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Research article

Antibiofilm activity of marine endophytic actinomycetes compound isolated from mangrove plant *Rhizophora mucronata,* Muthupet Mangrove Region, Tamil Nadu, India.

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Abstract

The antibiofilm activity of endophytic actinomycetes (EA) was isolated from mangrove plant of Rhizophora mucronata, Muthupet Mangrove region, South East Coast of Tamil Nadu, India. After surface sterilization, the leaves of the plants were ground by mortar and pestle for serial dilution method. The serial dilution of the mixed samples were added on the actinomycetes isolation agar (AIA) plate and spread on agar medium. After, the active EA strain and their activity were performed against biofilm forming pathogens. Further, the biofilm quantification was detected by using 96-well polystyrene plate. The antibiofilm potential of active EA strain was proved by using congo red agar plate method. Totally 50 strains of EA were isolated from internal tissue of the Rhizophora mucronata. The isolated strains were designated as endophytes by the validation process. Among the 50 strains, 5 strains were produced better antibiofilm ability against biofilm forming Pseudomonas aeruginosa (P. aeruginosa) and Klebsiella pneumoniae (K. pneumoniae). The complete biofilm inhibition was showed at the 100 µg/mL concentration and the exopolysaccharide production was detected in CRA plate after the EA 10 extract and named as NMS. Finally, the overall result was proved that the isolated EA NMS 10 strain has more antibiofilm activity.
Keywords: Biofilm, Mangrove system, Endophytic actinomycetes, biofilm inhibition assay, Congo red agar.

Introduction

Biofilms are highly complex structures encapsulated in an extracellular polymeric substance (EPS) of polysaccharides, protein and DNA (Anjugam et al. 2018). This complexity of biofilms makes the bacteria to adhere on medical devices or injured tissue for becoming persistent infections (Rajivgandhi et al. 2018). The bacterial biofilm can risen up to 1000 fold high resistant to antibiotics and biocide as compared to their planktonic counterparts (Rajivgandhi et al. 2018). In the biofilm formation, EPS play an important role against antibiotics due to reduced diffusion and penetration of antibiotics; it is in the form of acidic pH and lower oxygen level. All these factors performed are against the action of antibiotics. Almost, 80 % of the bacterial biomass as biofilm led to multi drug resistant (MDR) effect in bacteria (Rajivgandhi et al. 2014). Both gram positive (GP) and gram negative (GN) bacteria form biofilms including S*taphylococcus aureus*, *Escherichia coli*, *Proteus mirabilis, P. aeruginosa and K. pneumonia* and are often implicated in UTI (Delcaru et al. 2016). *P. mirabilis* and *E. coli* are the most frequent biofilm producing bacteria in UTIs (Mihankhah et al. 2017). The biofilm producing bacteria cause UTIs and linked with UTI obstruction, blockage of urinary catheters, bladder, kidney stone formation, and bacteriuria which may produce the most recurrent infection; it can be made by one or many bacteria which show high antimicrobial tolerance (Matsumoto et al. 2004).These bacteria also connected to all types of UTIs like catheter association, where it can affect recurrent including crystalline biofilms, urinary stones, pyelonephritis, and septicemia (Ranjbar-Omid et al. 2015). The pathogenicity was enhanced by various factors including quorum-sensing, urease, protease and haemolysins. Particularly, the important virulence factor quorum sensing (QS) plays important role in biofilm formation by communication and degradation

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(Hassan et al. 2016). Therefore, there is an urgent need to detect for effective, safe and affordable antimicrobial agents to overcome this problem.

Few years ago, marine actinomycetes and its derivatives are being applied as antibiofilm ability for infectious microbes (Houssam et al. 2015). Most researchers reported, marine actinomycetes had efficient antibiofilm activity against GP and GN (Ranjan et al. 2017). Actinomycetes are Gram positive, filamentous, spore forming bacteria with cell wall containing L-L diaminopimelic acid and with high G + C content (57–75%) in their DNA (Goudjal et al. 2014). In particular, the overlooked organisms is EA that inhabit within the tissues of higher plants and have interest on more concentration in recent years by synthesis of excellent antibiotics (Ganesan et al. 2017; Abdelfattah et al. 2016). Thus, the EA compounds act as alternatives to combat biofilm forming pathogens. The *Rhizophora mucronata* is a dominant mangrove plant used in modern scientific research has very low research. The aim of this study was to isolate EA from young, healthy mangrove plant and bring their potential activity against biofilm producing pathogens.

Materials and methods

Location and collection of plant samples

The mangrove plant *Rhizophora mucronata* was collected from Muthupet Mangrove region, (Lat.10°20'N & Long.79°35' E), which is the largest natural lake (360 KM) located in South East Coast, Tamil Nadu, India, where a unique ecological condition prevails. The collected plant was immediately packed with zip lock cover and maintained in ice box. The plant was taken to laboratory immediately and stored in ice box at 4 °C. The plant sample was clearly washed with tap water and followed by distilled water for removal of contamination and any other free-floating organisms. The 70 % ethanol was also used for washing to remove the epiphytes.

Culture media

Actinomycete isolation agar (AIA): Sodium caseinate, 2.0 g; L-Aspargine, 0.10 g; Sodium propionate, 4.0 g; Dipotassium phosphate, 0.5 g; Magnesium sulphate, 0.1 g; Ferrous sulphate, 0.001 g and Agar 20.0 g; distilled water, 1,000 ml; pH 7.0.Starch casein agar (SCA): Soluble starch, 10.0 g; casein,0.3 g; KNO3, 2.0 g; NaCl, 2.0 g; K2HPO4, 2.0 g;MgSO4.7H2O, 0.05 g; CaCO3, 0.02 g; FeSO4.7H2O,0.01 g; Agar, 20.0 g; distilled water, 1,000 ml; pH 7.0. Muller Hinton HiVeg™ agar No.2 (MHA): Hydrolysate, 17.50; HiVeg infusion, 2.00, Starch, soluble, 1.50; Agar 17, distilled water, 1,000 ml was used as production medium.

Test bacteria

The biofilm forming bacterial pathogens *P. aeruginosa* and *K. pneumonia* were used for this study and the bacterial cultures were obtained from Department of Marine Science, Bharathidasan University, Tiruchirapalli. All the media and chemicals were procured from Himedia Laboratory, Mumbai, India.

Isolation of endophytic actinomycetes

The EA was isolated from mangrove plant *Rhizophora mucronata* by plate inoculation method using serial dilution technique in AIA medium by previous report of (Rajivgandhi et al. 2018). Briefly, the collected plant leaves were grinded by mortar and pestle. After well ground, 1 g of the sample was mixed with 100 ml of sterile distilled water. Then, the samples were mixed well. After, 1 ml of the sample was taken and serially diluted upto 10^{-1} to 10^{-6} in sterile distilled water. All the plates were incubated at 28°C for 7 days. The pure culture of the isolates was obtained by repeated streaking on SCA plates. The pure cultures were transferred to fresh SCA slants and preserved at 4°C for further use.

Validation of endophytic actinomycetes

The isolated actinomycete strains whether identified as endophytes or not were validated by surface sterilization method (Rajivgandhi et al. 2018). The plant samples were thoroughly washed with tap water, and cut with small pieces of leaves, twigs, and buds (approximately 10 mm length) by using sterile scale pale. Then, the small pieces of plant was surface sterilized by using 70% ethanol for 2 min and followed by 1.3 M sodium hypochlorite for 35 min, after washed with 70% ethanol for 1 min. After completion of chemical sterilization, these surface sterilized pieces of plant were rinsed thoroughly in sterile double distilled water for 2 min, to remove unwanted surface sterilants. After, all the tissue samples were air dried for 1 min. The samples were rinsed thoroughly with double distilled water for 2 min, to remove unwanted surface sterilants. After, all the tissue samples were air dried for 1 min. For the proper sterilization, the last wash water was spread on to the MHA plates. For the parallel experiments, the unsterilized tissue pieces of plant were also placed on the AIA plate and incubated under the same conditions. The emerging colonies of actinomycetes without any contamination from surface treated tissue fragment was indicated as positive endophytes, fungal growth contamination of the unsterilized tissue of plant pieces as negative result were noted (differentiated morphologically by both macroscopic and microscopic evaluation) (Sangkanu et al. 2017).

Extraction and antibacterial activity of isolated EA

The isolated EA strains were taken in 1000 ml Erlenmeyer flasks containing 500 ml of starch casein broth (SCB) and incubated at 28°C for 20 days in a shaker (200 rpm). After 20 days, the

culture broth was centrifuged at 10,000 rpm for 30 min to eliminate the biomass. After recovering the culture filtrate, equal volume of various solvents n-butanol, ethyl acetate, dichloromethane and alcohol were added in the culture filtrate containing separating funnel. Then, the separating funnel was shaken vigorously for extracting the active metabolites. The experiment was performed in triplicate. The organic phase was kept in hot air oven at 45 °C till dry using rotary evaporator and done in vacuum. After the compound is finely powdered, the sample was diluted in dimethyl sulphoxide (DMSO) and stored in 4 °C for further biological activity. Further, the recovered compounds were tested against biofilm forming *P. aeruginosa* and *K. pneumoniae* by agar well diffusion method. Briefly, the primary screening of antibacterial activity of isolated EA was performed by agar well diffusion method (Ballav et al. 2015). The biofilm forming bacterial pathogens *P. aeruginosa* and *K. pneumonia* were used in this study. Briefly, the selected biofilm forming bacterial pathogens were swabbed on to the MHA plates. The wells were cut into the plates by using well borer. Then various concentration of the compound was added into the wells. All the plates were incubated at 37ºC for 24 h. After, incubation, the zone of inhibition was measured and noted.

Biofilm formation assay in 24-well polystyrene plates

The identified EA compound was used to determine the disruption of biofilm formation in *P. aeruginosa* and *K. pneumonia* by 24-well polystyrene plates (Rajivgandhi et al. 2018). Briefly, the staled 24h cultures of *P. aeruginosa* and *K. pneumonia* were inoculated in fresh TSB in the presence (treated) and absence (untreated) of the EA compound (10-150 µg/ml). The plates were incubated at 37ºC for 24hrs. After 24hrs, the plates were washed with sterile phosphate buffer saline (PBS) to eradicate the planktonic cells and allowed to air dry for staining. The 0.4% crystal violet solution (w/v) was used for identification of biofilm formation. After staining, the unstained dye was discarded, and the wells were rinsed twice with deionized water and allowed to air dry. Finally, 1 ml of absolute ethanol was added to each well. The optical density was determined at 570nm, and percentage of biofilm inhibition was calculated using the following formula:

$$\text{Percentage of inhibition} = \frac{\text{(Control OD570 nm-Test OD570 nm)}}{\text{Control OD570nm}} \times 100$$

The lowest concentration of the extract disrupts the growth of biofilm formation was referred as biofilm inhibitory concentration (BIC). The determination of visible reduction and disruption of biofilm formation by lowest concentration was analyzed and the results were compared with control wells reading at same OD570nm value. The Dist.H_2O containing well referred as blank. The determination of BIC was observed by both spectroscopic quantification and microscopic visualization.

Congo red agar plate method

The antibiofilm effect of isolated EA compound against test and reference strains (MTCC culture) of the bacteria were screened for the qualitative slime production by CRA plate assay (Neupane et al. 2016). The selected *P. aeruginosa* and *K. pneumoniae* and non-biofilm forming MTCC culture *P. aeruginosa* 424 and *K. pneumoniae* 7162 were inoculated on the CRA media and incubated at 37ºC for 24h. After incubation, the black color colonies of the plates indicate positive biofilm production, whereas pink colour colonies of the plates indicate negative biofilm production. The CRA medium is composed of TSB (30 g/L), sucrose (36 g/L), agar powder (18 g/L), and Congo red dye (0.8 g/L). Congo red stain was prepared as a concentrated aqueous solution, autoclaved separately and added to the media when the agar had cooled to 55◦C.

Result

Isolation and identification of actinomycetes

A total of fifty EA were isolated from *Rhizophora mucronata* based on colony morphology and microscopic appearance. Particularly, the actinomycetes were screened based on the utilization of enriched selective media supplemented with different antimicrobial agents (antibacterial and antifungal antibiotics). After four days incubation, the white, powdery, creamy white, pigment production with spore forming colonies were observed (Figure 1a). The isolated EA strains were further streaked in International Streptomyces Projected medium (ISP-2 agar) and showed clear white color fine growth of the colony were observed (Fig. 1b). The isolated colonies were named and stored in deep freezer for further use.

Validation of endophytic actinomycetes After execution of appropriate surface sterilization, the last wash water of the SCA plates was examined day-to-day to detect the EA growth. After 4 days, the endophytic colonies which grew on the AIA plates were observed and sub-cultured into ISP-2 slants for maintain the pure culture. The selected sample finger prints of the surface sterilized tissues were proved the isolated strains were endophytes.

In parallel, to secure actual surface sterilization and isolation of endophytes, the

unsterilized samples (washed only in tap water) were showed surface contaminating organisms (Various morphology was observed both in macroscopic and microscopic analysis (Fig. 2a, b). However, the result proved that the isolated strains were endophytes and it emerged from internal tissue of the algae.

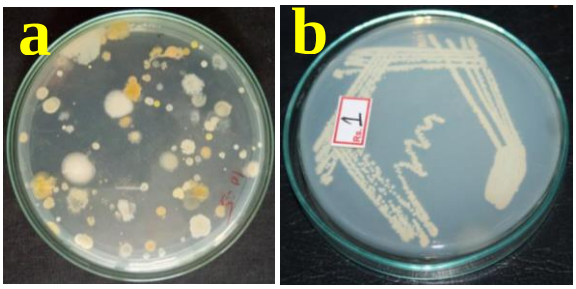

Fig.1. Isolation and identification of endophytic actinomycetes from *Rhizophora mucronata.* a) Isolation of EA from *Rhizophora mucronata* sample and b). Pure culture identification of selected EA strains in ISP-2 medium

Extraction and antibacterial activity of isolated EA

The secondary metabolites of isolated EA strains were recovered from liquid -liquid extraction. After the extraction, the crude compound performed against biofilm forming uropathogens *P. aeruginosa* and *K. pneumonia* and the result of all the strains were showed inhibition zone against both the uropathogens after 24 h incubation except NMS7, NMS 10, NMS 15, NMS 18, NMS 20. All the selected uropathoges were biofilm producers. The present study was highly focused on the actinomycete extract that had no effect on the growth of test pathogens, rather effectively controlled their virulence factors by acting upon the biofilms formed under QS.

Therefore, we have selected NMS 10 strain for this study. This study proved that the pathogens were highly sensitive to EA NMS 10 compounds. It possesses no bactericidal or bacteriostatic effect and no harm to the growth of the bacterial pathogens.

After careful observation of ethyl acetate extract from NMS 10 strains were showed no any antibacterial activity against selected biofilm forming uropathogens by agar well diffusion method. The displayed no antibacterial activity exhibited, the extract role in virulence of the pathogens (Fig. 4a, b). However, presence of some active molecules in the extract may have the capacity to influence the biofilm formation.

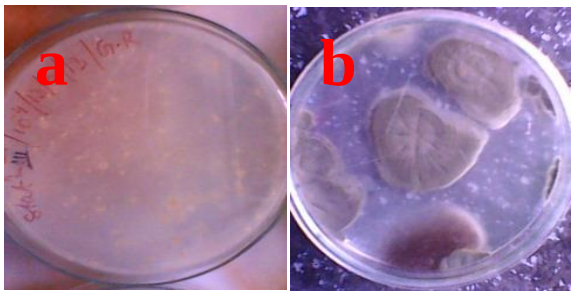

Fig. 2. Validation of EA from surface sterilized or unsterilized samples. a). The last wash water of the surface sterilization plate and b) unsterilized plant water inoculating plate

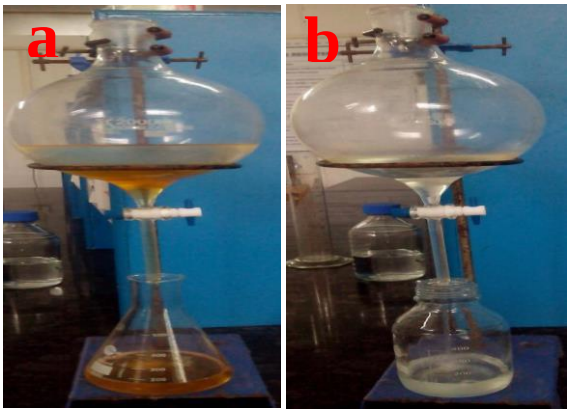

Fig.3. Extraction of antibiofilm metabolites from EA of NMS 10. a). Extraction of aqueous phase and b). Extraction of organic phase

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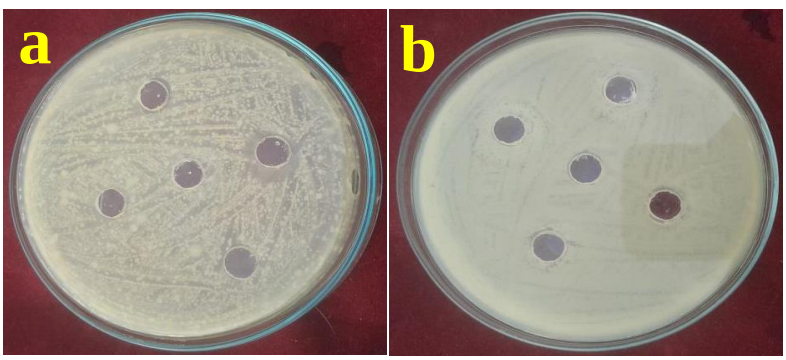

Fig. 4. Antibacterial activity of isolated EA strain NMS 10 extract against biofilm forming pathogens. a). *P. aeruginosa* and b). *K. pneumoniae*

Previously, the coral associated actinomycete extract has shown antibiofilm activity and inhibit the virulence of biofilm formation, not bacterial multiplication in MDR S. *aureus* (Rajivgandhi et al. 2014; Waturangi et al. 2016). The difference was noticed based on the actinomycetes strain and the pathogens against their antimicrobial ability (Sengupta et al. 2015). The chemical constituents of active extract from various marine sources interfere the bacterial multiplication and alter the metabolic pathway effectively, which leads to the virulence responses in bacteria (Kim et al. 2012). Hence, the result proved that the ethyl acetate extract of NMS 10 decrease the virulence in uropathogens and does not affecting the growth of cells. It has more antibiofilm effect instead of antibacterial.

Biofilm inhibition assay

The evaporated ethyl acetate extract was diluted in dimethyl sulphoxide for further biological studies. From the extract, we have chosen various concentrations (10-150) for biofilm inhibition assay against selected uropathogens and their virulence factor in terms of biofim formation was performed in 24-well microtiter plates. However, the NMS 10 crude compound showed better inhibition in (100 µg/mL) with 82% and 77 % of inhibition against both biofilms forming bacteria respectively (Fig. 5). Various concentration of the NMS 10 extract against both the biofilm forming pathogens result was summarized in Table 1. The 100 µg/mL concentration was fixed as a biofilm inhibition concentration (BIC). The result revealed that the crude compound of NMS 10 acted more potent against biofilm colonization and cell adherences. The mangrove plants have potential antibiofilm metabolites due to the different environmental condition with various nutrients (Maruthupandy et al. 2018; Naveen Kumar et al. 2018). Hence, our result confirms that biofilm was inhibited by the crude compound of NMS 10 when compared to control.

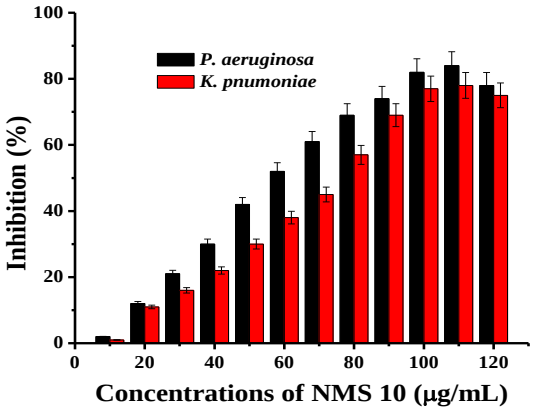

Fig.5. Inhibition of biofilm formation by NMS 10

Table. 1. Inhibition of biofilm formation by various concentration of NMS 10 extract

Various concentration of NMS 10 extract												
Pathogens	10	20	30	40	50	60	70	80	90	100	110	120
P. aeruginosa	2	12	21	30	42	52	61	69	74	82	84	78
K. pneumoniae	1	11	16	22	30	38	45	57	69	77	78	75

CRA method

The BIC of NMS 10 strain against biofilm producing *P. aeruginosa and K. pneumonia* was showed black color colonies in the CRA plates. Whereas, the pink color colonies of the CRA plates were observed against non-biofilm producing bacteria. The slime productions of the positive uropathogens were quantitatively examined on CRA method. The BIC of EA NM 10 extract interfere in slime production in both biofilm positive pathogens, therefore it exhibited black color colonies. The biofilm positive plates were showed in Figure 6 a, b. Hence, our result proved that the EA NM 10 extract has more inhibitory activity against biofilm forming bacteria.

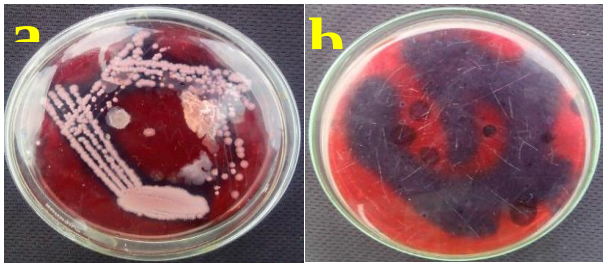

Fig. 6. Inhibition of biofilm formation by NMS 10 strain. a). Biofilm negative bacteria showed pink color colonies and b). Biofilm positive bacteria exhibited black color colonies

Conclusion

In the present study, we have confirmed that the EA NMS 10 has potent anti-biofilm activity against selected uropathogens at very low concentration. It has a increased hostile effect by damage the cell membrane of *P. aeruginosa and K. pneumoniae*. The crystal violet observation of the biofilm inhibition assay clearly indicates, the biofilm producing ability was decreased due to the effect of EA NM 10 extract. The CRA method also more evident that the extracted compound has antibiofilm metabolites. To conclude, the identified EA NM 10 strain has potential source to deal a biofilm forming uropathogens and may the source of novel drug synthesize. Further studies are needed to elucidate further biofilm inhibitory pathways and its efficacy in animal models.

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