Stress response of Actinomycetes to toxins in their bioremediation processes

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ABSTRACT

Besides the production of bioactive compounds, antitumour agents and immuno suppressants, Actinomycetes are popular for their powerful bioremediating properties. Actinomycetes, according to this particular study, are effective consumers of antibiotics and chemical complexes. They can degrade high doses of pesticides and chemical complexes. The study also explains the mechanisms by which the microbes respond to different xenobiotics in the environment and the stress proteins produced by them with respect to their detoxification properties in the environment. The study is significant as it explains about the powerful bioremediation potential of Actinomycetes and the production of novel stress related proteins. Actinomycetes can be employed for cleaning up toxins as they are utilized as carbon sources and in turn synthesize commercially viable enzymes and proteins.

Keywords – Actinomycetes, bioremediation, mutagens, protein expression, stress response

INTRODUCTION

In this modern era, by the advances in science and living, industrialization has crossed the values and concept of environmental naturality. Industries are meant for human welfare and to meet human needs, but their ecological consequences are high. The major industrial belt causes health hazards in the neighboring areas. Moreover the coastal ecosystems are highly degraded due to high population and industrial growth. Marine pollution includes a range of threats including farm land based sources, oil spills, untreated sewage, organic pollutants and radioactive substances, which destroys these habitats (Mc Cook, 1999, Bellwood et al., 2004).

Even though insecticides and toxic compounds in the ecosystem are successfully degraded by several microbes (Chetna and Madhuri, 2012) at sufficiently high concentrations; the pesticides in turn are known to modify the growth and activities of micro-organisms. These alterations are mediated through cytological and biochemical changes at the cellular level (Ruplal and Shivaji, 1984). Actinomycetes are one among the important group which effectively disintegrate and bioremediate the pesticides and other xenobiotics in the environment. Actinomycetes play important roles in the environmental fate of toxic metals with a multiplicity of physico-chemical and biological mechanisms effecting transformations between soluble and insoluble phases and produces significant levels of biosurfactants (Subhajit, 2012). Such mechanisms are important components of natural biogeochemical cycles for metals and metalloids with some processes being of potential application to the treatment of contaminated materials. Role of Actinomycetes in Bioremediation and stress related behavior has been extensively studied by Justin et al.,
Several Actinomycetes strains from composts are now being investigated to evaluate their capacity to degrade some petroleum hydrocarbons and to decolorize several synthetic dyes in order to reveal their potential application in bioremediation.

**MATERIALS AND METHOD**

- **Isolation and strain identification:**
  1 kg of soil was taken from the top surface soil (10 cm) from three different areas of the polluted coastal areas of Tamil Nadu. The serially diluted soil suspensions were pour plated in Potato dextrose agar and the plates were incubated at 28°C for 7 – 10 days. The isolated strains were identified by their morphological and physiological characteristics according to the classical biochemical approach. Morphological features such as spore morphology, aerial and substrate mycelia colourations were analysed. Biochemical tests such as Macromolecule hydrolysis, fermentation of various sugars and antibiotic resistance was studied. The observed result was compared with Bergey’s Manual of Determinative Bacteriology, 9th edition. Further confirmations were carried out with molecular characterization studies on the 16srRNA gene.

- **Estimation of sensitivity of Actinomycete to commercially available antibiotics and chemical complexes**
  The isolates were subjected to sensitivity assay using commercially available antibiotics such as Streptomycin, Tetracycline, Cefataxime, Roscillin, Cephalexin, Amoxycillin, Cefixime and Cypodexime in Actinomycete cultures grown on Potato Dextrose Agar plates. Filter paper discs incorporated with 5µl of each antibiotic containing 1mg/ml (w/v) were placed separately on plates swabbed with the strains and the changes (zone formation) were noted after 7 days. The Actinomycete isolates were tested against different chemical complexes such as Schiff’s base, copper complex, nickel complex, Azodye, Iron complex, Ethyl cyano acetate, Benzoyl acetone, Amino pyridine, B-naphthol, Benzoin, Salicyl aldehyde, Anthranilic acid, Copper complex, Nickel complex and Iron complex by repeating the same mechanisms followed previously for antibiotics. The mechanism of utilization of the chemical complexes by the isolates was estimated by the radius of the inhibition zone after seven days of incubation.

- **Treatment of Actinomycetes with pesticides**
  - **Treatment with Fenvalerate**
    Fenvalerate, a pesticide generally applied in agricultural farms and reported to be toxic to living beings was selected for the experiment. The Streptomycete strains were inoculated separately in the medium containing the pesticide at different concentrations (200, 300, 400, 500, 750, 1000 and 1500µl/100ml of the medium).
  - **Treatment with Malachite green**
    Malachite green, a dye which is normally used for treating fish diseases is a powerful intercalating agent. The Streptomycete strains were inoculated separately onto Potato Dextrose Agar plates containing 1000, 1500, 2000, 2500 µl Malachite green/100 ml medium. They were incubated for 7 days and the variations in growth, colourations and pigmentation were determined as mentioned above. The microbial cultures were screened for the traces of biodegraded products with Thin Layer Chromatographic technique.

- **Variations in physiological properties after chemical Treatment**
  - **Study of Antagonisms of untreated and treated cultures:**
    To test the variation in antagonistic property after screening, the untreated cultures (on agar slants) of Actinomycetes were studied using sloppy agar technique as described by Annie M. et al., (1994). The procedure was repeated for 10 Actinomycete isolates against 10 pathogenic bacteria before and after treatment and the results were compared. Butanol extracted cell filtrate and homolysate were coated onto filter paper discs and tested for antibiotic effect against pathogens like *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Salmonella sp.* and *Bacillus subtilis* by Kirby Bauer disc diffusion method.

The antifungal activity of exudates from selected strains of Actinomycetes was tested. All isolates which prevailed the screening were tested against four different pure cultures of fungi, viz. *Aspergillus sp.*, *Fusarium sp.*, *Trichoderma sp.* and *Rhizopus sp.* Butanol extracted cultures were coated onto filter paper discs and were placed onto the Potato Dextrose agar plates containing freshly swabbed fungal cultures. Plain filter paper discs with butanol served as the control. The inhibition zones produced were observed and measured for both the untreated as well as treated strains.

- **Changes in Protein and Amino acid contents**
The total proteins present in the intracellular and extra cellular fluids of treated and untreated strains were determined by Lowry’s method (Lowry et al., 1951). The total amino acids were estimated by Thin Layer Chromatography as described by Ninhydrin method of Moore and Stein, 1948. The amount of different proteins after and before the treatment with the pesticide were analysed by Polyacrylamide Gel Electrophoresis.

- **Changes in Sodium Chloride tolerance**
  A separate experiment was carried out to analyze the differences in tolerance after and before subjecting the strains to the chemicals. Each Streptomycete strain was inoculated separately into Tryptone Yeast Extract broth containing 1, 5, 10, and 15% of NaCl concentrations. Plain Tryptone Yeast Extract Agar without NaCl inoculated with treated and untreated Actinomycetes served as the control. These cultures were incubated for 7 days and the growth, colour and dry biomass were recorded.

- **Changes in protein concentration in the untreated and treated strains at varied pH levels**
  The isolates treated with the chemicals were analyzed for their changes in protein production at varied pH levels such as 6.5, 7 and 7.5. The aerial and substrate mycelial colorations and variation in exuded pigments were studied according to standard protocols.

- **Changes in Protease activity**
  Protease activity was measured for all the strains assayed individually with different test tubes. 6ml of enzyme (culture filtrate) was added to all the tubes. They were incubated at 37°C for 10 minutes. After incubation, 1ml of 12% TCA was added, and the mixture was cooled rapidly in ice, and centrifuged at 4000 rpm for 10 minutes of the supernatant was collected. A blue colour was developed and read at 650nm. The OD values were plotted in along with the standard values to obtain the enzyme activity of extracellular protease in the samples.

- **Variation in the production of L-Asparaginase**
  The treated and untreated strains of Actinomycetes were inoculated separately into test tubes containing Tryptone soy Broth with 1% L-Asparagine. The growth was measured after 7 days. To the filtrate of each treated and untreated strain, 0.5ml of Nessler’s reagent was added. The precipitate was removed after centrifugation and the clear supernatant was used to measure the optical density at 450nm. Tryptone Yeast extract broth with 1% asparagine and Nessler’s reagent served as the control.

**RESULTS**

- **Isolation of Actinomycetes and strain identification**
  Soil samples were isolated from different regions of the coast of cuddalore, in Tamil Nadu which is reported to be among the most polluted industrial sites of south India. The site is heavily laden with chemical pollutants and pesticides. These soil samples were used for the isolation of Actinomycetes, with respect to their pigmentation and sporulation properties. Soil samples from the coast produced more number of colonies with better pigmentation and sporulation properties proving their bioactivity. A total of ten strains of distinct characteristics were isolated and stored. Band profiles in phylogenetic clustering of 16srRNA sequences for the strains SC4, SC14, SC48 showed high level of similarity to (98%) to Streptomycetes and clustered with *Streptomyces fradiae*, *S. lividans* and *S. erythraea*. The identified organisms, SC7, SC15, SC19, SC26 and SC45 showed high percentage (96%) of similarity with *Frankia alni*, *Actinomyces turicensis*, *Actinomyces radingae*, *Actinomyces israeli* and *Nocardia asteroides*. SC31 and SC33 showed a less percentage of identity to the profiles i.e. 89% towards *Rhodococcus fascians* and *Actinomyces israeli*. Biochemical tests carried out on the different isolates revealed that all the ten isolates of study belonged to the group of Actinomycetes. Further confirmation of the isolates was done with molecular characterization of the 16srRNA gene. Related sequences were retrieved from the databases aligned and subjected to phylogenetic analysis. All the details are included in the supplementary data section.

- **Treatment of the strains with chemicals**
  The selected strains were subjected to treatment by chemical agents, fenvalerate and malachite green. The strains were very hardy that they could tolerate upto 2500 µg/ml of the chemicals which was considerably high when compared to the bacterial strains maintained as controls (*Salmonella sp.*, *E coli* and *Bacillus sp.*). Lower concentration of pesticide (upto 500µg/ml) had no impact on the viability of Streptomycete colonies whereas higher concentrations could produce notable changes such as altered spore colour, variation in colony size, NaCl tolerance.
etc. in the strains. But even higher concentrations up to 2000µl/100ml could be tolerated by some of the hardy varieties. For details view the supplementary data.

- **Changes in physiology after treatment with pesticides and dyes**
  Antagonism was checked at three different experimental conditions viz. in situ colony analysis with untreated and the treated organisms. Streptomycete cultures were found to exhibit the maximum antagonistic properties after treating with the chemical substances, producing inhibition zones against many pathogenic bacteria up to a diameter of 6.2 cm. Among the isolated strains, the maximum resistance was noted in SC7, SC15, SC19, SC26, SC31 and SC45. Antifungal activity of untreated cultures of all the Streptomycete strains was tested against four different fungal strains. The Streptomycetes could effectively kill all of the fungal strains tested except for one of them, *Fusarium sp.* by Streptomycete strain SC48 after treatment with the pesticides.

On testing the resistance of the isolates against commercial antibiotics, most of the isolates proved to be resistant. It was surprising to note that the strains utilized the antibiotics and hence heavily colonized the filter paper strips coated with the commercial antibiotics. The details are evident in Figure 1. The chemical complexes were disintegrated by the strains as they overgrew the filter papers coated with them. Colonies clustering around the antibiotic and chemical coated discs curiously indicated that antibiotics promoted the growth of these Streptomycetes species and may serve as nutrients for the production of many important cellular components.

The strains of Streptomycetes were treated with 13 chemical complexes. The results were compared with those of bacterial strains (*Salmonella sp.*, *E coli* and *Bacillus sp.*). The chemical complexes inhibited bacterial growth as proved by inhibition zones. The Streptomycetes were insensitive, hardy and were capable of accepting the complexes and degrading them so that the complexes were, utilized as substrates or raw materials for the production of various metabolites and bioactive compounds as evidenced by an enhanced pigmentation and spore coloration all around the colonies. Profuse growth was observed near and around the chemical coated strips, indicating their higher preference for the chemicals. Thin Layer chromatographic studies revealed that there were no traces of the complex in the medium after consumption by the Actinomycetes, indicating that the compounds have been disintegrated totally to their elemental components and absorbed into the cellular substratum. It is evident from the above observations that the Streptomycetes utilize this quality to protect and adapt themselves to the highly polluted environment. This marks the possibility that these Streptomycetes colonise in the soil and disintegrate the toxic chemicals present in the environment.

Mycelial growth diminished at higher concentrations of NaCl in the strains treated with the pesticide unlike those of the untreated strains. The strains which were subjected to mutagens, showed a decrease in the growth and tolerance levels to NaCl. The growth was maximum at the 0% levels of NaCl and 8 among the 10 strains were found to survive at 15% levels of NaCl in case of the untreated strains. The pesticide treated strains had a diminished growth and most of them were nonviable at 15% levels. The viable strains at these high salt concentrations exhibited a very low percentage of growth. The isolated strains could tolerate extreme levels of NaCl, pesticide and dye concentrations. This proves that these organisms could be used as biotechnological extremophiles which can survive even at extreme environments and the property of utilizing them as nutrient sources proves convincing that the strains act as powerful bioremediants. Such changes might be involved in protein switching actions and better stress enzyme production in the Actinomycetes studied. Jayabarath et al., 2010 analyzed the biodegradation properties of carbofuran pesticides by saline soil Actinomycetes. The observations in the present study also support and augment this view. This property could be easily exploited for manipulating culture conditions and setting a suitable environment of the bioremediating strains of the species.

- **Changes in protein expression after treatment with pesticides**
  The change in physiology was attributed to increased protein and amino acid production, better pigment production, decrease in protease activity and lowering of sodium chloride tolerance. The protein production increased after treatment with toxic chemicals and the maximum range was observed at the pH 7. Different amino acids were found to be expressed after the treatment. The protease activity of the strains showed marked variations after the treatment. The untreated strains expressed a better protease activity when compared to the treated strains as depicted by the Table 1 & 2. Expression of various proteins liked after the treatment which proves the fact that upregulated expression of the genes has occurred to cope up with the external stress factors involved. This rise in protease expression inhibits the enzyme L-asparaginase, thus a lowered level of L-asparaginase is reported.

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Enzyme producing properties as that of L-asparaginase, decreased when these strains were treated with pesticides. L-Asparaginase activity diminished to a considerable level after subjecting to chemical mutagens. The growth of the mycelium was also seen to be reduced after chemical treatment. The details are depicted in Figure 2. Bands corresponding to heavy proteins (97 kDa) similar to heat shock proteins were observed in treated strains. Protein bands corresponding to the molecular weight of protease (55kDa) enzymes were noted from the study. Similar results were obtained elsewhere. (Boutibonnes, 1993 and Bailly, 1991). The data indicates that there are differences in the band characteristics expressed by the strains treated with the pesticide (Figure 3). All these characteristics imply that Actinomycetes are capable of surviving adverse environmental conditions and are powerful strains to utilize any harmful or toxic xenobiotics in the environment.

Table 1. Identification of Amino acids present in the exudates

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Actinomycete strains</th>
<th>U</th>
<th>Aminoacids</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>SC4</td>
<td>Proline</td>
<td>Proline, Methionine</td>
</tr>
<tr>
<td>2.</td>
<td>SC7</td>
<td>Proline</td>
<td>Proline, Leucine</td>
</tr>
<tr>
<td>3.</td>
<td>SC14</td>
<td>Methionine</td>
<td>Methionine</td>
</tr>
<tr>
<td>4.</td>
<td>SC15</td>
<td>Leucine</td>
<td>Proline</td>
</tr>
<tr>
<td>5.</td>
<td>SC19</td>
<td>Glutamine</td>
<td>Glutamme</td>
</tr>
<tr>
<td>6.</td>
<td>SC26</td>
<td>Alanine</td>
<td>Nil</td>
</tr>
<tr>
<td>7.</td>
<td>SC31</td>
<td>Glutamine</td>
<td>Glutamme, Leucine</td>
</tr>
<tr>
<td>8.</td>
<td>SC33</td>
<td>Glutamine</td>
<td>Glutamme, Methionine</td>
</tr>
<tr>
<td>9.</td>
<td>SC45</td>
<td>Glutamine</td>
<td>Glutamme, Alanine</td>
</tr>
<tr>
<td>10.</td>
<td>SC48</td>
<td>Methionine</td>
<td>Methionine</td>
</tr>
</tbody>
</table>

U - Untreated Strains    T - Treated strains (with pesticides)

Table 2. Studies on the changes in protease activity of the extracellular products at different incubation periods

<table>
<thead>
<tr>
<th>S.No</th>
<th>Actinomycete strains</th>
<th>Protease activity (U/ml)</th>
<th>At the end of 92 hrs of growth</th>
<th>At the end of 148 hours of growth</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td>U</td>
<td>T</td>
<td>U</td>
</tr>
<tr>
<td>1</td>
<td>SC4</td>
<td>10.9</td>
<td>10.0</td>
<td>18.1</td>
</tr>
<tr>
<td>2</td>
<td>SC7</td>
<td>5.5</td>
<td>5.1</td>
<td>9.5</td>
</tr>
<tr>
<td>3</td>
<td>SC14</td>
<td>4.2</td>
<td>3.0</td>
<td>7.3</td>
</tr>
<tr>
<td>4</td>
<td>SC15</td>
<td>9</td>
<td>4.2</td>
<td>10.8</td>
</tr>
<tr>
<td>5</td>
<td>SC19</td>
<td>3.3</td>
<td>4.2</td>
<td>5.6</td>
</tr>
<tr>
<td>6</td>
<td>SC26</td>
<td>4.9</td>
<td>5.3</td>
<td>6.8</td>
</tr>
<tr>
<td>7</td>
<td>SC31</td>
<td>5.5</td>
<td>3.9</td>
<td>3.7</td>
</tr>
<tr>
<td>8</td>
<td>SC33</td>
<td>8.9</td>
<td>7.6</td>
<td>8.7</td>
</tr>
<tr>
<td>9</td>
<td>SC45</td>
<td>4.7</td>
<td>3.6</td>
<td>6.5</td>
</tr>
<tr>
<td>10</td>
<td>SC48</td>
<td>4.7</td>
<td>4.1</td>
<td>8.3</td>
</tr>
</tbody>
</table>

U - Untreated Strains    T - Treated strains (with pesticides)

Fig. 1. Graphical representation of the change in L-asparaginase activity before and after pesticide treatment

Modulations in expression of L-asparaginase activity on mutagenesis of various Actinomycetes

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This hint at the activation of certain genes which are ‘switched on’ to express proteins that may be needed to combat the stress conditions, akin to heat shock proteins. Justin et al., 2008, proved that number of genes whose expression changed during stress condition was eight times greater than that in the control experiment. The results depict that Induction and production of useful bioactive compounds such as antibiotics could be enhanced by combining UV irradiation with chemical treatments.

Fig. 2. Colonization of Actinomycetes over the filter papers coated with Antibiotics

![Control](image1)

- Aerial mycelia
- Substrate mycelia

![Control](image2)

Fig. 3. Variations in the expression of proteins before and after pesticide treatment.
Fresh Strains) Lane- 1              SC 4
Lane- 2              SC 14
Lane- 3              SC 19
Lane- 4              SC 31
Lane- 5              SC 48
Lane- M              Marker

(Pesticide treated strains) Lane- 6            SC 4
Lane- 7              SC 14
Lane- 8              SC 19
Lane- 9              SC 31
Lane-10             SC 48

CONCLUSION

Biochemical tests and 16srRNA analysis carried out on the different Actinomycete isolates revealed that all the ten isolates of study belonged to the group of Actinomycetes. The strains were very hardy that they could tolerate higher concentrations upto 2000µl /100ml of pesticides, dyes and chemical complexes. These Actinomycete cultures were found to exhibit antagonistic properties after treating with the chemical substances, producing inhibition zones against pathogenic bacteria to a diameter of 6.2 cm. The chemical complexes supplied were disintegrated by the strains and used as food source by the strains. Subjecting the strains to toxins was assisted with the production of varied proteins. L-asparaginase activity decreased and protein protease activity increased. A better protease activity was observed after pesticide treatment consecutively lowering the L-asparaginase activity. Proteins similar to heat shock proteins and proteases with a molecular weight of 92kDa and 55kDa were observed to be produced by the stressed Actinomycete strains.

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REFERENCES