Potential bioactive compound from marine actinomycetes against biofouling bacteria

Bavya M1, P Mohanapriya1, R Pazhanimurugan1, & R Balagurunathan2*

1Department of Microbiology, Sri Sankara Arts & Science College, Kanchipuram - 631 561, Tamil Nadu, INDIA
2Department of Microbiology, Periyar University, Salem - 636 011. Tamil Nadu, INDIA
*[E mail: r-balaguru@yahoo.com]

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In the present study, 25 bacterial isolates were recovered from fouling samples collected from Parangipettai coastal area. Based on their adherence property, three isolates were characterized and identified as Bacillus sp. (BB11), Serratia sp. (BB13) and Alteromonas sp. (BB14). Six out of 20 actinomycete extracts showed inhibition of biofouling bacteria in disc diffusion method. Strain R1 extract showed maximum inhibition against all the biofouling bacteria tested. Culture filtrate and ethyl acetate extract from strain R1 showed 13-15 mm inhibition against biofouling bacteria. Reduction in biofilm formation was also observed in cover slip method. Active antifouling compound was purified using TLC and detected by bioautography. Based on chemical screening, the active compound was tentatively identified as sugar containing molecule. Potential actinomycete strain R1 was characterized and identified as Streptomyces filamentosus (R1). Present study concluded that the marine actinomycete, Streptomyces filamentosus (R1) will be a potential source for the development of eco-friendly antifouling compounds. This will be a better alternative to the pollution causing synthetic antifoulants.

[Keywords: antifouling compound, marine actinomycetes, bioautography, Streptomyces, chemical screening]

Introduction

In the marine environment any solid surface submerged in seawater will become covered by a complex layer consisting of an organic conditioning film, microfouling and macrofouling organisms such as marine bacteria, fungi, diatoms, protozoa, barnacles, mussels, algae and tube worms. These fouling organisms cause serious technical problems by settling on ships’ hulls, power plant cooling systems, aquaculture, fishing nets, pipe line and other marine infrastructures. In most of the countries, the biofouling was prevented by using antifouling paints with organotin like tri-n-butyltin (TBT), in which copper and organonitrogen compounds. The increasing concerns about the negative effects of TBT led the International Maritime Organization (IMO) and Marine Environmental Protection Committee (MEPC) to decide to ban the usage of TBT or other substances containing tin as biocides in antifouling paints beginning in January 2008. An effective alternative to TBT is not currently available; therefore, there is an urgent demand for the development of environment- and human-friendly non-toxic antifouling compounds.

Actinomycetes are primarily recognized as a source for high value metabolites such as antibiotics, antivirals, anticancers, enzymes and recombinant products in which most of the antibiotics are of terrestrial origin. As marine environmental conditions are extensively different from terrestrial ones, the marine actinomycetes are different from those of terrestrial strains in producing different types of bioactive compounds. The unique compounds like salinosporamide and abyssomycin from marine actinomycete genera Salinispora and Verrocosispora add a new dimension to marine natural product research. Unfortunately actinomycetes in general and marine actinomycetes in particular are less/unexploited for antifouling compounds. The first step in laboratory antifouling research work is the isolation of biogenic compounds and their testing on the growth of fouling organisms. Till date, antifouling compounds are reported from marine organisms like bacteria, algae, sponges and certain plants; but such reports from marine actinomycetes are scanty. You et al., studied inhibition of Vibrio biofilm formation by a marine actinomycete A66, Streptomyces albus. An antifouling diketopiperazines was reported from
deep sea bacterium Streptomyces fungicidicus. Xu et al. isolated five structurally similar antifouling compounds from marine Streptomyces isolated from deep-sea sediments. In India, there are few reports on antifouling compounds. Unfortunately actinomycetes in general and marine actinomycetes in particular are unexploited for antifouling compounds. The present study was carried out for the isolation of antifouling compounds from marine actinomycetes.

Materials and Methods
Isolation, characterization and identification of biofouling organisms

This work was carried out during September 2008 to April 2009. Microfouling samples were scraped from boat and other marine structures around Parangipettai coastal area (Lat.11°29′N; Long. 79°47′E), Tamil Nadu and collected in sterile polythene bags. Biofouling bacteria were isolated by standard spread plate method using Zobell’s marine agar. Plating was done in duplicate and incubated at 28°C for 3-5 days. After enumeration, morphologically different bacterial colonies were selected, purified and sub-cultured on nutrient agar slants supplemented with 2% NaCl.

All the bacterial isolates were tested for adherence property by inoculating them into sterile seawater containing glass cover slips in the beaker. After 6 hours, the cover slips were removed and stained with 0.4% crystal violet to check the adherence of bacteria. Bacterial isolates which form a slimy layer on the cover slips were selected for further characterization.

Characterization and identification of biofouling bacteria

Phenotypic characteristics such as micro morphology (gram staining, capsule staining, endospore staining and motility), cultural, biochemical and salt tolerance characteristics of the selected isolate were studied by adopting standard procedures. Based on the studied phenotypic characteristics, the selected biofouling bacteria were identified at genus level.

Screening of marine actinomycetes for antifouling activity

Totally 20 crude ethyl acetate extracts were tested for antifouling activity against selected bacteria isolated from fouling samples. The ethyl acetate extracts were prepared from cell free supernatant of soybean meal medium cultured with marine actinomycetes. All the 20 marine actinomycetes were isolated from sediment samples collected from Rameswaram marine ecosystem. All the cultures are available in the actinomycetes repository at Department of Microbiology, Sri Sankara Arts & Science College, Kanchipuram. Antifouling activity of actinomycete extracts were tested against biofouling bacteria by disc diffusion method. About 0.25 mg of crude extract was impregnated on filter paper disc (5 mm diameter) and placed on nutrient agar plates inoculated with selected biofouling bacteria isolated from fouling samples. All the plates were incubated at 28°C for 24 hours and observed for zone of inhibition.

Production of antifouling from potential strain

Based on the results of preliminary screening, one potential strain was selected for further investigations. All the media used in this study were prepared in 50% filtered seawater. Spores of potential actinomycete strain were scraped from yeast extract malt extract agar (ISP2 medium) and inoculated in to 50 mL of soybean meal inoculation medium (soybean meal 1%; glucose 1%; glycerol 1%; NaCl 0.5%; pH 7.0±0.2) in 250 mL conical flask and kept in rotary shaker at 120 rpm for 48 hours at 28°C. Then 10% of inoculum was transferred into 100 mL of soybean meal production medium (soybean meal 1.5%; glucose 1.5%; glycerol 0.5%; NaCl 0.5%; CaCo3 0.10%; pH 7.0±0.2) and kept in rotary shaker at 120 rpm for 120 hours at 28°C. After fermentation, mycelium and supernatant were separated by centrifugation at 10,000 rpm for 30 minutes at 4°C. Antifouling activity of culture broth was tested by agar well diffusion method. The extra cellular compounds from culture supernatant were extracted by liquid-liquid extraction method using ethyl acetate and concentrated by evaporation. Antifouling activity of the crude extract was tested by disc diffusion method as described earlier.

Partial purification of crude compound by thin layer chromatography (TLC)

Partial purification of antifouling compound was carried out using readymade silica gel coated TLC sheets (Silica gel 60 - F254 nm). Crude extract was spotted at the bottom of TLC sheet using capillary tube and placed in a glass tank with solvent system. To find out the best solvent system to separate the crude compound, solvents such as chloroform, methanol, acetic acid, n-butanol, n-hexane and water
were used in different proportions. After running the chromatogram, the TLC plate was air dried and placed in closed iodine chamber to clearly visualize the separated compounds as spots.

Detection of active compound by bioautography

The bioautography method described by Rahalison et al. was followed for the detection of active compound separated in ready-made silica gel coated TLC sheets (10 cm × 5 cm size). Chromatogram developed, as described above, was placed over bioassay chamber (nutrient agar containing petri plate inoculated with biofouling bacteria) and incubated at 28°C for 24 hours. Active spot was indicated by zone of inhibition around the spot. The active compound was further purified by preparative TLC (20 cm × 20 cm) and tested against biofouling bacteria by disc diffusion method.

Chemical screening

Chemical screening of partially purified compound was performed by adopting the method described by Fiedler. The active spot separated from preparative TLC was spot inoculated at the bottom of silica gel coated TLC sheet and separated with chloroform: methanol (10:90) solvent system. After drying, the TLC sheets were sprayed with following chemical reagents viz., Ehrlich reagent, blue tetrazolium, vanillin - sulphuric acid, naphthoresorcin - sulphuric acid and kept in hot air oven at 120°C to visualize the colour change.

Inhibition of biofilm formation

About 10 mL of sterilized (autoclaving at 121°C for 15 mins) seawater was taken in two beakers (control and test) and each 1 mL of biofouling bacterial culture (BB11) and two clean glass slides were placed in both the beakers. In the inhibition beaker, about 200 µl of ethyl acetate extract (dissolved in 1% dimethyl sulfoxide (DMSO)) was added and closed with aluminium foil and kept for 24 hrs. Two hundred µl of 1% DMSO was added in to control beaker as solvent control. After incubation, the glass slides were removed from both the beakers, stained with 0.4% crystal violet and observed under bright field microscope.

Characterization of actinomycete strain

Microscopic, cultural and physiological characteristics such as pH, temperature & salt tolerance and carbon utilization of potential strain were studied by adopting the method described by Shirling and Gottlieb. Based on phenotypic characteristics, the potential actinomycetes were identified at species level with the help of Nonomura’s key and Actinobase database.

Results and Discussion

Isolation, characterization and identification of biofouling bacteria

After incubation, morphologically different colonies were observed and bacterial populations estimated as 4.0×10⁷ cfu/gram of sample. Totally 25 bacterial colonies were recovered from Zobell’s marine agar plates. Based on the adherence property, three isolates (BB11, BB13, and BB14) were selected and their microscopic, cultural and biochemical characteristics are given in Table 1. Based on phenotypic characteristics, the biofouling bacterial strains were identified as Bacillus sp. (BB11), Serratia sp. (BB13), and Alteromonas sp. (BB14). Among the three bacteria, Bacillus and Alteromonas were frequently reported from fouling samples and Serratia sp. was less reported. Further, in salt tolerance studies, all the three isolates showed growth up to 7.5% NaCl which confirmed their marine origin.

Table 1—Characteristics of selected biofouling bacteria

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>BB11</th>
<th>BB13</th>
<th>BB14</th>
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</thead>
<tbody>
<tr>
<td>Gram staining</td>
<td>G+</td>
<td>G+</td>
<td>G</td>
</tr>
<tr>
<td>Motility</td>
<td>Motile</td>
<td>Motile</td>
<td>Motile</td>
</tr>
<tr>
<td>Endospore staining</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Nutrient agar</td>
<td>colorless</td>
<td>Pink</td>
<td>Orange</td>
</tr>
<tr>
<td>MacConkey agar</td>
<td>-</td>
<td>-</td>
<td>NLF</td>
</tr>
<tr>
<td>Cetrimide agar</td>
<td>No growth</td>
<td>-</td>
<td>No growth</td>
</tr>
<tr>
<td>TCBS agar</td>
<td>No growth</td>
<td>-</td>
<td>No growth</td>
</tr>
<tr>
<td>MSA agar</td>
<td>No growth</td>
<td>-</td>
<td>No growth</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Oxidase</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>IMViC</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>NaCl tolerance</td>
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</tr>
<tr>
<td>0 %</td>
<td>+</td>
<td>+</td>
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<tr>
<td>1 %</td>
<td>++</td>
<td>++</td>
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<tr>
<td>2.5 %</td>
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<td>++</td>
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<tr>
<td>5 %</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>7.5 %</td>
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<tr>
<td>10 %</td>
<td>-</td>
<td>+</td>
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</table>

‘+’ Positive, ‘-‘ Negative, NLF – Non Lactose Fermenting; ‘ND’ Not Determined
Antifouling activity

Of the 20 actinomycete extracts tested, 6 extracts inhibited biofouling bacteria. Ethyl acetate extract obtained from the actinomycete strain R1 which showed maximum inhibition against all the three biofouling bacteria was selected as potential strain for further investigations. Strain R1 was previously isolated from sediment samples collected from Rameswaram coastal area, Tamil Nadu. During fermentation strain R1 showed good growth on soybean meal medium. Many workers used soybean meal medium for the production of bioactive compounds from marine actinomycetes.\textsuperscript{21,22,23,10} The culture filtrate showed 7-9 mm inhibition while the ethyl acetate extract showed 13-15 mm inhibition against the three biofouling bacteria tested. This result evidenced that antifouling compound produced from strain is extra cellular in nature. In most of the studies, ethyl acetate was used as the best solvent for the extraction of bioactive compounds from actinomycetes.

Partial purification, bioautogram and chemical screening

Among various solvent systems tested, 2 spots were observed when chloroform: methanol (10:90) was used as solvent system and the Rf value was measured as 0.58 and 0.86. In bioautography, the second spot showed activity against biofouling bacteria. In disc diffusion method the active compound showed 16 mm inhibition zone against BB11 and BB13 and 20 mm inhibition zone against biofouling bacteria. The active spot showed brown color spot when Naphthoresorcin – Sulphuric acid reagent used as spraying reagent. Therefore the functional group of the active compound present in R1 was identified as sugar containing molecule. Screening programme for biologically active natural products require the right bioassays. Detection of compounds with the desired activity in crude extracts depends on reliability and sensitivity of test systems used. Bioassays are also essential for monitoring the required effects throughout activity guided fractionation. In this study, TLC based bioautography method was used to detect the active antifouling compound. Bioautography is a very convenient and simple way of testing natural products and pure substances for their effects on human and plant pathogenic microbes.\textsuperscript{24} This TLC based bioassay allows in-situ detection of active compound. Further, for the first time the bioautographic assay was used for the detection of antifouling compounds from actinomycetes.

Biofilm inhibition by actinomycete extracts

Ethyl acetate extract from strain R1 inhibited biofilm formation by the strain BB11. Microscopic examination of the inhibition cover slip showed less number of organisms than that of control cover slip. Field testing for inhibition of biofilm formation was needed to prove the effectiveness of the actinomycete extracts.

Characterization and identification

Under bright field microscope, both the substrate and aerial mycelial structure of strain R1 was observed. The substrate mycelium is non fragmented. The aerial mycelium is long and rectiflexible (RF) in arrangement. Under SEM observation, the spores showed smooth surface. The colonies of strain R1 appeared powdery in consistency with gray mycelium, brownish pink reverside pigment. Strain R1 showed good growth on ISP2, ISP3, ISP4, ISP5 and ISP7 medium, and moderate growth on ISP1 medium. No growth was observed on ISP6 medium. Good growth was also observed at 20°C to 40°C, 0-5% NaCl concentration and pH range between 7 and 11. The NaCl tolerance of strain R1 confirmed its marine origin. Strain R1 utilized glucose, fructose, rhamnose, mannitol, arabinose and xylose.

Based on phenotypic characteristics, the potential actinomycete strain was identified as \textit{Streptomyces filamentous} (R1). An anthracycline group of antibiotic named requinomycin with antiphage activity is known to be produced by a strain of \textit{Streptomyces filamentosus}\textsuperscript{25}. \textit{Streptomyces filamentosus} (R1) isolated in this study also showed good antifouling activity. In general, antibiotic production is a strain specific process but not species or genus specific.\textsuperscript{26} Further characterization, structure elucidation and field evaluation is needed to prove its potential. The present study concluded that the marine actinomycete, \textit{Streptomyces filamentosus} (R1) will be a potential source for the development of eco-friendly antifouling compounds, which will be a better alternative to the pollution causing synthetic antifoulants.

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