Antibiofouling activity of marine actinobacterial mediated titanium dioxide nanoparticles

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Present study is focused on the synthesis of titanium dioxide nanoparticles (TiO$_2$ NPs) from marine actinobacteria and to check their antibiofouling activity against the isolated biofouling bacteria. A total of five actinobacterial strains were isolated from Chennai marine sediments, Tamilnadu, India, which were designated as SV1-SV5. Two biofouling organisms designated as BSV1 and BSV2 were isolated from Chennai port, Tamilnadu India. All the isolated actinobacterial strains (SV1-SV5) showed positive results for the synthesis of TiO$_2$ NPs, which was confirmed by UV analysis. Further characterization of the synthesized TiO$_2$ NPs was done by using Atomic force microscopy (AFM), X-ray diffraction (XRD) and Fourier transform infrared radiation (FTIR). Isolated biofouling bacteria BSV1 and BSV2 were identified as *Staphylococcus* sp. Antifouling activity of actinobacterial extract and the combination of actinobacterial extract and TiO$_2$ nanoparticle were tested by using agar well diffusion method. Maximum zone of inhibition was shown by SV2 (15 mm) and SV3 (18 mm) with TiO$_2$ synthesized supernatant against biofouling bacteria. Potential actinobacterial strain SV2 and SV3 were identified as *Streptomyces* sp. These results shows marine actinobacteria can be used as a potential source for obtaining novel secondary metabolites with important ecological role, including preventing from fouling organisms.

[Keywords: Marine Actinobacteria, Biofouling, Titanium dioxide, Photocatalyst]

Introduction

Biofouling or biological fouling is the undesired deposition of materials on surfaces; the deposited particles can multiply on expense of nutrients. In the motile state bacteria may release exopolysaccharides to form a thick matrix, which results in the formation of a biofilm and cell may alter their morphology and grow at different rates. Biofouling is economically significant on ship hulls, where high levels of fouling can increase damage, reducing the overall hydrodynamic performance. Hence, despite of a natural process it also causes huge economic losses to marine industries. The fouling causing community can be broadly divided into two groups, Micro fouling and Macro fouling. They produce certain adhesive substances which are necessary for their attachment to solid surfaces such as ship hull. Micro fouling community results in biofilm formation and bacterial adhesion. Whereas macro fouling community results in attachment of layer organisms such as branacles, arthropod in sea, mussels, sea weeds etc. In general Biofouling affects ships severely. It leads to decreased carrying capacity, increased (30%) fuel consumption, increased cost of maintenance of shipping industry, increased (50%) marine transport cost. Hence, in order to reduce such problems certain appropriate strategies such as antifouling strategies should be employed. In most of the countries biofouling is prevented by antifouling paints. It can be done by removing accumulation or by preventing accumulation. Antifouling agents can be used against biofouling such as organotins like TBT (Tri Butyl Tin), TPT (Tri Phenyl Tin). TBT was one of the most commonly used antifouling agent for coating in ships and it was found to be very effective. Now organotins have been banned because in order to be effective it should be used in a higher concentration and its coating was found to be toxic to marine environment and not effective in ships that travel long distance. The development of environment and human friendly non-toxic antifouling compound is very important.

Titanium dioxide (TiO$_2$) is also known as titanium oxide or titania, is the only naturally accruing oxide of titanium. It has wide range of application in different industries like in paint
industries and different products like sunscreen etc. TiO$_2$ is produced in varying particles size, oil and water dispersion and with varying coatings for the cosmetics industry. TiO$_2$ in its pigment form is known as white pigment. This pigment is used extensively in plastics and other application for its UV resistance properties $^9$, where it act as a UV absorber. TiO$_2$, particularly in the anatase form is a photocatalyst under UV light. TiO$_2$ is added to paints, cements, windows, tiles or other products for its sterilizing, decolorizing and antifouling properties and is used as a hydrolysis catalyst. Titanium dioxide has the highest refractive index of any material known to man, greater even than diamond. To take advantage of this property, titanium dioxide must be mined, refined and ground to a fine, uniform particle size. TiO$_2$ has potential application in removing of all types of organic pollutants in water $^{10}$. The photocatalytic activity of TiO$_2$ was discovered by Akiria Fujishima in 1976 $^{11}$. This process on the surface of the TiO$_2$ was called as Honda-Fujishima effect.

Marine Actinobacteria are an important source of the marine environment. They have different properties such as antibacterial, antiviral, anticoagulant and antifouling properties. Actinomycetes are a group of bacteria which possess many important and interesting features. Actinomycetes were originally considered to be an intermediate group between bacteria and fungi but now recognized as prokaryotic organisms $^{12}$. They are filamentous Gram positive, non motile, sporulating, non capsulated bacteria having high G+C (>55%) content in their DNA. They can be terrestrial or aquatic $^{13}$. They have complex life cycles which are widely distributed in terrestrial ecosystems. In the last decades, actinobacteria are increasingly being isolated from marine environments demonstrating that actinobacteria are ubiquitous in marine sediments, however lower numbers than in soils. The majority of actinomycetes are free living and saprophytic bacteria. They are producers of a large number of natural products, many of them with clinical, pharmaceutical or agricultural application $^{14}$. It is therefore assumed that marine actinomycetes might produce different types of bioactive compounds $^{15, 16}$. In 2012, Karthik et al reported first marine actinobacterial mediated TiO$_2$ NPs synthesis followed by Priyaragni et al in 2013 $^{17, 18}$. So far, these two studies are available for marine actinobacterial mediated TiO$_2$ NPs synthesis. Hence, the present study is focused on Marine Actinobacteria mediated nanoparticle synthesis that have important ecological roles, including preventing from fouling organisms and photocatalyst.

**Materials and Methods**

All the chemicals and media used in the study were purchased from Sisco Research Laboratories (SRL) Pvt. Ltd, Mumbai, India and Hi Media Laboratories, Mumbai, India. Actinobacteria were isolated from the sediments of Chennai region (Latitude: 11°00’N Longitude: 78°00’E), Tamilnadu, India by spread plate technique on Starch-casein agar after serial dilution in 50% sea water. To minimize fungal contamination, all agar plates are supplemented with 50 µg/ml of nystatin. Actinobacteria colonies that appear on the starch-casein petriplates are counted from 5th day onwards upto 28th day. Powdery and leathery colonies of isolated Actinobacteria were stored at 4°C until further use $^{19}$. For the synthesis of TiO$_2$ NPs, isolated actinobacterial colonies were inoculated in SS broth, production medium (soluble starch-25 g, glucose-10 g, yeast extract-2 g, CaCO$_3$- 3 g, trace elements-1 mL and distilled water-1000 mL) and incubated in shaker incubator for 7 days at 28°C. After incubation, crude sample was centrifuged and pellet was discarded. To the supernatant TiO(OH)$_2$ was added aseptically and incubated for 24 h at room temperature.

**Characterization of nanoparticles**

After 24 hrs of incubation, 1 ml of supernatant was withdrawn from TiO(OH)$_2$ added culture broth and absorbance was taken using UV-Visible spectrophotometer (U-2800, Japan) in the range of 400-600 nm. Qualitative testing of supernatant by UV-visible spectrophotometer, confirmed the reduction of TiO(OH)$_2$ and synthesis of TiO$_2$ NPs. Biosynthesized TiO$_2$ NPs were heat dried and powder form was obtained. X-ray diffraction measurements were carried out on D8 advance diffractometer (BRUKER, Germany). The scanning was done in the region of 20 from 30° to 80° at 0.02 min and the time constant was 2s. The mean particle diameter of TiO$_2$ NPs was calculated from the XRD pattern using the Scherrer equation:

\[
D = \frac{K \lambda}{\beta \cos \theta}
\]
Where, $K$ is the shape constant, $\lambda$ is the wavelength of the X-ray, $\beta_{1/2}$ and $\theta$ are the half width of the peak and half of the Bragg angle, respectively.

The powdered sample was diluted in sodium dodecyl sulphate solution and then subjected for ultrasonification. Prepared sample was coated on a glass slide and then used for the analysis. AFM analyses of the TiO$_2$ NPs were performed under ambient condition using Nano Surf Easy Scan2, Switzerland.

Two milligrams of TiO$_2$ NPs powder sample were mixed with 200 mg KBr (FT-IR grade). The mixture was ground subsequently for 3-5 mins. The pellet was then prepared using die-set and quick-hand press. The pellet was placed into the sample holder and FTIR spectra were recorded in the range 4000-450 cm$^{-1}$ in FT-IR spectroscopy (AVATAR 300 FT-IR, Thermo Nicolet, USA).

**Antibiofouling activity**

Biofouling samples were collected from Chennai port (Latitude: 11°00’N Longitude: 78°00’E) Tamilnadu, India during March 2012. The samples were scrapped from boat surface and transferred to sterile polythene bags (Fig. 1). Biofouling organisms were isolated by using spread plate method on Zobells marine agar media and incubated at 28°C for 3-5 days. After enumeration morphologically distinct and discrete bacterial colonies were selected and purified on nutrient agar plates.

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**Application of TiO$_2$**

Method of self cleaning is a multi step processes. Surface of a glass slide was rinsed with alcohol and then slides were divided into two half. The first half is cleaned with distilled water and the second half is coated with the titanium synthesized broth, followed by drying in which the coated area will be dried off. After drying the coated areas were washed with distilled water to check the self cleaning property of TiO$_2$. Photocatalytic activity of TiO$_2$ was determined by examining the discoloration of the Rhodamine dye. Seven sets of five testubes were prepared and then 0.5mL of biosynthesized nanoparticle was inoculated into each testubes consisting of 5 mL of rhodamine (RB) dye individually. Later all the seven sets of test tubes were exposed to UV radiation at varying time interval such as 5, 10, 20, 30, 40, 50 and 60 minutes and the concentration changes of the dye solution were measured using a UV-vis spectrometer at 553 nm corresponding to the maximal absorption of the dye. Before irradiation the suspension was kept in darkness for 15 minutes to ensure sufficient adsorption of the dye.

A Langmuir-Hinshelwood kinetic model is widely used to describe the kinetics of the photodegradation of many organic compounds are widely described by

$$r = -dC/dt = kKC/(1+KC)$$

Where $r$ is the rate of reaction (mol/L.min), $C$ is the equilibrium concentration of reagent (mol/ L)
$t$ is the time (min)
$k$ is the rate constant (1/min)
$K$ is the Langmuir constant (L/mol).

This equation is simplified to a pseudo-first-order expression, when the concentration of reagent being reacted is too low, as

$$r = -\frac{dC}{dt} = kC$$

Equation (2) can be integrated, resulting in

$$\ln C_0/C = kt$$

Where $C$ is the dye concentration at instant $t$ (mg/L)
$C_0$ is the dye concentration at $t = 0$ (mg/L)
$k$ is the pseudo-first-order rate constant (1/min)
$t$ is the irradiation time (min).

**Identification of potential marine Actinobacteria**

The genus of strains with good antifouling activity against the microfouling organisms was identified using cell wall composition analysis and micromorphological studies. Species was identified based on methods described by Shirling and Gottlieb (1966) and the key of Nonomura (1974).

**Results and Discussion**

A total of twenty five marine Actinobacteria strains were isolated from marine sediment samples collected from Chennai and among them only five strains of actinobacteria synthesizes TiO$_2$ nanoparticle and were designated as SV1- SV5. Only few reports are available on eco-friendly biological synthesis of TiO$_2$ NPs. Absorbance of TiO$_2$ NPs was found to be in the range of 400-420 nm (Fig. 2). The crystalline nature of TiO$_2$ NPs was analyzed using XRD. The XRD spectrum was matched with JCPDS card No.89-8303 which exhibits the TiO$_2$ NPs peaks at 20 as 27.44°, 32.04°, 45.06°, 56.64° and 76.37°. Mean particle diameter of TiO$_2$ NPs was calculated from the XRD pattern (Fig. 3) using the Scherrer equation. Average particle size obtained from XRD data was found to be about 3.5 nm.

TiO$_2$ NPs were found to be of spherical in shape of about 37.54 nm length at 15 µm image length (Fig. 4).
Similarly, the extracellular synthesis of TiO$_2$ NPs has been investigated using the marine actinobacteria, *Streptomyces* sp LK-3. Particle size and morphology was characterized using UV-visible, XRD, AFM, SPM techniques and found to be in the range of 3.5-92 nm with spherical and oval shape$^{31}$. The different peaks shown in the Fig. 5 represents different functional groups like Aldehyde, Alkenes, Nitro and Alkynes groups. The peaks at 2065.76 cm$^{-1}$ represents the C-H Aldehyde stretching, 1637.56 cm$^{-1}$ as C=C conjugate, 1384.89 cm$^{-1}$ as NO$_2$ Conjugate and 644.22 cm$^{-1}$ as Alkynes. As TiO$_2$ NPs is metal it will never possess any functional groups, thus presence of various functional groups showed the TiO$_2$ NPs synthesizing ability of the isolate SV1-SV5.

![Fig. 5—FT-IR spectrum of TiO$_2$](image)

Totally two biofouling bacterial colonies were recovered from from Chennai port (Latitude: 11°00'N Longitude: 78°00'E) Tamilnadu on Zobell’s marine agar plates which were designated as BSV1 and BSV2. Both isolated biofouling bacteria showed adherence to the glass coverslip implying the formation of biofilm by the bacteria. Based on morphological and biochemical characteristics these biofouling bacteria were identified as *Staphylococcus* sp. In previous studies *Bacillus* sp, *Alteromonas* sp and *Staphylococcus* sp were isolated as a dominant flora$^{32,33}$. Out of the five actinobacterial extracts tested, two extracts inhibited biofouling organisms. Based on the antifouling activity the supernatant added with TiO$_2$ showed maximum zone of inhibition when compared to the supernatant without added TiO$_2$ (Table 1).

<table>
<thead>
<tr>
<th>BIOFOULING STRAINS</th>
<th>WITH TiO$_2$</th>
<th>WITHOUT TiO$_2$</th>
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</thead>
<tbody>
<tr>
<td>BSV1</td>
<td>15mm</td>
<td>10mm</td>
</tr>
<tr>
<td>BSV3</td>
<td>18mm</td>
<td>11mm</td>
</tr>
</tbody>
</table>

Maximum zone of inhibition was noticed against biofouling bacteria by SV2 and SV3. SV3 which showed maximum zone of inhibition against *Staphylococcal* sp. was selected as potential strain for further investigation.

This result evidenced that SV3 has more antifouling property than SV2. Hence, the combined effect of the extract and TiO$_2$ will be more potent and can be used for the production of antifouling paints.

The two potential actinobacterial strains SV2 and SV3 were identified as *Streptomyces* sp based on the morphological and biochemical characteristic$^{34}$. According to Kutzner (1972) for proper identification of genera and species of actinomycetes, besides morphological and physiological properties, various other biochemical properties such as cell wall chemo type, whole-cell sugar pattern, peptidoglycan type, phospholipids type and G+C% of DNA should be determined$^{35}$.

In the self cleaning process, the area coated with TiO$_2$ synthesized broth showed more clarity compared to the area coated with distilled water. The Previous studies other than TiO$_2$ manganese pervskite, zirconium oxide, yttrium iron garnet and palladium also showed photocatalytic activity$^{24}$. Hence the deposition of organic pollutants over the glass surfaces can be prevented.

Photodegradation of Rhodamine occurs in presence of TiO$_2$ when UV irradiated. For the maximum degradation, the dye solution was kept in the dark for 15 minutes before irradiation (Fig. 7 and Fig. 8). The photodegradation of RB increases with increasing time interval when exposed to UV radiation. Photodegradation is indicated by the discoloration of the dye. The

![Figure 6—Graph showing photodegradation of Rhodamine](image)
discoloration of RB occurs when the free radicals of TiO₂ binds with the positively charged dye. The photocatalytic activity of the materials was monitored on a UV-vis spectrophotometer by measuring absorption spectra at 553 nm. The absorbance of RB decreased to almost zero within 60 min, i.e. 95% degradation of the dye occurs at 60 minutes with SV3 strain (Table 2). According to the previous studies Methylene blue showed maximum absorption at \(\lambda=664\) nm and gradually decreases during the irradiation time.

![Fig. 6—Photodegradation effect synthesized TiO₂ NPs using isolated marine actinobacteria](image)

![Fig. 7—Photocatalytic degradation kinetics of synthesized TiO₂ NPs using isolated marine actinobacterial where X-axis represents ln (Co/C) and Y-axis time interval](image)

Table 2—Photodegradation of RB at 60min

<table>
<thead>
<tr>
<th>Strain</th>
<th>Degradation in %</th>
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<tbody>
<tr>
<td>SV1</td>
<td>89%</td>
</tr>
<tr>
<td>SV2</td>
<td>90%</td>
</tr>
<tr>
<td>SV3</td>
<td>95%</td>
</tr>
<tr>
<td>SV4</td>
<td>92%</td>
</tr>
<tr>
<td>SV5</td>
<td>87%</td>
</tr>
</tbody>
</table>

Hence experimental data indicates that TiO₂ significantly affect the degradation of positively charged dyes. The present study concluded that the Actinobacteria 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.

**Conclusion**
Controlling the menace of biofouling has become a major thrust area in microbiological research. The aim is to provide an antibiofouling agent which is not only cost effective, but also non-toxic to the marine organisms. In the present study, a combination of marine actinobacterial extract and titanium dioxide nanoparticles showed potential antibiofouling activity against the biofouling bacteria. Therefore, the findings of this study suggest that a combination of marine actinobacterial extract and titanium dioxide nanoparticle based paint can be further optimized to form a potentially less toxic, non-corrosive and cost effective remedy for biofouling.

**Acknowledgement**
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References


35. Kutzner, E., Simple working key for the classification and identification of named taxa included in the international Streptomyces project, *IJSEM*, 22(1972) 139-148