri* can produce the biocompounds pyrrolo[1,2-a pyrazine-1,4 dione hexahydro-3-(phenylmethyl)-,2(Acetoxymethyl)-3-(methoxycarbonyl)biphenyl,Phenol,2,5-bis(1,1 dimethylethyl) in their medium. These marine bacteria are expected to be potential resources of natural antibiotic products. It can be concluded that isolation of associated marine bacteria can provide a number of microbial strains as a source of new marine biomolecules. Further studies are required for the purification and chemical characterization of the bioactive products.

Keywords: Antimicrobial potential, Epibiont, *Pseudomonas stutzeri*, *Staphylococcus vitulinus*, Secondary metabolites, Supernatant.

1. INTRODUCTION

Each surface in the sea is quickly covered by a biofilm.M ost marine organisms are relatively free of macro fouling, although some may be covered by a thin film of epiphytic bacteria. Little attention has been given to the role of these bacteria in maintaining the host's health. These epibionts can play a protective role by releasing compounds into the surrounding seawater to prevent extensive surface fouling. The secondary metabolites produced by marine organisms have more novel and unique structures compared to terrestrial organisms due to the complex living circumstances and species diversity, and the bioactivities are much stronger. There may also be industrial and medical applications for these compounds. Compared to other bacteria that produce active compounds, the relative ease of cultivating these microbes suggests that marine-associated bacteria are useful in bioprocess applications, such as antimicrobial or antifouling compound production. Competition in the marine environment among microbes for space and nutrients is a sturdy selection pressure that endows marine microorganisms with a lot of natural products of medical and industrial attention [1-3]. Bacteria have highly efficient extracellular enzyme systems such as proteases, amylases, lipases and phosphatases, in order to catabolize complex materials into simpler fractions. Hence bacteria and other microorganisms play a significant role in the ocean as they do in terrestrial environments but only meager information is available about their occurrence and activities.

Previous studies found a high percentage of marine epiphytic bacteria producing antimicrobial metabolites compared to the number of planktonic isolates producing these metabolites [1].

Most algae have a higher growth rate in the presence of bacteria than in their absence. Many algal species need specific vitamins to grow, and bacteria may be partly

responsible for these substances. Furthermore, several seaweeds may synthesize antiviral, antibacterial and antifungal compounds against several pathogens, and bacteria living on algae are thought to be the producers of these active compounds [4]. A new thread for research is the analysis of marine bacteria and their potential role in the development of metabolites. Several investigations have supplied an increasing number of biologically active and structurally unique compounds. In the marine environment, bacteria and other micro-organisms are ubiquitous. They are taxonomically diverse, biologically active, and colonize all marine habitats, from the deep oceans to the shallowest estuaries. Most of the existing antimicrobial drugs are the earlier generation derivatives and the need for new drug development has been further exacerbated by microbial resistance against them. There are acceptable options for biocompatible, biodegradable and non-toxic metabolites of plants or animals. There has been an ongoing search for new drugs from the sea for more than two decades [5]. This description seems to fit anti-microbial peptides (AMPs), known as the new generation native peptide molecules isolated from a wide range of organisms and species from bacteria to humans. As a result, they were named "organic antibiotics" because they are active against a wide range of micro-organisms, including bacteria and filamentous fungi, as well as protozoan and metazoan parasites. Even though many seaweeds have reported antifouling compounds, the genus *Sargassum* (Phaeophyceae) is known to generate antibacterial, antitumoral, antimalarial, antiherbivorous and antifouling compounds [6].This work reports the potential of bacteria associated with the marine seaweed *Sargassum wightii*, collected from the coast of Vizhinjam (8°22′45″N 76°59′29″E) and Varkala (8.73°N 76.71°E)for the production of secondary metabolites against fungal pathogens, *R.solani*, *F.monoliforme* and *C.purpurae*.

2. MATERIAL AND METHODS

2.1. Collection of Seaweeds and Isolation of Epiphytic Bacteria

Seaweed samples were collected from two study sites, Vizhinjan and Varkala. They were handpicked and immediately washed with seawater and transported to the laboratory on ice. Different species of *Sargassum* were identified with the help of experts. From the different species, *S.wightii* was taken and its surface was washed with fresh water. A bacterial sample was taken from the surface with a sterile cotton swab. The serial diluted samples were spread on Zobell marine agar plates. The plates were incubated for 24-48hr at 37°C.

2.2. Crude Extract Preparation and Screening of Antagonistic Activity

The pure bacterial cultures obtained were maintained on Nutrient Agar slants. Overnight bacterial culture (100 ml) in marine broth was centrifuged at 4°C at 7000rpm for 20min. The supernatant was collected and extracted with ethyl acetate. Organic layer was concentrated with a vacuum rotator. The concentrated solutions were used for bioassay against *R. solani*, *F. monoliforme* and *C. purpurae*. Mueller Hinton agar plates were prepared and uniformly swabbed with pathogen. Thereafter, it was punched with 5mm diameter wells and filled with 30µl of the concentrated bacterial extracts. The petri-dishes were incubated at 37°C for 24h. After incubation, plates were examined for inhibition zones. Clear inhibition zone developed around wells was regarded as an indication of antimicrobial activity. The culture of *R. solanii*, *F. monoliforme* and *C. purpurae* was collected from National Chemical laboratory, Pune, India.

2.3. Identification of most active isolates by 16s rRNA profiling and phylogenetic analysis

The bacteria with wide antimicrobial spectrum were identified to the species level by PCR amplification of the 16S rRNA gene, BLAST analysis, and comparison with sequences in the GenBank nucleotide database. The 16S rRNA gene sequences were analyzed and the relative phylogenetic positions were determined by searching GenBank database using BLASTn algorithm [7]. Phylogenetic tree was constructed from evolutionary distances using neighbor-joining DNA distance algorithm [8]. Tree topologies were evaluated by bootstrap analysis of data sets with MEGA5 [9].

2.4. Separation of EPS by HPTLC and GC-MS Analysis

The HPTLC separated fraction of Extra Polymeric Substance was analyzed by Gas Chromatography-Mass Spectrometry (GC-MS) for elemental analysis. The data were obtained with an Argilent 6890 N Network GC system CKL/ANL/GC-001/5975 Inert x L Mass selective Detector at 70 eV and 20°C.

3. RESULTS AND DISCUSION

3.1. Epiphytic strains

The eleven isolates obtained from *S.wightii* were represented as VS1-VS5 (site 1) and RS1-RS6 (site2)

respectively. Morphological, Gram staining and Biochemical tests such as IMVIC (indole, methyl red, voges - proskauer and citrate) tests, sucrose fermentation test, lactose fermentation test, catalase test , oxidaze test and urease test were carried out and the results are given in Tables 1 and 2.

Table 1: Morphological, Gram staining and Biochemical properties of the isolates from Site 1

Isolates	Fermentation		Indole	Methyl red	Voges Proskaur	Citrate	Urease	Catalase	Oxidase	Morphology and Gram Staining
	Sucrose	Lactose								
VS1	-	-	-	-	-	-	+	+	-	Gram positive cocci
VS2	-	-	-	-	-	-	+	+	-	Gram positive cocci
VS3	+	-	-	-	-	+	-	+	+	Gram negative rods
VS4	-	-	-	-	-	+	-	+	+	Gram negative rods
VS5	+	-	-	-	-	+	-	+	+	Gram negative rods

+activity, −no activity

Table 2: Morphological Gram Staining and Biochemical Properties of the Isolates from Site 2

Isolates	Fermentation		Indole	Methyl red	Voges Proskaur	Citrate	Urease	Catalase	Oxidase	Morphology and Gram Staining
	Sucrose	Lactose								
RS1	+	-	-	-	-	-	-	-	+	Gram positive cocci
RS2	-	-	-	-	-	-	-	+	+	Gram negative rods
RS3	+	-	-	-	-	-	-	+	+	Gram positive rods
RS4	+	-	-	+	+	+	-	+	-	Gram positive rods
RS5	-	-	-	-	-	+	-	+	+	Gram negative rods
RS6	-	-	-	-	+	-	+	+	-	Gram positive cocci

+activity, −no activity

According to biochemical properties most of the isolated strains belong to Proteobacteria and fermicutes. 55% of the isolates were Gram negative and 45% Gram positive. In some of the earlier studies on the bacterial population of Cochin estuary water and sediment samples, gram-negative forms are reported to predominate over gram-positive forms [10, 11]. Of the dominant bacterial classes identified there was significant seasonal variation but, Alpha proteo bacteria accounted for ~40 % of the total 16S sequences, Betapro teobacteria were ~5 % and Gammaproteobacteria were ~20%; Bacteroidetes (~20%) throughout the year [12]. In our study from the isolates 55% were *Pseudomonas* sp.33% were *Staphylococcus* sp. and 9% belongs to *Bacillus* sp. *Pseudomonas* was the predominant genus in this study, followed by *Staphylococcus*. *Bacillus* is obtained in least number (Table 3). Philip found that *Vibrio* and *Pseudomonas* were the predominant genera in the Cochin estuary [10]. The abundance of *Bacillus* was also mentioned. According to Gopinath, the prevalent genera identified were *Pseudomonas, Vibrio, Bacillus* and *Staphylococcus* [11].

Table 3: Classification of the Strains according to morphology and biochemistry

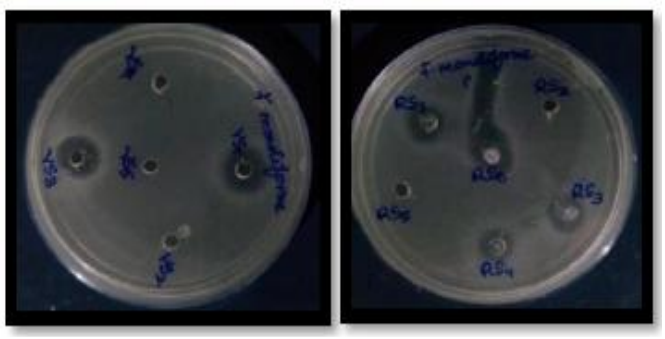
Sl No	Isolates	Phylum	Family	Strains
1	VS1	Firmicules	Staphylococcaceae	*Staphylococcus* sp
2	VS2	Firmicutes	Staphylococcaceae	*Staphylococcus* sp
3	VS3	Proteobacteria	Pseudomodacaeae	*Pseudomodas* sp
4	VS4	Proteobacteria	Pseudomodacaeae	*Pseudomodas* sp
5	VS5	Proteobacteria	Pseudomodacaeae	*Pseudomodas* sp
6	RS1	Firmicules	Staphylococcaceae	*Staphylococcus* sp
7	RS2	Proteobacteria	Pseudomodacaeae	*Pseudomodas* sp
8	RS3	Proteobacteria	Pseudomodacaeae	*Pseudomodas* sp
9	RS4	Firmicules	Bacillaceae	*Bacillus* sp
10	RS5	Proteobacteria	Pseudomodacaeae	*Pseudomodas* sp
11	RS6	Firmicules	Staphylococcaceae	*Staphylococcus* sp

3.2. Antogonistic epiphytes

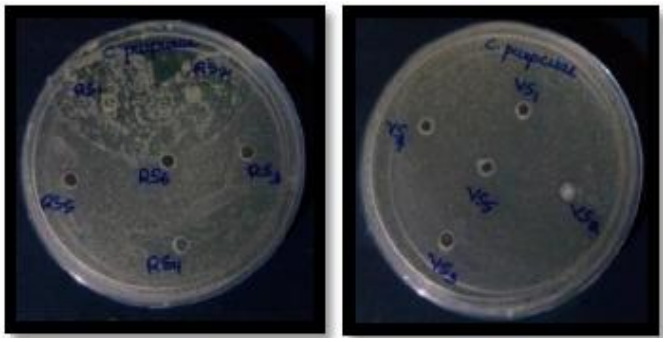
The pure culture of the bacterial epibionts were maintained in agar slants and subsequently tested for their antimicrobial potential against the plant pathogens like *R. solani*, *F. monoliforme* and *C. purpurae* by agar well diffusion method. The inhibition zone obtained was shown in Table 4 and Fig1.From the eleven isolates eight showed antagonistic activity. Interestingly, *Pseudomonas* sp. are the most extensively studied group of biocontrol bacteria because they have many traits that make them effective biocontrol agents of plant and animal pathogens [13, 14]. Among the various species of *Pseudomonas*, *P. aeruginosa* have been used widely in agriculture as microbial control agents due to their high abundance, growth rate and low environmental sensitivity. Non pigmented bacterial isolates dominate the surface colonization of most marine algae as microbial control agents due to their high abundance, growth rate and low environmental sensitivity [13]. Non pigmented bacterial isolates dominate the surface colonization of most marine algae.

Table 4: Zones of Inhibition Formed by Epiphytic Strains

Bacterial isolates	Plant pathogen (inhibition zone in mm±Std err)		
	Fungal pathogens		
	R solani	*C purpurae*	*F. monoliforme*
VS1	-	-	5±0.22
VS2	-	5±0.27	-
VS3	8±0.33	-	9±0.22
VS4	-	-	-
VS5	-	-	-
RS1	-	-	5±0.27
RS2	-	-	-
RS3	5±0.27		5±0.27
RS4	-	-	5±0.27
RS5	4±0.29	-	-
RS6	-	-	9±0.31



A



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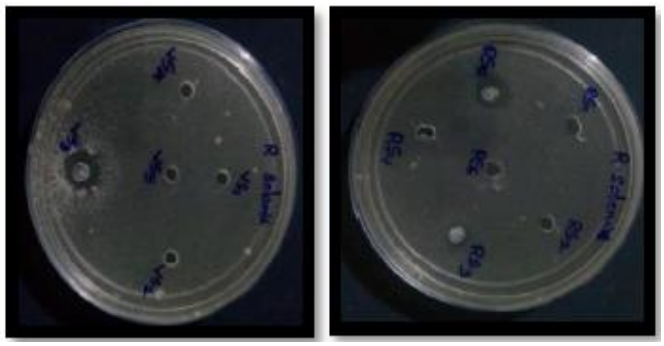


C

Fig. 1: Screening of antimicrobial potential by agar well diffusion method

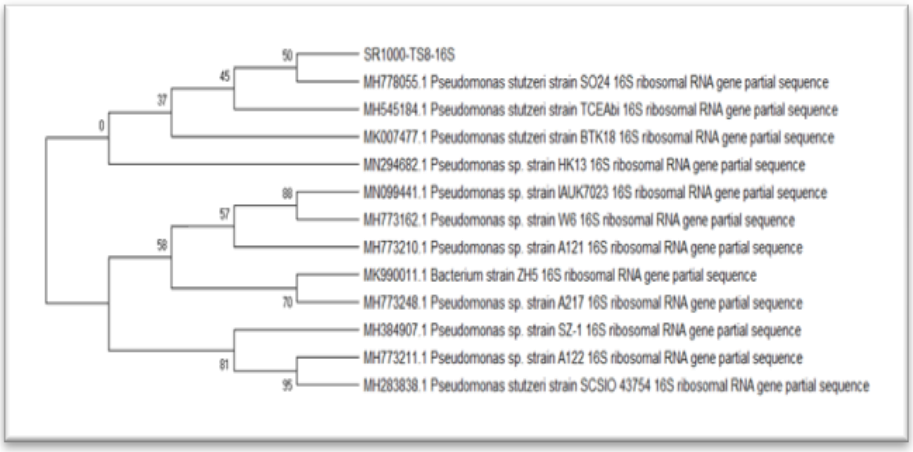
A) Inhibition zones of T&R strains against R. solanii
B) Inhibition zones of T&R strains against C. purpurae
C) Inhibition zones of T&R strains against F. monoliforme

3.3. Characterization of most active isolates

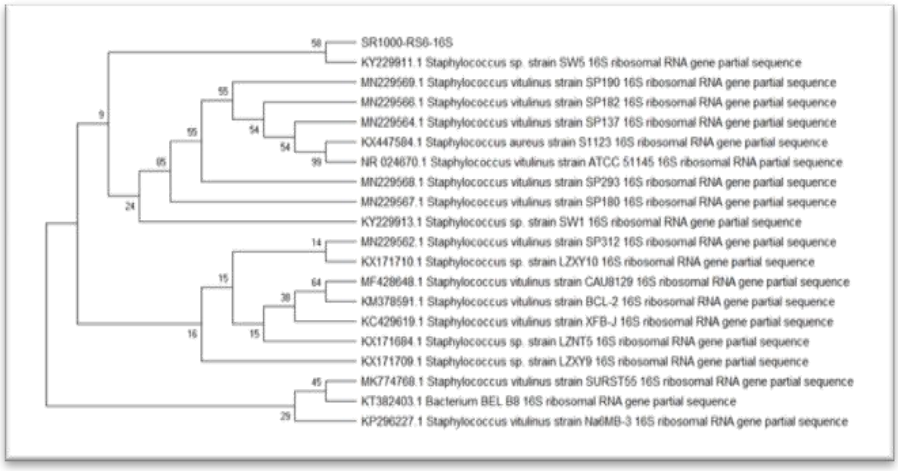
The most active isolates from Site1 *i.e.,* VS3 (9±0.22) and from Site2 (9±0.31) *i.e.* RS6 were identified upto species level by 16S rRNA profiling and a phylogenic tree were constructed (Fig 2 [A, B]). According to the sequences analyzed, VS3 is identified as *P. stutzeri* and RS6 is *S. vitulinus.* The antagonistic principle obtained from ethyl acetate extract of the most active isolates were further separated using HPTLC and the active parts of the spots were checked using GC -MS analysis (Fig. 3[A,B] and Fig.5 [A, B]).

S. vitulinus produced the bioactive compounds pyrrolo[1,2-a pyrazine-1,4 dione hexahydro-3-(phenylmethyl) compound, 2(Acetoxymethyl)-3-(methoxycarbonyl)biphenyl compound and *P. stutzeri* produced the biocompounds pyrrolo[1,2-apyrazine-1,4-dionehexa hydro-3-(phenylmethyl), 2(Acetoxymethyl)-3-(methoxy carbonyl)biphenyl, Phenol, 2,5-bis(1,1-dimethylethyl) in their culture. These biocompounds are responsible for the antimicrobial properties of the strains. *S vitulinus* produced the bioactive compounds pyrrolo [1,2-a pyrazine-1, 4 dione hexahydro-3-(phenylmethyl)-compound, 2(Acetoxymethyl)-3-

(methoxycarbonyl) biphenyl compound and *P stutzeri* can produce the biocompounds pyrrolo[1,2-a pyrazine-1,4 dione hexahydro-3-(phenylmethyl)-2(Acetoxymethyl)-3-(methoxycarbonyl)biphenyl, Phenol, 2,5-bis(1,1-dimethylethyl) in their culture. In this study *S. vitulinus* and *P. stutzeri* produced antimicrobial compounds such as Phenol, 2, 4-bisdimetyl ethyl)-ester and Pyrrolo [1,2-a] pyrazine- 1,4-dione. Antimicrobial properties of these compounds were recorded earlier [15, 16]. Marine *Vibrio* sp. is highly capable of synthesising Phenol, pyrrolo [1,2-a]pyrazine-1,4-dione, Pyrrolidinone derivative with pharmacological properties[17]. Based on earlier findings and as observed in the present study, Bacillales and Pseudomonadales are efficient producers of Phenol and Pyrrolidinone derivatives.

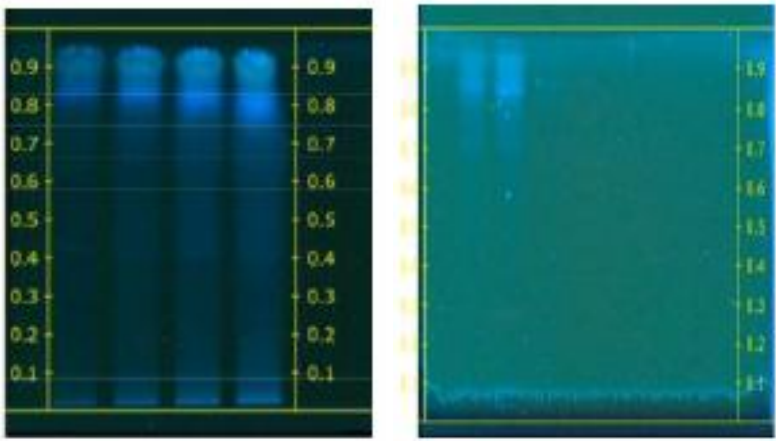


A



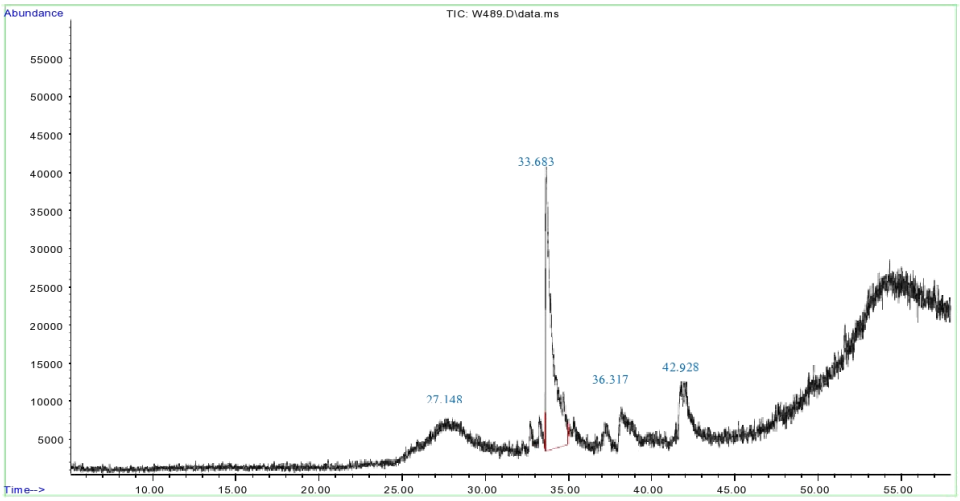
B

Fig. 2: A) Phylogenic Tree of *P. stutzeri*, B) Phylogenic Tree of *S.vitulinus*



A **B**

Fig. 3: A) HPTLC separation of *P.stutzeri*, B) HPTLC separation of *S.vitulinus*



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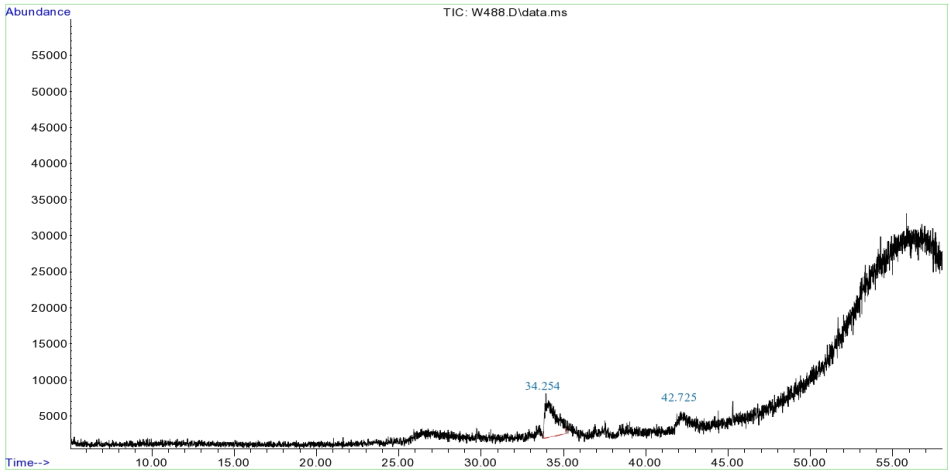

B

Fig4: A) GC-MS profiling of *P. stutzeri,* B) GC-MS profiling of *S. vitulinus*

In the present study, the compounds produced from potential isolates *S. vitulinus* and *P. stutzeri* having effective antimicrobial properties, could be further studied for other activities. Such isolates could be potential candidates for new antimicrobial compounds to combat pathogens. The biological properties of this pyrrole compound are of special interest in relation to its great activity. The importance of antimicrobial substances for chemical ecology of the sea is obvious, in view of the inhibitory properties of *Pseudomonas* for many bacteria isolated from the marine environment. The selective activities of the pyrrole compound and other substances produced by marine organisms probably exert special controls in the complex population dynamics of marine microorganisms. Bioactive metabolites produced by marine *Pseudomonas* species have been reported [5, 18]. Marine *Pseudomonas* sp. are potential sources for medically relevant bioactive substances 19]. Anand *et al*. observed bioactivity in *Staphylococcus* bacteria isolated from four species of sponges [5]. Previously, these compounds have been reported for antimicrobial properties. Pyrrole was used for numerous medical uses and was used as a prevention agent for antibiotics, antitumor, antifungal, anti-inflammatory drugs and cholesterol reducing agents. Pyrrole was also able to suppress HIV-1 and DNA polymerases, as well as protein kinase [20, 21]. Pyrrole and its substitute pyrazine prevent seizures and are used as a potent anticonvulsant medication. A *streptomyces* strain isolated from mangrove soil was reported to produce pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro with an antioxidant property of 32% in 1 mg ml^{-1} [22]. While the antioxidant property of the MSI45 compound was 81% higher in 0.1 mg ml^{-1}. The total inhibition of *Staphylococcus aureus* in the time kill

assay between 24 and 2 hours of incubation was obtained at 1x, 4x and 8xMIC. Hexahydropyrrolo [1,2-a]pyrazine-1,4-dione was identified as an algicidal agent against *Microcystis aeruginosa* [23].

These bacteria species are generally not really symbiotic to the host but can instead be regarded as associated bacteria with consanguineous relationship with their hosts [24]. On the one hand, these bacteria could acquire from their host plant the necessary nutrition such as vitamin, polysaccharide and fatty acid while, they could excrete products such as amino acids, antibiotics and toxins that are conducive to the development and metabolism of the hosts or improve the hosts ' chemical defense capacity [3]. The bacterial diversity detected in this study may constitute a fraction of the total diversity of associated bacteria. Only a small percentage of the bacteria can be grown using the medium and fermentation techniques currently available [6].The results indicated that some marine bacteria were likely to release various antibiotic compounds to provide themselves with superiority in survival competition. In order to find more novel structures, new ways of screening of these compounds should be applied. The idea is that the organisms will lyse and perish if cell wall formation is blocked, but if metabolism or protein synthesis is blocked, the organisms will merely slow down. While this is true to some extent, the concentration of the antibacterial agent often depends on bactericidal or bacteriostatic outcomes. A low dose of a "bactericidal" antibacterial agent may only inhibit bacterial growth, while a high dose significantly affect the anti-cell wall antibiotics, in which case anti-ribosomal antibiotics would be more effective. Biocontrol agents have received a considerable amount of attention for the control of soilborne and airborne plant diseases. Biocontrol is environmental friendly, safe and can protect the crop for a long time. R. *solanii, F. monoliforme* and *C. purpurae* are serious fungi affecting yield and product quality of many susceptible hosts. It is demonstrated in numerous studies that successful disease control was achieved using bacteria [25, 26] in many cropping systems. The most efficient bacteria used for agricultural management belonged mainly to the genera *Bacillus* [27-29] *Pseudomonas* [30], *Enterobacter* [31], *Serratia* [32], and at a lesser extent *Streptomyces*, *Burkholderia*, *Pantoea*, and *Paenibacillus* [30]. These results indicate that certain species are selective in response to certain pathogens. It can be stated that epiphytic bacteria of the marine algae used in this study had different defense mechanisms, which create an ecological and biotechnological interest in their antimicrobial activity. Development of economically feasible standard operating procedures for the production of extracts in large scale with reproducible antibacterial efficiency is necessary.

4. CONCLUSION

We have demonstrated *S. wightii* associated bacteria have a great potential in producing antimicrobial compounds. The present study confirms the potential of *P. stutzeri and S. vitulinus* for the production of anti-pathogenic agents such as as pyrrolo[1,2-a pyrazine-1,4 dione hexahydro-3-(phenylmethyl)-compound, 2(Acetoxymethyl)-3-(methoxycarbonyl)biphenyl compound and *P stutzeri* can produce the biocompounds pyrrolo[1,2-a pyrazine-1,4 dionehexahydro-3-(phenylmethyl)-,2(Acetoxymethyl)-3-(methoxycarbonyl)biphenyl, Phenol, 2,5-bis(1,1-dimethylethyl) in their culture. Development of economically feasible standard operating procedures for the production of extracts in large scale with reproducible antibacterial efficiency is necessary. The bioactive potential of epiphytic bacteria could be a potential source of marine bioprospecting in future. Because of emerging and re-emerging infections to important economic crops, further studies are required for the documentation of different bioactive compounds from seaweed associated bacteria against plant pathogens.

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