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Isolation, Screening and Optimization of Culture Conditions for Enhanced Antibacterial Activity by a Marine Epibiotic Bacterium *Bacillus flexus* APGI against Fouling Bacterial Strains

Ramasamy Ramasubburayan[1], Susan Titus[2], Pradeep Kumar Verma[2], Grasian Immanuel[1] and Arunachalam Palavesam[1]*

[1]Centre for Marine Science and Technology, Manonmaniam Sundaranar University, Rajakkamangalam - 629502, India
[2]Naval Material Research Laboratory, DRDO, Ambernath, Maharastra, India.

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A total of 21 epibiotic bacterial strains were isolated from the surface of seaweeds *Chaetomorpha antennina* and *Ulva fasciata* collected from Kanyakumari coast, Tamilnadu, India. The isolated epibiotic bacterial strains were screened for antibacterial property through cross streak method against six fouling bacterial strains. Cross streak assay result inferred that, seven (33%) out of 21 epibiotic bacterial strains retained antibacterial activity. Of the seven bacterial strains tested, UF-4 showed broad spectrum antagonistic activity. Based on 16S rRNA sequencing, UF-4 showed the highest similarity (99%) to the member of the species *Bacillus flexus* APGI. Further this strain was cultured in five different production media (PM1-PM5), among these PM1 supported for the highest antibacterial activity against fouling bacterial strains. Optimization of various culture conditions revealed that, the active strain *B. flexus* APGI had grown well at pH 8.0, 40°C temperature, 3% inoculum size, mannitol and meat extract as the best carbon and nitrogen sources, respectively towards achieving maximum antibacterial activity. Results of present study highlighted the striking inhibitory activity of *B. flexus* APGI against fouling bacterial strains and proved to be a promising source of antibacterial compounds.

Key words: Seaweeds, Epibiotic bacteria, Optimization, Antibacterial activity.

Materials submerged in seawater are rapidly colonized by diverse range of biofouling organisms which includes microfoulers such as bacteria, fungi, diatoms, protozoa and macrofoulers such as mussels, barnacles, bryozoans and tube worms[1,2]. Settlement of these fouling organisms often cause huge material damage and economic losses in maintenance of mariculture, shipping industries, naval vessels, underwater pipelines, etc.[3]. Application of antifouling coating is one of the most widely employed strategies to control recruitment of fouling organisms on ship hulls and other marine infrastructures[4]. Traditionally, biocides such as tributyltin (TBT) and copper were broadly used in antifouling paints to combat problems pertained to fouling. Though these biocides are much effective against fouling organisms, they are reported to affect the non-targeted marine organisms adversely[3]. This has significantly necessitated the search for the development of environmentally benign antifouling compounds.

Marine natural product based antifoulant is one of the promising alternative for classical

* To whom all correspondence should be addressed.
Mob.: +91-9443545411;
Email : plavesh06@gmail.com

antifoulant. Rittschof[5] stated that, for marine antifouling research, bioactive substances of particular interest should be one that show deterrence properties and can be used for the development of antifouling coatings. But till today, one of the major bottleneck problems faced by antifouling research is that, lack of consistent supply of bioactive compounds from marine organisms. According to Dobretsov *et al.*[2], it is highly impossible to obtain adequate quantity of natural product from macroorganisms to meet out the requirements of commercialization of bioactive ingredients. This holds particularly for natural resources that are rare or endangered. Therefore to overcome the problems related to supply, intense research is now being progressed towards identifying new antifouling metabolites from marine microorganisms. The major advantage of using microorganisms as the source of natural product antifoulant (NPA) is that the compounds can be produced more rapidly and fairly in huge quantities by their unlimited reproductive capability using bioreactors[4]. This could often be achieved by optimizing the culture conditions such as medium components, carbon sources, nitrogen sources, temperature, pH etc.

It was reported that, marine epibiotic bacteria associated with living surfaces such as sponges, corals, seagrasses and marine invertebrates are acquiring increasing attraction towards isolation of novel antifouling compounds[6-9]. Epibiotic bacteria isolated from the surface of several seaweeds were reported to produce potent bioactive compounds, which effectively deter the settlement of fouling bacterial strains, thus suggesting that they may protect the seaweed from fouling by other organisms[10]. Dobretsov and Qian[11] showed that, *Vibrio* sp. isolated from the surface of *Ulva reticulata* had effectively prevented settlement of larvae of tube worm *Hydroides elegans* and inhibited the growth of benthic fouling diatom *Nitzschia paleacea.* Recently, Kumar *et al.*[12] isolated bacterial strains belonging to the member of the genus *Pseudoalteromonas, Bacillus, Vibrio* and *Shewanella* spp. from the surface of *U. lactuca* which were showed significant antibacterial and antidiatom activities. Increasing evidences clearly emphasize that, associated microorganisms are the real source of many marine organisms-derived compounds. It is widely reported that preventing settlement of fouling bacterial strains over substrata was found to be one of the most desirable ways for breaking the fouling chain. Thus considering the importance of above mentioned topics, in the present study an attempt has been made to isolate, screen and identify promising epibiotic bacterium from the surface of seaweeds and to optimize suitable culture conditions to enhance its bioactivity against fouling bacterial strains.

MATERIALS AND METHODS

Isolation of epibiotic bacteria from seaweeds

For the present study, seaweeds such as *Chaetomorpha antennina* and *Ulva fasciata* were collected from the Kanyakumari coast, Tamilnadu, India and brought to the laboratory in sterile polythene bags in an ice box. Collected seaweeds were washed gently using sterilized seawater to remove loosely adhered bacterial strains. The surfaces of seaweeds were swabbed individually with sterile cotton swabs and spreaded over Zobell marine agar (ZMA) plates. The plates were then incubated for seven days at 37°C[13]. Based on the colony morphology, 21 distinct bacterial isolates were selected, restreaked and purified and then stored in ZMA slants at 4°C for further study.

Primary screening of epibiotic bacterial strains

Epibiotic bacterial strains isolated from the chosen seaweeds were tested individually for their antagonistic activity against fouling bacterial strains through cross streak method[14] with little modification of expression of the results in percentage of inhibition[15]. Marine fouling bacterial strains such as *Pseudomonas aeruginosa* (JN979983), *Aliivibrio fischeri* (JN979986), *Vibrio alginolyticus* (JN979984), *Aeromonas hydrophila* (JN561697), *Shigella flexneri* (JN979987) and *Pantoea agglomerans* (JN979985) previously isolated from marine substrata submerged at Chinnamuttom fisheries harbour were collected from Microbial culture collection center, Centre for Marine Science and Technology, M.S.University, Rajakkamangalam, Tamilnadu, India. In brief, the isolated epibiotic bacterial strains were individually streaked on ZMA plates as a single streak and incubated at 28°C for 48 h. Then the test fouling bacterial strains were applied as single streak perpendicular to epibiotic bacterial strains, without touching it. Control plates without epibiotic

bacterial strains were also maintained separately. All the plates were incubated for 24 h at 28°C. Thereafter, the plates were observed and the zone of inhibition of test fouling bacterial strains (reduced growth or lack of fouling bacterial growth) was compared with control plate and recorded as high (81 – 100% inhibition), moderate (21 – 80% inhibition), low (1 – 20% inhibition) and no inhibition or activity (0% inhibition). On the basis of cross streak assay result, the broad spectrum inhibitory activity rendering epibiotic isolate UF-4 was alone selected for further study.

Molecular identification of promising epibiotic bacterial isolate

Extraction of genomic DNA

The promising epibiotic bacterium (UF-4) was grown in Zobell Marine Broth (ZMB) for 24h at 28ºC. Then it was centrifuged at 10000 x g for 10 min at 4ºC. The supernatant was discarded and the pellet was resuspended in TE buffer. Then 8 mg/ml concentration of lysozyme was added and the entire set up was incubated at 37ºC for 1 h. After the specified incubation period, 100 µl of 0.5 M EDTA (pH 8.0) was added. Besides, 60 µl of 10% SDS and 3 µl of proteinase K was added and then incubated at 55ºC overnight. The supernatant was then extracted two times with phenol: chloroform and once with chloroform: isoamylacohol (24:1) and precipitated with ethanol. The genomic DNA obtained was then resuspended in sterile distilled water and stored at 4ºC[16].

Amplification of 16S rRNA gene

The 16S rRNA gene of UF-4 was amplified from the extracted genomic DNA using universal primers: FP (5'-AGAGTTTGATCCTGGCTCAG-3') and RP (5- GGTTACCTTGTTACGACTT-3)'. PCR was executed in 50 µl of reaction mixture containing 2 µl (10ng) of DNA, 0.5 µM of both primers, 1.5 mM $MgCl_2$ and 50 µM of deoxynucleoside triphosphate (DNTP), IU of *Taq* polymerase and buffer as mentioned by the manufacturer (MBI Fermentas). Amplification was performed in Eppendorf gradient mastercycler, as per the following procedure: an initial denaturation for 3 min at 95°C, followed by 40 amplification cycles consisting of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min. After amplification, the PCR product was purified and then sequenced by Genetic Analyzer (Applied Biosystems, USA).

Nucleotide sequence analysis and Phylogenetic tree construction

The nucleotide sequence of UF-4 was matched with previously published 16S rRNA gene sequences in NCBI GenBank using BLAST algorithm[17]. The 16S rRNA sequences that are closely related to UF-4 were retrieved from NCBI GenBank and aligned through multiple sequence alignment using CLUSTAL X software[18]. A Phylogenetic tree was constructed by Neighbor-Joining (NJ) and Kimura two pair method and topologies were evaluated by performing bootstrap analysis of 1000 sets by using MEGA 4.0 software[19]. The identified 16S rRNA sequence of UF-4 was then deposited in the NCBI GenBank Database

Selection of suitable production medium for promising epibiotic bacterium

In order to find out the suitable production medium (PM) for the enhanced antibacterial activity, UF-4 was cultured in five different production media (PM1-PM5). Before the start-up of experiment, UF-4 was enriched in ZMB at 37°C for 12h. Then 2% seed culture was transferred individually into 100 ml seawater containing five different production media *viz.* PM1 (Yeast extract-0.2%, Beef extract-0.5%, Peptone-0.2%, Dextrose-2%, $MgSO_4$-0.01% and $C_2H_3NaO_2$-0.01%); PM2 (Starch-1.5%, Soybean meal-1%, Yeast extract-0.5%, NaCl-0.25%, $CaCO_3$-0.2%); PM3 (Starch-0.3%, Yeast extract- 0.2% and Peptone-0.2%); PM4 (Peptone-0.3%, Yeast extract-0.15%, Na_2HPO_4-0.1% and $FeSO_4$-0.01%) and PM5 (Glycerol-1%, Sucrose-1%, Yeast extract-0.25% and Peptone-0.2%) at pH 7 and then incubated at 37°C for 72h in a rotatory shaker (120 rpm) as described by Ramasubburayan[20]. The culture broth was then centrifuged at 10000 rpm for 15 min and then filtered in 0.22 µm membrane filter for the complete removal of bacterial cells. The culture free supernatants (CFS) were then checked individually for their significant bioactivity against fouling bacterial strains through agar well diffusion method.

Antibacterial activity

Antibacterial activity of CFS of UF-4 was determined by agar well diffusion method. Briefly, overnight cultures of test fouling bacterial strains were prepared and aseptically spreaded over Muller Hinton Agar (MHA) plates using sterile cotton swabs. Thereafter wells of 6 mm diameter were punched over MHA plates using a sterile gel

puncher and filled with 100 µl of CFS. The plates were then incubated at 37°C for 24h. The growth inhibitory activity in terms of zone of inhibition (mm) formed around each well was measured and recorded. The assay was carried out in triplicates.

Optimization of various culture conditions for enhanced antibacterial activity

Effect of different carbon and nitrogen sources

Based on the screening results of different PM's on antibacterial activity, the prominent result rendering production medium (PM1) was selected and used for optimization study. To investigate the effect of carbon sources on antibacterial activity, various carbon sources such as fructose, galactose, lactose, maltose, mannitol and xylose were tested individually by replacing dextrose present in PM1 at 2% concentration each. Medium devoid of any of the above carbon source (MCS) was used as control. Likewise to find out the influence of nitrogen sources on antibacterial activity, the PM1 was individually substituted with various organic nitrogen sources such as beef extract, peptone, meat extract and yeast extract and also various inorganic nitrogen sources such as ammonium nitrate (NH_4NO_3), ammonium hydrogen carbonate [$(NH_4)HCO_3$], ammonium sulphate [$(NH_4)_2SO_4$)] and potassium chloride (KCl) at 0.5% concentration each. Medium without any of the above nitrogen source (MNS) was taken as control. The whole set up was incubated at 37°C for 72 h in a rotatory shaker (120 rpm). Then CFS of all samples was then tested against fouling bacterial strains through agar well diffusion method.

Effect of different pH and temperature

The effect of pH on antibacterial activity was determined by adjusting the PM1 at nine different pH values ranged from 3.0 to 12.0 before inoculating UF-4. The entire experimental media were incubated at 37°C for 72h in a rotatory shaker (120 rpm). Similarly, to test the effect of temperature on antibacterial activity, UF-4 was grown in PM1 at different various temperature ranged from 10 to 50°C for 72 h in a rotatory shaker (120 rpm). The CFS of each sample was then tested against fouling bacterial strains through agar well diffusion method.

Effect of different inoculum size (%)

Effect of inoculum size (%) on the antibacterial activity was determined by growing UF-4 in PM1 at varying level of inoculum sizes such as 1, 2, 3, 4 and 5%. Then the whole set up was incubated at 37ºC for 72 h in a rotatory shaker (120 x g). The CFS from each sample was then tested against fouling bacterial strains through agar well diffusion method.

Statistical analysis

The results of present study were subjected to Two-way Analysis of Variance using SPSS-16 version, SPSS Inc, Chicago, USA and was expressed at varying significant levels ($P < 0.05$; $P < 0.01$; $P < 0.001$ and $P > 0.05$).

RESULTS

Percentage of epibiotic bacterial strains isolated from selected seaweeds

In the present investigation, based on the colony morphology 21 epibiotic bacterial strains were isolated. Of the 21 isolates, maximum of 12 isolates (57%) were from the seaweed *C.*

Table 1. Inhibitory activity of isolated epibiotic bacterial strains through cross-streak method

Strain code of seaweed epibacterial isolates	Relative Inhibition of test fouling bacterial strains					
	P. aeruginosa	*A. hydrophila*	*P. agglomerans*	*A. fischeri*	*V.alginolyticus*	*S. fleneri*
CA-3	30	40	20	-	-	-
CA-7	-	-	20	20	40	-
CA-10	30	40	-	20	-	-
UF-1	10	10	-	-	20	30
UF-4	40	85	85	50	50	50
UF-7	-	25	30	40	30	-
UF-9	50	25	-	30	25	30

10 -20% = Low activity; 21 - 80% = Moderate activity; 81 – 100% = High activity; - = No activity

antennina; whereas, minimum of 9 isolates (43%) were from *U. fasciata*. The isolated epibiotic bacterial strains were individually designated (*C. antennina*: CA-1 to CA-12 and *U. fasciata*: UF-1 to UF-9) and used for further study.

Primary screening of epibiotic bacterial strains

Primary screening results showed that, out of 21 epibiotic bacterial strains, seven (33%) displayed antagonistic activity; whereas other tested epibiotic bacterial strains (67%) did not show any significant inhibitory activity; hence their results were not represented in Table 1. Among the seven epibiotic bacterial strains, UF-4 was found to be the most active strain, which strongly inhibited the growth of all the tested fouling bacterial strains. It registered the highest antagonistic activity (85%) against *A. hydrophila*, *P. agglomerans* and moderate activity (40-50%)

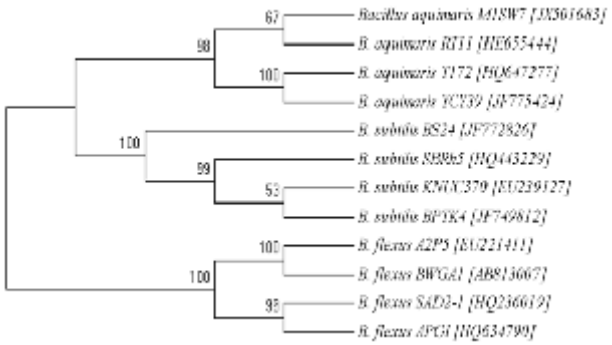

Fig. 1. Phylogenetic position of candidate bacterium *Bacillus flexus* APGI with closely related sequences retrieved from NCBI GenBank

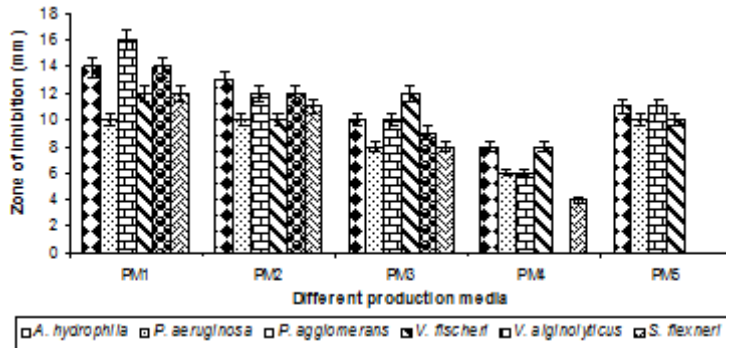

Fig. 2. Effect of different production media on growth inhibition of fouling bacterial strains

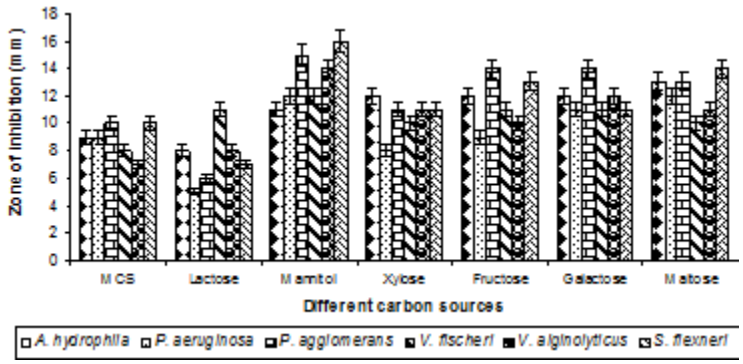

Fig. 3. Effect of different carbon sources on growth inhibition of fouling bacterial strains

against *P. aeruginosa*, *A. fischeri*, *V. alginolyticus* and *S. flexneri*. Next to this, epibiotic bacterium UF-9 exhibited moderate antagonistic activity (25-50%) against all the fouling bacterial strains except against *P. agglomerans*. Epibiotic bacterial strains such as CA-3, CA-7, CA-10, UF-1 and UF-7 showed low to moderate activity against tested fouling bacterial strains.

Identification of active epibiotic bacterium through 16S rRNA analysis

The 16S rRNA gene sequence analysis (1450bp) of UF-4 revealed that, it had highest sequence similarity (99%) with *Bacillus flexus* SAD-1 (Accession number: HQ236019) in the NCBI Database. Hence, the 16S rRNA gene sequence of UF-4 was deposited in the NCBI GenBank under the name of *Bacillus flexus* APGI with the accession number: HQ634790 (Fig. 1).

Selection of suitable production medium

Fig. 2 shows the result on the effect of different production medium on growth inhibition of fouling bacterial strains. It inferred that, *B. flexus* APGI cultivated in PM1 had exhibited broad spectrum antibacterial activity (100%) against the tested fouling bacterial strains. It exhibited maximum growth inhibitory activity of 16.0 ± 0.57mm against *P. agglomerans* and minimum of 10.0 ± 0.20mm against *P. aeruginosa*. The candidate bacterial strain grown in PM2 and PM3 had also recorded 100% growth inhibitory activity; nevertheless they displayed a consistent reduction in zone of inhibition ranged from 10.0 ± 0.18 to 13.0 ± 0.42 and 8.0 ± 0.15 to 12.0 ± 0.42mm, respectively. At last, *B. flexus* APGI cultivated in PM4 and PM5 attributed 83.3 and 66.6% antagonistic activity with least level of zone of inhibition (4.0 to 8.0 and 10.0 to 11.0mm). Two-way analysis of variance revealed that, the data on antibacterial activity as a function of variation between different production media (F_{PM}) as well as variation between fouling bacterial strains (F_F) were statistically significant (F_{PM} = 8.234463; P<0.05 and F_F = 2.614878; P<0.05). Based on the above result it was found that, *B. flexus* APGI had grown well in PM1 and showed promising fouling bacterial growth inhibitory activity; hence this medium was selected for further optimization study.

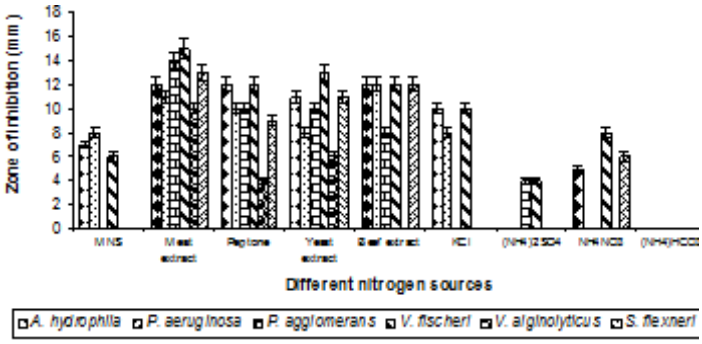

Fig. 4. Effect of different nitrogen sources on growth inhibition of fouling bacterial strains

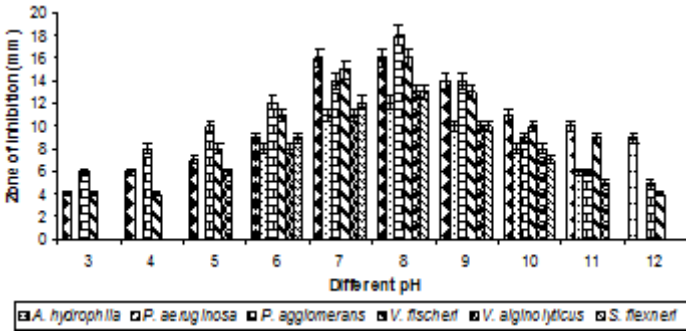

Fig. 5. Effect of different media pH on growth inhibition of fouling bacterial strains

Effect of different carbon sources on growth inhibition of fouling bacterial strains

The result on the effect of different carbon sources on antibacterial activity of *B. flexus* APGI is represented in Fig. 3. PM1 supplemented with almost all the carbon sources exhibited 100% growth inhibitory activity. However, PM1 containing mannitol showed pronounced fouling bacterial growth inhibitory activity. It recorded maximum antagonistic activity of 16.0 ± 0.64mm against *S. flexneri* and minimum of 11.0 ± 0.33mm against *A. hydrophila*. The next best carbon sources in the order of merit were: galactose > maltose > fructose which expressed the zone of inhibition ranged from 11.0 ± 0.18 to 14.0 ± 0.47; 10.0 ± 0.16 to 14.0 ± 0.60 and 9.0 ± 0.11 to 14.0 ± 0.49mm, respectively. PM1 supplemented with the carbon sources such as xylose and lactose recorded comparatively lesser bioactivity (8.0 ± 0.16 to 12.0 ± 0.65 and 5.0 ± 0.20 to 11.0 ± 0.50mm) than the other tested carbon sources. Two-way ANOVA test indicated that, the data on antibacterial activity as a function of variation between different carbon sources (F_C) as well as variation between different fouling bacterial strains (F_F) were statistically significant (F_C = 11.70429; P<0.01 and F_F = 2.75395; P<0.05).

Effect of different nitrogen sources on growth inhibition of fouling bacterial strains

Among the various organic nitrogen sources tested, PM1 amended with meat extract showed predominant growth inhibitory activity (100%). It recorded markedly higher antagonistic activity of 15.0 ± 0.42mm (*V. fischeri*) and lesser antagonistic activity of 10.0 ± 0.23mm (*V. alginolyticus*). Besides meat extract, supplementation of yeast extract and peptone had also recorded 100% growth inhibitory activity; but they showed comparatively less zone of inhibition and were: 6.0 ± 0.15 to 13.0 ± 0.48 and 4.0 ± 0.10 to 12.0 ± 0.42mm, respectively. At last, PM1 supplemented with beef extract recorded 83.3% antagonistic activity with the zone of inhibition ranged between 8.0 ± 0.21 and 12.0 ± 0.47mm. Among the different inorganic nitrogen sources tested, PM1 supplemented with ammonium nitrate

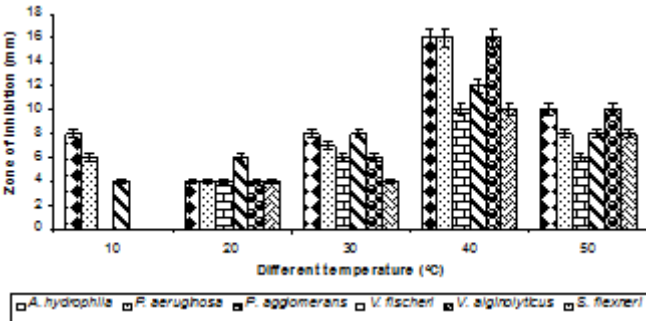

Fig. 6. Effect of different temperature (°C) on growth inhibition of fouling bacterial strains

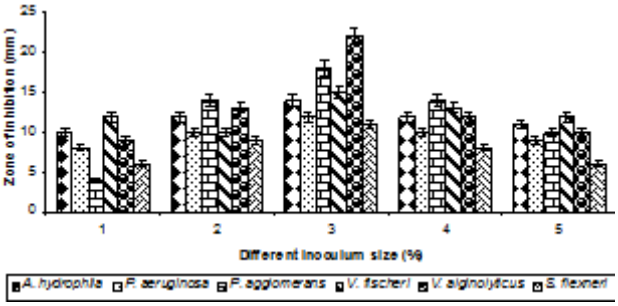

Fig. 7. Effect of different inoculum size (%) on growth inhibition of fouling bacterial strains

and potassium chloride exhibited maximum of 50% antagonistic activity and related zone of inhibition was ranged from 5.0 ± 0.12 to 8.0 ± 0.20 and from 8.0 ± 0.33 to 10.0 ± 0.46mm, respectively. PM1 supplemented with ammonium sulphate registered marginally lesser growth inhibitory activity of 33.3%; whereas, medium with ammonium hydrogen carbonate showed a negative impact on growth inhibitory activity. PM1 without any nitrogen source supported least level of zone of inhibition, which were ranged between 6.0 ± 0.10 and 8.0 ± 0.18mm (Fig. 4). Two-way analysis of variance for the data on antibacterial activity as a function of different nitrogen sources (F_N) as well as fouling bacterial strains (F_F) indicated that the variance due to the tested variables were statistically significant (F_N = 17.34992; P<0.01 and F_F = 7.133407; P<0.05).

Effect of different pH on growth inhibition of fouling bacterial strains

The effect of different pH on antibacterial activity by *B. flexus* APGI is shown in Fig. 5. Results showed significant variation in antibacterial activity when *B. flexus* APGI was cultivated under different pH levels (3.0 to 12.0). It was noticed that, at initial pH (3.0), the antibacterial activity recorded by *B. flexus* APGI was too low (4.0 ± 0.10 to 6.0 ± 0.16mm). As the pH of media increased, the antimicrobial activity was also showed linear increase and reached its maximum at pH 8.0. At this pH, it recorded 100% growth inhibitory activity with higher bioactivity of 18.0 ± 0.69mm against *P. agglomerans* and lower bioactivity of 12.0 ± 0.42mm against *P. aeruginosa*. Similarly, *B. flexus* APGI grown at pH9 had also expressed obvious inhibitory zones and was ranged between 10.0 ± 0.37 and 14.0 ± 0.53mm. Nevertheless, further increase in media pH i.e., from 10.0 to 12.0 resulted in decrease of antimicrobial activity. ANOVA (Two-way) test indicated that, the data on antibacterial activity as a function of variation due to different pH (F_{pH}) as well as variation due to different fouling bacterial strains (F_F) were statistically significant (F_{pH} = 47.08351; P<0.001 and F_F = 22.88549; P< 0.01).

Effect of different temperature on growth inhibition of fouling bacterial strains

The result on the effect of different temperatures on antibacterial activity by *B. flexus* APGI is shown in Fig.6. Among the tested temperatures, the candidate bacterial strain *B. flexus* APGI was grown well at 40ºC and attributed broad spectrum antibacterial activity (100%) with the maximum zone of inhibition of 16.0mm each against *A. hydrophila*, *P. aeruginosa* and *V. alginolyticus*; whereas, it displayed minimum zone of 10.0mm against *P. agglomerans*. Further at lower or higher incubation temperature a consistent reduction in growth inhibitory activity was noticed. For instance, *B. flexus* APGI grown at 50ºC had registered moderate growth inhibitory activity and it ranged between 6.0 ± 0.18 and 10.0 ± 0.38mm. On the other hand, *B. flexus* APGI grown at lower temperatures (10, 20 and 30ºC) registered much lesser zone of inhibition, which was ranged between 4.0 ± 0.13 and 8.0 ± 0.24mm, respectively. Two-way analysis of variance test revealed that the data on antibacterial activity as a function of variation due to different temperatures (F_T) as well due to different fouling bacterial strains (F_F) were statistically significant (F_T = 28.76471; P<0.001 and F_F = 3.157354; P<0.05).

Effect of different inoculum size (%) on growth inhibition of fouling bacterial strains

Result on the antibacterial activity by varying volume of *B. flexus* APGI inoculum size (%) inferred a gradual increase in growth inhibitory activity with respect to increase in inoculum size from 1 to 3% (Fig. 7). For instance, medium inoculated with 1 and 2% inoculum size exhibited growth inhibitory activity ranged from 4.0 ± 0.10 to 12.0 ± 0.34 and 9.0 ± 0.31 to 13.0 ± 0.42mm, respectively. PM1 inoculated with 3% inoculum size evidenced higher antagonistic activity and registered the maximum zone of inhibition of 22.0 ± 1.27mm against *V. alginolyticus* and minimum zone of 11.0 ± 0.46mm against *S. flexneri*. Further increase in inoculum size (4 and 5%) did not show notable increase in antagonistic activity. Analysis of variance (Two-way) for the data on antibacterial activity as a function of variation due to different inoculum size (F_I) as well as different fouling bacterial strains (F_F) were statistically significant (F_I = 9.064471; P<0.05 and F_F = 3.918711; P< 0.05).

DISCUSSION

In marine environment, certain seaweeds are fouled heavily; while some others remain relatively free from fouling. Lack of fouling on the surface of seaweeds primarily reflects the ability

of epibiotic bacterial strains to produce fouling inhibitory compounds.

In the present investigation, inhibitory activity of the epibiotic bacterial strains was determined through cross streak method. Cross streak is one of the more reliable methods to find out active strains based on their varying degree of inhibitory activity. This result showed that, out of 21 epibiotic bacterial strains 33% (seven) retained inhibitory activity against one or more fouling bacterial strains. This agrees with the results of Ma *et al.*[21], who reported that, 34% of the epibiotic bacterial strains isolated from the surfaces of seaweeds and marine invertebrates displayed promising antibacterial activity against fouling bacteria isolated from net-cages in coastal waters in Dalian. Likewise Kanagasabapathy *et al.*[13] inferred that 20% of epibiotic bacterial strains isolated from the surface of various brown seaweeds had shown significant growth inhibition on fouling bacterial strains. In the present study, among the seven epibiotic bacterial strains tested, UF-4 was the only bacterium which showed strongest inhibitory activity; nevertheless other epibiotic bacterial strains showed low to moderate inhibitory activity. The successful growth inhibitory activity rendered by UF-4 evidently substantiated its antifouling potentials. The inhibitory activity shown by UF-4 may be due to the release of polar antimicrobial substance from the surface of UF-4 into media that has significantly inhibited the growth of test fouling bacterial strains. Results of the present study corroborated by the findings of Strahl *et al.*[14], who demonstrated that water soluble compounds released by the marine brittle star (*Amphipholis gracillima*) associated bacteria BE37 had diffused into the medium and inhibited the growth of the test bacterial strains at a significant distance from the BE streak and produced a clearly defined zone of inhibition when tested through cross streak. Thus the observed results infer that, the growth inhibitory activity was mainly because of release of active metabolites into the media.

In the present study, the 16S rRNA sequence of most active epibiotic bacterium UF-4 showed highest similarity (99%) with *Bacillus flexus* SAD2-1 (Accession number-HQ23619). Several studies that have been carried out earlier substantiated the occurrence of various species of the genus *Bacillus* in marine habitats and its potential to control growth of fouling strains. For example, bacterial strains belonging to the genus *Bacillus* spp. such as *B. pumilus, B. licheniformis, B. subtilis, B. mojavensis, B. firmus* isolated from various seaweeds *viz. Fucus serratus, Laminaria* sp., *Pulmaria palmate, U. lactuca* and turtle grass *Thalassia testudinum* were found to exhibit promising antifouling activity against fouling bacterial strains and diatoms[22,9,23]. To the best of our knowledge, this is the first report which demonstrates the antifouling efficacy of marine epibiotic bacteria *B. flexus* APGI.

It is well known that designing a suitable culture medium for production of bioactive metabolite is highly tedious and requires much knowledge on cultivation parameters. Among the five different PM's tested, PM1 was found to be the most suitable medium for the production of antimicrobial substance. It showed remarkable inhibitory activity with higher level of zone of inhibition (10.0 and 16.0mm) than other PM's tested (PM2 > PM3 > PM4 > PM5). Higher inhibitory activity attributed by *B. flexus* APGI in PM1 clearly indicated the suitability of media components towards enhancing growth and synthesis of biologically active secondary metabolites. Most often, variations in media composition may show significant alteration in zone of inhibition. This observation supports the work of Xiong *et al.*[24], who investigated antibiotic and antifouling compound production by marine derived fungus *Cladosporium* sp. using eleven different culture media and showed media dependent antibacterial activity. Krassilnikov[25] stated that, the ability of microorganisms to produce bioactive compound is not a fixed property; whereas, it can either be greatly increased or completely lost depending on the conditions in which they are grown.

Carbon is one of the most important sources in PM, which acts as sources of precursor and energy for synthesizing both primary and secondary metabolites by microorganisms. In general carbon source required for achieving maximal growth and antagonistic activity seems to be differed among bacterial strains. In the present study, effect of different carbon sources on antibacterial activity revealed that, PM1 amended with mannitol recorded higher degree of antagonistic activity. In accordance to the result

of the present study, Todorova and Kozhuharova[26] reported that, *B. subtilis* has effectively assimilated mannitol as the best carbon source and displayed promising antagonistic activity against both gram positive and gram negative bacterial strains. Rezuanul Islam *et al.*[27] observed a similar result, wherein medium substituted with mannitol had significantly enhanced the growth and bioactivity of *B. subtilis* subsp. *subtilis* C9 against fungus *Rhizoctonia solani*. In the present study, next to mannitol, PM1 amended with galactose, maltose and fructose also supported consistent increase in bioactivity; whilst medium with xylose and lactose had shown substantial reduction in antagonistic activity. The findings of the present study are also correlated with the work of El-Banna[28] and Joshi *et al.*[29], who had reported that antagonistic activity of *Corynebacterium xerosis* NB-2 and *Bacillus* sp. was not significantly influenced when medium was amended with lactose and xylose as carbon source. Thus the present study clearly manifested that, *B. flexus* APGI require mannitol as sole carbon source for its better growth and antimicrobial metabolite production.

Nitrogen is the second major source which supports growth and various metabolic activities of microorganisms, because it involves in the synthesis of cell structural and functional proteins. Among various organic nitrogen sources tested, PM1 supplied with meat extract was found to display superior antagonistic activity against the test fouling bacterial strains. Besides meat extract, PM1 amended with yeast extract and peptone also registered maximum antagonistic activity. This finding is in agreement with the result of Nishanth Kumar *et al.*[30], who reported that, meat extract and yeast extract were the best nitrogen sources for *Bacillus* sp. towards achieving maximum antibacterial activity. In the present study, of various inorganic nitrogen sources tested, PM1 supplemented with ammonium nitrate and potassium chloride exhibited maximum antagonistic activity and it ranged from 5.0 to 8.0 and 8.0 to 10.0mm, respectively. Nevertheless, the other tested inorganic nitrogen sources were not supported the candidate bacterial strain in exhibiting bioactivity. In accordance to this result Abushady *et al.*[31] reported that, medium supplemented with ammonium nitrate and sodium nitrate had profoundly increased the production of bioactive lipopeptide surfactin by *B. subtilis*. Thus the result on the effect of different nitrogen sources inferred that, *B. flexus* APGI had the preference of utilizing organic nitrogen sources better than inorganic nitrogen sources.

The pH and temperature of PM is the most important physical factors which affect the growth and biosynthesis of secondary metabolites during fermentation. In the present study, result on the effect of different media pH on antibacterial activity by *B. flexus* APGI showed a linear increase in growth inhibitory activity, which ranged from 4.0 to 6.0 and from 11.0 to 16.0mm with respect to increase in pH from 3.0 to 7.0. At pH 8.0, the maximum antagonistic activity was noticed with the zone of inhibition range of 12.0 and 18.0mm. However, further increase in media pH beyond 8.0 showed a consistent reduction in growth inhibitory activity. The observed results indicated that, *B. flexus* APGI require pH 8.0 for its optimum growth and antimicrobial compound production. Also it was noticed in the present study that, neutral pH (7.0) had also supported consistent increase in antagonistic activity. Similar to the present result, Muaaz *et al.*[32] inferred that, *B. subtilis* had shown higher antimicrobial activity, when it was grown between pH 7.0 and 8.0. This also agrees with the result of Awais *et al.*[33], who reported pH 8.0 as optimum for production of antimicrobial metabolite by *Bacillus* sp.

Concerning the effect of different incubation temperature on antibacterial activity, it was noticed that *B. flexus* APGI was grown well at 40°C and resulted better inhibitory zone range of 10.0 ± 0.33 to 16.0 ± 0.59mm. Nevertheless, at temperatures higher or lower than 40°C registered a reduction in zone of inhibition. Lesser inhibitory activity recorded by *B. flexus* APGI at lower (10, 20 and 30ºC) temperatures may be due to retardation in metabolic activity; whereas at higher temperature (50ºC) it may be due to inactivation of bacteria. Thus this study indicated that, *B. flexus* APGI requires 40°C as its optimum temperature for enhancing growth and secondary metabolite biosynthesis. Result of the present study corroborated by the findings of Kumar *et al.*[12] and Joshi *et al.*[29], who reported that *Bacillus* sp. grown at 40ºC had attributed wide spectrum of antagonistic activity against the tested pathogenic bacterial strains.

Inoculum plays an important role in enhancing production of secondary metabolites during fermentation process. In the present study, result on the effect of different inoculum size (%) of *B. flexus* APGI showed significant variation on antibacterial activity. Of the different inoculum size tested, medium with 3% inoculum was found to be optimum for exhibiting maximum antibacterial activity. Further increase (4 and 5%) or decrease (1 and 2%) in inoculum size resulted in gradual reduction in antibacterial activity. Often, it is highly essential to provide optimum inoculum size to PM for achieving maximal bioactivity. Supporting the result of the present study, Abushady *et al.*[31] reported that the production of bioactive lipopeptide surfactin A from *B. subtilis* was higher at 2% inoculum size which was found to be optimum for maximum yield. The findings of the present result fall in line with the observations of Mudgetti[34], who pointed out that, lesser inoculum size may often result in insufficient biomass, causing reduced product formation; on the other hand, higher inoculum size may produce too much biomass leading to poor product formation. Results of the present study clearly emphasized that, the epibiotic bacterium *B. flexus* APGI isolated from the surface of seaweed *U. fasciata* could effectively inhibit the growth of fouling bacterial strains. The optimization process significantly enhanced the antibacterial property of *B. flexus* APGI and thus substantiated existence of promising antibacterial compounds within CFS. Also it indirectly indicates the defensive role of *B. flexus* APGI against settlement of potential competitors on the seaweed surface itself. Further studies will provide noteworthy information on the utilization of this epibiotic bacterium as a potent source of novel antifouling agent towards conserving marine organisms from the toxic effect of biocides.

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