Chapter III

Identification and characterization of antibiofilm agents from seaweed surface associated bacteria against Group A Streptococcus through culture dependent approach
S. pyogenes, a β- haemolytic bacterium belonging to group A type among the streptococci (Group A Streptococcus, GAS), is a highly successful exclusive human pathogen (Fiedler et al., 2015). Diseases caused by GAS range from mild superficial, self-limiting conditions such as impetigo and pharyngitis to life-threatening invasive diseases such as necrotizing fasciitis and streptococcal toxic shock syndrome (Walker et al., 2014). Untreated infections of GAS can even lead to severe sequelae in the form of glomerulonephritis and rheumatic heart disease in susceptible individuals (Tan et al., 2014). GAS is ranked as the ninth leading infectious cause of human mortality by World Health Organization manifesting its important status among bacterial pathogens. An estimate of 111 million cases of pyoderma, 616 million cases of pharyngitis, and over 5,00,000 deaths due to severe systemic diseases and sequelae are reported to be caused by GAS per year (Ralph and Carapetis, 2013).

GAS is equipped with a large and diverse set of virulence determinants, which enable it to attach to host tissues, evade the immune system and spread infection (Carapetis et al., 2005). Important virulence factors include surface associated M protein, hyaluronic acid capsule, lipoteichoic acid, pyrogenic exotoxins (A, B and C), streptokinase, streptodornase, streptolysin S, streptolysin O, and biofilm formation (Krzysiak et al., 2013).

Biofilm is an assemblage of surface-associated microbial cells encased in thick extracellular polymeric substance matrix (Lembke et al., 2006). Biofilm formation of GAS has been proposed to play a key role in pathogenesis by protecting it from host immune cells and antibodies (Thenmozhi et al., 2011). Streptococcal biofilm was found to be present in tonsilar reticulated crypts of nearly one-third of patients who underwent tonsillectomy (Roberts et al., 2012). Nearly 90 % among the 289 GAS strains isolated from different clinical sources were found to have the capacity to form
biofilm (Baldassarri et al., 2006). GAS was also proven to form biofilm-like bacterial communities during soft tissue infection in zebra fish (Cho and Caparon, 2005). Failure of biofilm mutants to cause infection further augments the clinical significance of biofilm in pathogenicity (Cvitkovitch et al., 2003).

Biofilm mode of GAS growth is attributed as a decisive factor contributing to antibiotic resistance development and therapeutic treatment failure. For instance, clinical isolates from pharyngitis patients who failed to respond to antibiotic treatment were all prolific biofilm formers. These strains were also found to have increased minimum microbial eradication concentration for all contemporary antibiotics used for treatment (Conley et al., 2003). Yet another study reported that biofilm formation was predominant among macrolide susceptible GAS isolates rather than resistant ones. The susceptible isolates were speculated to use biofilm as a barrier against the entry of antibiotics, resulting in treatment failure (Baldassari et al., 2006). Hence, even in cases where the pathogen is susceptible to antibiotics, biofilm formation complicates the treatment.

Antibiofilm agents, which inhibit biofilm formation and thereby aid in antibiotic penetration is believed to be a promising alternative to combat this menacing problem (Taraszkiewicz et al., 2012). Several independent studies have shown that seaweed and seagrass surface associated bacteria are capable of producing bioactive compounds (Egan et al., 2008; Singh et al., 2015). The likelihood of identifying bioactive producing bacteria from seaweed and seagrass associated community is higher, since they harbour beneficial epiphytic bacterial community as their first line of defense against harmful pathogens (Penesyan et al., 2010). With this background, the present study aims to explore the epiphytic bacterial community of
seaweed (*Gracilaria* sp.) and seagrass (*Cymodaceae* sp.) of Palk Bay origin for antibiofilm compounds against GAS.

**Results and Discussion**

**Screening of culturable bacterial isolates**

Infections caused by GAS often fail to respond to antibiotic therapy, which is attributed mainly to its ability to form biofilm *in vivo* (Fiedler *et al.*, 2015). This has driven a need for agents that can act against GAS biofilm formation. In the present study, culturable bacterial isolates associated with the surface of red seaweed, *Gracilaria* sp. (N = 60) and seagrass, *Cymodaceae* sp. (N=28) of Palk Bay origin were screened for their ability to inhibit the biofilm formation of GAS. The crude CFCS of 48 h old cultures were used for screening antibiofilm activity with an aim to target their secreted metabolites. Among the isolates screened, R60 (seaweed associated bacterium) significantly inhibited the biofilm formation of the reference strain *S. pyogenes* SF370 (Figure 3.1). The isolate R60 was identified (to the species level) as *Bacillus subtilis* (Genbank accession number- KM067452) by 16S rRNA gene sequencing.

![Figure 3.1. Light microscopic images (400X) showing antibiofilm activity of untreated and proteinase K treated CFCS of R60 (*B. subtilis*) against *S. pyogenes* SF370.](image)

**Figure 3.1.** Light microscopic images (400X) showing antibiofilm activity of untreated and proteinase K treated CFCS of R60 (*B. subtilis*) against *S. pyogenes* SF370.
Purification and characterization of active lead

In order to identify the nature of active lead, the CFCS of R60 was subjected to proteinase K treatment and analyzed for its ability to inhibit biofilm formation. Proteinase K treated supernatant showed antibiofilm activity similar to that of untreated supernatant, indicating the active principle to be non-proteinaceous (Figure 3.1). Hence, successive solvent extraction of cell free culture supernatant of R60 was carried out to purify and identify the active compound responsible for antibiofilm activity. The active principle was found to have a polarity soluble in ethyl acetate. Subsequently, crude ethyl acetate extract was subjected to bioactivity guided partial purification using thin layer chromatography. Various solvent systems were experimented as mobile phase, among which chloroform: ethyl acetate: methanol in the ratio of 9:10:1 showed well resolved bands when developed in iodine vapour (Figure 3.2).

Figure 3.2. TLC chromatogram of R60 crude extract separated using various solvent systems. Top panel shows TLC plates visualized under long wavelength UV and the bottom panel shows plates developed in iodine vapour (CHL- chloroform; EA-ethyl acetate; MET-methanol).
The TLC chromatogram of R60 crude extract separated using chloroform: ethyl acetate: methanol (9:10:1) solvent system was divided into four fractions (Figure 3.3). Silica was scrapped from each fraction and the compounds present in it were extracted using methanol and their antibiofilm activity was evaluated (Figure 3.4). Among the fractions, fraction 1 (F1) exhibited significant antibiofilm activity against GAS. The compounds present in the active fraction (F1) were analyzed and detected using gas chromatography coupled with mass spectrometer (Figure 3.5). The results pertaining to the GC-MS analysis are given in Table 3.1.

Figure 3.3. TLC chromatogram of R60 crude extract separated using chloroform: ethyl acetate: methanol (9:10:1) solvent system. Right panel exhibits the TLC chromatogram visualized under long wavelength UV and the left panel shows chromatogram developed with iodine vapour. The separated fractions were marked as F1, F2, F3 and F4.

Figure 3.4. Light microscopic images (400X) showing the effect of TLC separated fractions of R60 crude extract on the biofilm formation of GAS. Fraction 1 exhibited antibiofilm activity.
Figure 3.5. GC-MS chromatogram of partially purified ethyl acetate extract of R60.

Table 3.1. Compounds present in the partially purified extract of R60 - as identified by GC-MS analysis

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Retention time (min)</th>
<th>Name of the compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.9</td>
<td>Trans-2-undecen-1-ol</td>
</tr>
<tr>
<td>2</td>
<td>9.8</td>
<td>2-Decen-1-ol</td>
</tr>
<tr>
<td>3</td>
<td>10.1</td>
<td>Phenol,2,4-bis (1,1-dimethylethyl)</td>
</tr>
<tr>
<td>4</td>
<td>11.5</td>
<td>5H-dibenzazepine 10,11-dihydro-5-nitroso</td>
</tr>
<tr>
<td>5</td>
<td>13.8</td>
<td>Norvaline, N-(2-methoxy ethoxy carbonyl) undecyl ester</td>
</tr>
<tr>
<td>6</td>
<td>14.5</td>
<td>Tridecanoic acid, methyl ester</td>
</tr>
<tr>
<td>7</td>
<td>17.3</td>
<td>Cyclooctasiloxane hexadecamethyl</td>
</tr>
<tr>
<td>8</td>
<td>20.4</td>
<td>Cyclononasiloxane, octadecamethyl</td>
</tr>
<tr>
<td>9</td>
<td>22.6</td>
<td>Tetracosamine-cyclododecasiloxane</td>
</tr>
</tbody>
</table>

Interestingly, GC-MS analysis revealed the presence of phenol,2,4-bis(1,1-dimethylethyl), which is also called as 2,4-Di-tert-butylphenol (Figure 3.6), referred as DTBP in this Chapter. A number of studies have reported the antibacterial (Dehpour et al., 2012), antifungal (Sang et al., 2011; Rangel-Sanchez et al., 2013; Dharni et al., 2014), antioxidant (Choi et al., 2013) and anticancer (Sathuvan et al., 2014),...
2012; Varsha et al., 2015) activity of DTBP isolated from various bacterial, fungal and plant sources. More recently, DTBP has been shown to reduce the biofilm formation of Serratia marcescens, a Gram negative nosocomial human pathogen (Padmavathi et al., 2014). However, DTBP has seldom been explored for its antibiofilm activity against Gram positive bacteria. With this milieu, the present investigation was directed towards analyzing the efficacy of DTBP in inhibiting the biofilm of GAS.

![Figure 3.6. Structure of 2,4-Di-tert-butylphenol](image)

**Dose dependent antibiofilm activity of DTBP**

Preliminary studies to evaluate the antibiofilm potential of DTBP was carried out using the reference strain S. pyogenes SF370 which belongs to M1 serotype. DTBP exhibited a concentration dependent biofilm inhibition with a maximum of 79% inhibition at 48 µg mL⁻¹ concentration (Figure 3.7). DTBP was found to have no effect on the growth of GAS up to 60 µg mL⁻¹ concentration, above which a mild bacteriostatic effect was observed. Hence, all the assays were performed at < 60 µg mL⁻¹ concentration. In comparison, studies with limonene showed 83% of biofilm inhibition at a much higher concentration of 400 µg mL⁻¹ (Subramenium et al., 2015a), manuka honey showed 75% reduction in biofilm formation at 10% concentration (Maddocks et al., 2012) and morin hydrate reduced only 50% of biofilm formation at 225 µM concentration (Green et al., 2012).
Figure 3.7. Percentage of *S. pyogenes* SF370 biofilm formation in the presence of increasing concentrations of DTBP. Bottom panel represents microtiter plate wells showing crystal violet stained biofilms of *S. pyogenes* SF370 grown in the absence and presence of DTBP at increasing concentrations. * indicates p < 0.005.

**DTBP inhibits biofilm formation of different clinical M serotypes of GAS**

GAS strains are classified into serotypes based on the antigenic differences observed in the N-terminal region of the surface associated M protein (McMillan *et al.*, 2013). Large-scale epidemiological studies have revealed that certain M serotypes are specifically associated with a particular disease and their distribution differs significantly across the globe (Esposito *et al.*, 2015). On the other hand, clinical isolates are generally more potent in biofilm formation (Marks *et al.*, 2014). Hence, the present study was extended further to evaluate the ability of DTBP in inhibiting the biofilm formation of different clinical M serotypes of GAS.

The clinical isolates (SP5, SP7, SP11, SP22, SP30 and SP31) and the reference strain (SF370) were individually treated with increasing concentrations of DTBP and observed under light microscope. The M types differed in their ability to form biofilm which is in concordance with the studies by Baldassarri *et al.* (2006) and
Lembke et al. (2006). Among the clinical isolates analyzed, SP22 was the strongest biofilm former, followed by SP31, SP5, SP7, SP30 and SP11. DTBP was able to significantly inhibit the biofilm formation of all the strains tested (Figure 3.8). BIC was considered as the lowest concentration which showed a maximum breach in biofilm and micro-colony formation. The BIC of the tested strains varied between 16 - 48 µg mL⁻¹. However, no correlation could be observed between the capacity of biofilm formation and the BIC of DTBP. For instance, the BIC of SP30 was 32 µg mL⁻¹, whereas that of SP31 was 16 µg mL⁻¹, which is a proficient biofilm former than SP30. Similar results were observed in the study carried out by Nithyanand et al. (2014) using usnic acid and they have ascribed it to the varying amount of EPS layer surrounding each strain. DTBP could share similar grounds for this observed variation. Considering the link between M protein and specific clinical syndrome, the ability of DTBP to inhibit the biofilm formation of different M serotypes of GAS may have significant impact on medical applications.

**Non-bactericidal antibiofilm activity of DTBP**

It is ideal for an anti-biofilm agent not to impose any growth inhibitory effect on the pathogen (Dharmaprakash et al., 2015). Interfering biofilm formation without affecting growth is expected to overcome resistance, since the effect is non-lethal and would only allow clearance of the pathogen by host immune system and antibiotics. Hence, growth curve was assessed for all the GAS strains (SF370 and clinical isolates) grown in the absence and presence of DTBP at their respective BIC. No significant difference in the growth pattern was observed between the control and treated samples of all the tested strains (Figure 3.9). This result clearly evidences that the reduction in biofilm formation observed upon DTBP treatment was not a consequence of growth inhibition and attests DTBP as an ideal antibiofilm agent.
Figure 3.8. Light microscopic visualization of antibiofilm activity of increasing concentrations of DTBP (16 - 48 µg mL⁻¹) against *S. pyogenes* SF370 and six different clinical M serotypes. Biofilm inhibitory concentrations (lowest concentration which shows maximum biofilm inhibition) of DTBP against GAS isolates are highlighted in the image.
Figure 3.9. Effect of DTBP at their BIC on the growth of (A) SF370, (B) SP5, (C) SP7, (D) SP11, (E) SP22, (F) SP30 and (G) SP31.
DTBP reduces biofilm biomass and affects cell surface architecture

The characteristic architecture of streptococcal biofilm plays a crucial role in preventing the penetration of antibiotics and host immune cells (Chao et al., 2015). Confocal Laser Scanning Microscopic (CLSM) analysis was done in order to get a detailed view about DTBP induced changes in the biofilm architecture of reference strain *S. pyogenes* SF370. The results depicted a remarkable decrease in the biofilm biomass and micro-colony formation. Affirming the liquid assay, a dose dependent biofilm inhibition was observed with maximum inhibition at 48 µg mL\(^{-1}\) concentration of DTBP (Figure 3.10). Biofilm thickness measured using z-stack analysis, also showed reduced thickness of treated biofilm (10 µm) compared to control (35 µm). The reduced biofilm biomass and thickness upon DTBP treatment directly implies an efficient penetration of antibiotics.

![Figure 3.10. CLSM images (400X) showing inhibition of *S. pyogenes* SF370 biofilm formation by increasing concentrations of DTBP (16 - 48 µg mL\(^{-1}\)). Scale bar = 50 µm.](image)

Scanning Electron Microscopic (SEM) images were taken to explicate the morphological changes induced by DTBP (BIC) on the reference strain *S. pyogenes* SF370. The results revealed a decreased biofilm biomass with reduced extracellular polymeric substance (EPS) in treated sample. Strikingly, the cell surface of treated sample was found to be disturbed, contrary to the control which had an intact cell
surface with uniform cell boundary (Figure 3.11). This observation is in total agreement with the earlier report, in which the phenolic nature of DTBP was found to change the cell membrane integrity of \textit{Phytophthora cinnamomi} (Rangel-Sanchez \textit{et al.}, 2013). The cell surface of GAS attributes to many of its virulence factors particularly, those involved in colonization and evasion of host immune response such as opsonisation. Hence, perturbed cell surface of GAS upon DTBP treatment, entails reduced ability to colonize as well as an enhanced susceptibility to phagocytosis.

![Control vs Treated](image)

**Figure 3.11.** Scanning electron micrographs of \textit{S. pyogenes} SF370 biofilms formed in the absence and presence of DTBP (48 µg mL\textsuperscript{-1}) after incubation for 24 h. Scale bar = 2 µm.

**DTBP affects initial adhesion stage of biofilm formation**

EPS serves a plethora of functions such as promoting the initial attachment of cells to solid surfaces, formation of mature biofilm architecture and increasing the mechanical stability of biofilm. In addition, it profoundly contributes to antibiotic resistance, either by precluding the access of the antimicrobial agents or by substantially reducing their concentration (Shafreen \textit{et al.}, 2011; Packiavathy \textit{et al.}, 2014). DTBP treatment considerably (33-46 %) reduced the EPS production of all the M serotypes tested (Figure 3.12).
On the other hand, EPS is also known to profoundly influence the cell surface hydrophobicity, by altering the physicochemical characteristics of cell surface (Ras et al., 2013). Cell surface hydrophobicity is a major determinant important for the initial adherence and colonization of bacteria to the substratum, because it masks the repulsive force between the cell surface charge and the substratum (Courtney et al., 2009). Hence, decreased EPS production observed in DTBP treated samples was also expected to reduce the cell surface hydrophobicity of GAS. Results of MATH assay revealed that DTBP treatment greatly reduce (60-70 %) the cell surface hydrophobicity of all the clinical M serotypes of GAS (Figure 3.12).

Figure 3.12. Percentage inhibition of EPS production and cell surface hydrophobicity by DTBP (at their BIC) against different M serotypes of GAS. Samples with methanol served as vehicle control. Data are presented as mean ± SD. Student t test demonstrate significant difference between the tests and control (p < 0.005).

Together, the results of EPS quantification and MATH assay suggest that DTBP inhibits the earlier stages of biofilm formation, in particular, at the initial adherence stage. To validate this hypothesis, mature biofilm dispersion assay was performed, which evaluates the ability of an agent to disperse pre-formed biofilm. DTBP was found to have no effect on the pre-formed biofilms of GAS (Figure 3.13).
further manifesting the hypothesis that DTBP acts on the initial stages of biofilm formation. Even though, GAS strains differ in their ability of forming biofilm, the process relies primarily on the adhesion of cells to the substratum at every instance (Limsuwan and Voravuthikunchai, 2008; Kreikemeyer et al., 2011). Hence, affecting cell surface hydrophobicity explains the antibiofilm activity of DTBP against GAS irrespective of its M serotype.

**Figure 3.13.** Effect of DTBP (at their BIC) on mature biofilm dispersion of different M serotypes of GAS. Samples with methanol served as vehicle control. Data are presented as mean ± SD of OD.

**DTBP has no effect on cysteine protease**

Cysteine protease is another important virulence factor of GAS, since it cleaves fibronectin, vitronectin, extracellular matrix proteins and human interleukin-1b into active form thereby causing inflammation, shock and tissue destruction (Honda-Ogawa et al., 2013; Krzysciak et al., 2013). However, DTBP was found to have no effect on the protease production of all the serotypes analyzed (Figure 3.14).
Figure 3.14. Effect of DTBP (at their BIC) on the protease production of different M serotypes of GAS. Samples with methanol served as vehicle control. Data are presented as mean ± SD of OD.

**DTBP renders GAS susceptible to phagocytosis by inhibiting hyaluronic acid production**

Another important virulence factor of GAS is hyaluronic acid capsule, a carbohydrate-based bacterial capsule surrounding the bacterium, which protects GAS from phagocytosis. In addition, the capsule is an important adherence factor in the pharynx, since it binds to CD44 on epithelial cells (Krzysciak et al., 2013; Falaleeva et al., 2014). Acapsular mutant strains had reduced colonization capacities in animal models demonstrating its importance in virulence (Stollerman and Dale, 2008; Tan et al., 2014). Biochemical estimation of hyaluronic acid confirmed that DTBP treatment reduced hyaluronic acid synthesis by 55-65% (Figure 3.15).

To validate the reduction in hyaluronic acid synthesis, blood survival assay was performed, which measures the susceptibility of GAS to phagocytosis. Group A streptococcal clinical isolates grown in the absence and presence of DTBP at their BIC were evaluated for their ability to evade phagocytosis, by enumerating the
number of cells that survive after exposure to healthy human blood. The total number of CFU in DTBP treated samples were 4 - 6 fold less compared to the untreated cells, manifesting that DTBP affects hyaluronic acid mediated anti-phagocytosis (Table 3.2).

**Figure 3.15.** Percentage inhibition of hyaluronic acid synthesis by DTBP (at their BIC) against different M serotypes of GAS. Samples with methanol served as vehicle control. Data are presented as mean ± SD. Student t test demonstrate significant difference between the tests and control (p < 0.005).

**Table 3.2.** Effect of DTBP at their BIC on the survival of clinical isolates of GAS in healthy human blood

<table>
<thead>
<tr>
<th>GAS strains</th>
<th>CFU mL⁻¹</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
</tr>
<tr>
<td>SF370</td>
<td>2.5 x 10³</td>
<td>4.4 x 10²</td>
</tr>
<tr>
<td>SP5</td>
<td>2.7 x 10³</td>
<td>5 x 10²</td>
</tr>
<tr>
<td>SP7</td>
<td>3.5 x 10³</td>
<td>6.6 x 10²</td>
</tr>
<tr>
<td>SP11</td>
<td>1.8 x 10³</td>
<td>3.1 x 10²</td>
</tr>
<tr>
<td>SP22</td>
<td>1.9 x 10³</td>
<td>4 x 10²</td>
</tr>
<tr>
<td>SP30</td>
<td>3.4 x 10³</td>
<td>8.2 x 10²</td>
</tr>
<tr>
<td>SP31</td>
<td>1.1 x 10³</td>
<td>2.3 x 10²</td>
</tr>
</tbody>
</table>
DTBP reduces the MIC of standard antibiotics

Conventionally GAS infections are treated using antibiotics, with penicillin being the preferred choice. For patients allergic to penicillin, antibiotics such as erythromycin and tetracycline are administered (Steer et al., 2012; Ralph and Carapetis, 2013). However, the biofilm mode of GAS growth was found to cause antibiotic resistance, leading to therapeutic treatment failure. This is mainly attributed to the complex three dimensional architecture of biofilm that precludes antibiotic entry. In some cases, the varied physiological and metabolic status of biofilm cells compared to planktonic cells was found to contribute to the antibiotic resistance (Mah, 2012; Subramenium et al., 2015b). Since DTBP inhibits biofilm formation, it is expected to enhance the susceptibility of GAS to antibiotics. This was evaluated by determining the MIC of erythromycin and tetracycline in the absence and presence of DTBP (at their BIC) against the reference strain and the clinical M serotypes of GAS (Table 3.3).

Table 3.3. MIC values of erythromycin and tetracycline against different clinical M serotypes of GAS in the absence and presence of DTBP (at their BIC)

<table>
<thead>
<tr>
<th>GAS strain</th>
<th>MIC of erythromycin (µg mL⁻¹)</th>
<th>MIC of tetracycline (µg mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without DTBP</td>
<td>With DTBP</td>
</tr>
<tr>
<td>SF370</td>
<td>0.5</td>
<td>0.01</td>
</tr>
<tr>
<td>SP5</td>
<td>1</td>
<td>0.01</td>
</tr>
<tr>
<td>SP7</td>
<td>0.5</td>
<td>0.0001</td>
</tr>
<tr>
<td>SP11</td>
<td>0.1</td>
<td>0.0005</td>
</tr>
<tr>
<td>SP22</td>
<td>0.1</td>
<td>0.001</td>
</tr>
<tr>
<td>SP30</td>
<td>0.05</td>
<td>0.0005</td>
</tr>
<tr>
<td>SP31</td>
<td>7</td>
<td>0.1</td>
</tr>
</tbody>
</table>
In general, the tested strains displayed higher resistance towards tetracycline than erythromycin. Among the analyzed clinical isolates, SP31 displayed high MIC values against both erythromycin and tetracycline, while SP30 displayed least MIC values. Nevertheless, a significant decrease in MIC value was observed in the presence of DTBP (at their BIC) against all the strains tested. This result highlights the suitability of using DTBP in combination with antibiotics to treat drug resistant streptococcal biofilms by increasing the susceptibility of bacteria to antibiotics.

**Fourier Transform Infrared (FTIR) Spectroscopic analysis**

FTIR was used to explore the changes in the cellular architecture upon DTBP treatment. Control and DTBP treated *S. pyogenes* SF370 pellets were scanned between the range of 4000-400 cm\(^{-1}\) with a spectral resolution of 4 cm\(^{-1}\). The FTIR spectrum showed significant changes in regions corresponding to fatty acids (3000-2800 cm\(^{-1}\)), amide I and II bonds of protein and peptides (1700-1500 cm\(^{-1}\)) and carbohydrates of microbial cell wall (1200-900 cm\(^{-1}\)) which are highlighted as I, II and III respectively in Figure 3.16A. These differences are ascribed to the biofilm EPS, which is a polymer of proteins, lipids and carbohydrates. This result goes in unison with the biochemical quantification of EPS and SEM analysis wherein, EPS was reduced in DTBP treated sample. The changes in carbohydrates region of microbial cell wall (1200-900 cm\(^{-1}\)) could also be attributed to hyaluronic acid, a carbohydrate based capsule, which was also inhibited upon DTBP treatment. Principal component analysis also complemented the observed variation. In all the three analyzed spectral regions, the control and treated sample lie in different quadrants (Figure 3.16B). Altogether, the FTIR result augments the hypothesis of decreased hydrophobicity as a consequence of reduced EPS production.
Figure 3.16. FTIR analysis of GAS. (A). FTIR spectra of *S. pyogenes* SF370 grown in the presence (48 µg mL⁻¹) and absence of DTBP. Regions showing high variations are highlighted: (I) 3000-2800 cm⁻¹: fatty acid region; (II) 1700-1500 cm⁻¹: amide I and II bonds of proteins and peptides; (III) 1200-900 cm⁻¹: carbohydrates of microbial cell walls. (B). Magnified FTIR spectra (a, b, c) of highlighted regions: I, II and III in (A) and their respective principal component analysis (d, e, f).
Non-toxic DTBP reduces virulence *in vivo*

Biochemical assays carried out in the present study suggests decreased virulence of GAS upon DTBP treatment. To further manifest this, *in vivo* studies were performed using *Caenorhabditis elegans* as model organism. *C. elegans* was selected for *in vivo* studies, since it is a facile, well-established and widely accepted model to study the pathogenesis of GAS (Watson *et al.*, 2016). In addition, the nematode shares a conserved molecular and cellular pathway with humans. In-depth genome analysis has confirmed that orthologs of majority of genes involved in disease pathways in human are also present in *C. elegans* (Balla and Troemel, 2013). The effect of DTBP on the virulence of GAS was assessed by infecting the worms in the absence and presence of DTBP. As a representative, reference strain, *S. pyogenes* SF370 alone was taken for survival analysis. GAS caused complete killing (100 %) of worms at 96 h, whereas only 33 % of worms were dead in the presence of DTBP (Figure 3.17). This result clearly affirms the decreased virulence of GAS in the presence of DTBP. An earlier study have demonstrated that treating GAS with antibiofilm agent, 3-furan carboxaldehyde increases its virulence (Subramenium *et al.*, 2015b) making it incompatible for clinical use. Unlike 3-furan carboxaldehyde, DTBP treatment inhibits biofilm as well as virulence, which augur well for its therapeutic application.

A compound should be non-toxic for its application in clinical settings. Since, *C. elegans* is also popularly used as a bio-monitor for eco-toxicological studies (Leung *et al.*, 2008; Harlow *et al.*, 2016), the same has been used for evaluating the toxicity of DTBP. The toxicity was assessed by comparing the survival rate of worms grown in the presence of DTBP with that grown in the presence of vehicle control (methanol). No significant difference in the survival rate was observed between the
two groups (Figure 3.17), evidencing the non-toxic nature of DTBP and its suitability for clinical application.

![Figure 3.17](image)

**Figure 3.17.** Inhibitory effect of DTBP (48 µg mL⁻¹) on the virulence of *S. pyogenes* SF370 as assessed by *C. elegans* survival rate. The decreased virulence upon DTBP treatment was found to be statistically significant (P <0.005).

**In vivo colonization assay**

*In vivo* colonization of GAS aids in establishing a successful infection in the host (Nobbs *et al.*, 2009). Since, GAS uses biofilm as its mode for colonization, DTBP was anticipated to inhibit the *in vivo* colonization. This was evaluated by calculating the intensity of intestinal bacterial colonization in *C. elegans* using CLSM and CFU assay. CLSM analysis which quantifies fluorescence as a measure of colonization revealed that the intensity of colonization was greatly reduced in DTBP treated samples (50 units) compared to control (150 units) (Figure 3.18). Similar observation was made in the CFU assay, wherein the control had 1.1 x 10⁴ CFU compared to the treated in which only 2.3 x 10³ CFU were present. This corresponds to nearly 5 fold decrease in internalization. Both the results make it apparent that
DTBP treatment effectively inhibits the adherence of GAS to the intestinal tract of the nematode.

**Figure 3.18.** Intensity profile and CLSM images of *C. elegans* exposed to *S. pyogenes* SF370 in the absence and presence of DTBP (48 µg mL⁻¹).

To conclude, the obtained results demonstrate the antibiofilm potential of DTBP against different clinical M serotypes of GAS. The study suggests that DTBP targets the initial adhesion stage of the biofilm formation cascade by affecting cell surface hydrophobicity and EPS production. Reduced hyaluronic acid synthesis was hypothesized to be the prime reason for the impaired virulence. Furthermore, DTBP treatment increased the susceptibility of GAS towards antibiotics, attesting its suitability to be used in combination with antibiotics. The decreased virulence and biofilm formation was confirmed using *in vivo* studies in *C. elegans*. Nevertheless, the results needs to be further validated in higher eukaryotes. Considering its non-bactericidal and nontoxic nature, DTBP could be a promising candidate to evade the onset of GAS pathogenesis.
Summary

Epiphytic bacterial isolates of the red seaweed (*Gracilaria* sp.) and seagrass (*Cymodaceae* sp.), were screened for their ability to inhibit biofilm formation by GAS. Among the bacterial isolates screened, seaweed surface associated bacterium R60 (*Bacillus subtilis*) was found to have greater potency in inhibiting the biofilm of GAS. Bioactivity guided purification revealed 2,4-Di-tert-butyl-phenol (DTBP) as the active principle. DTBP exhibited a dose dependent antibiofilm activity against GAS (SF370 and six different clinical M serotypes). Microscopic analysis revealed changes in cell surface architecture and reduced thickness upon DTBP treatment. Results of extracellular polymeric substance quantification, microbial adhesion to hydrocarbon assay and fourier transform infrared spectroscopic analysis suggested that DTBP probably interferes with the initial adhesion stage of biofilm formation cascade. Reduction in hyaluronic acid synthesis goes in unison with blood survival assay wherein, increased susceptibility to phagocytosis was observed. *In vivo* studies using *C. elegans* manifested the reduction in adherence and virulence, which prompts further investigation of the potential of DTBP for the treatment of GAS infections.