Chapter 3B

Role of $\beta$ 1-4 linked polymers in biofilm structure of marine *Pseudomonas* sp CE-2 on 304 - stainless steel coupons.

Exopolymers play an important role in biofilm formation and structure.
3B.1 Introduction

Microorganisms readily attach to surfaces immersed in aquatic environments, and form biofilms enmeshed in extracellular polymeric matrix (Costerton et al., 1999). Although the appearance and functions of biofilm in various environments may be different, the steps leading to biofilm formation are the same (Escher & Characklis, 1990). The first step is the development of the conditioning film (Loeb & Neihof, 1975; Bhosle et al., 2005). Microorganisms then colonize the conditioned surfaces. Attached microorganisms grow and develop into biofilms with the help of exopolysaccharides (EPS) and/or other cellular appendages (Busscher et al., 1995).

Bacteria produce different types of EPS that vary in size, configuration and linkages (Allison, 1998). Several functions have been ascribed to EPS. However, protection and maintenance of biofilm structure appear to be the most important. For example, colonic acid ($\beta$, 1-3 linkage), alginate ($\beta$, 1-4 linkage), and polysaccharide intercellular adhesin (PIA) ($\beta$, 1-6 linkage) produced by *Escherichia coli*, *Pseudomonas areuginosa*, and *Staphylococcus aureus* and *Staphylococcus epidermidis*, respectively are known to play important role in the formation and maintenance of the biofilm (Danese et al., 2000; Kaplan, 2004; Chang et al., 2007). Similarly, production of calcofluor binding ($\beta$, 1-4 linked) polymers or cellulose type polymers by *Acetobacter xylinus*, *Agrobacterium tumefaciens*, *Rhizobium* sp, *Hyphomonas* sp, *Escherichia coli*, *Salmonella typhimurium*, and *Salmonella enteritidis*, and the role of these polymers in biofilm formation have been reported earlier (Ross & Witter, 1983; Quintero, 1995; Zogaj...
et al., 2001; Solano et al., 2002). From these studies, it appears that the linkages between the subunits of the polymers give functional specificity to biofilm EPS. Nevertheless, our understanding of the role of such polymers in biofilm formation and maintenance is far from complete. It is possible that bacteria may produce polymers with different linkages for adhesion and biofilm formation. Therefore, in order to better understand the process of biofilm formation, it is essential to characterize the polymer linkages involved in biofilm formation onto surfaces. The objectives of the present research were to study the biofilm formation and EPS production by the CE-2 biofilms, and to identify the involvement of polysaccharide linkages in biofilm formation.

3B.2 Materials and methods

3b.2.1 Chemicals and reagents
Calcofluor, protease (Bacillus polymyxa), lipase (Candida rugosa), and cellulase (Trichoderma reesei) were purchased from Sigma- Aldrich, USA.

3b.2.2 Coupon preparation
Coupons of 304-stainless steel (SS) (1.2 mm diameter) were prepared following the method described in section 3a.2.2 of the chapter 3A.
3b.2.3 Growth media, bacteria and inoculum preparation

Basal salt solution (BSS) medium was used in the present study. The medium composition was described in the section of 2.2.7 of the chapter 2. *Pseudomonas* sp CE-2 was used in the present study.

3b.2.4 Biofilm formation by CE-2

The culture CE-2 biofilms were formed on the SS coupons (1.2 mm diameter), over a period of time, using the method described in the section 3a.2.4 of the chapter 3A.

3b.2.5 Quantification of CE-2 biofilm formation

Biofilm formed on the SS coupons was quantified by estimating total bacterial cell counts using the procedure described in section 3a.2.5 of the chapter 3A.

3b.2.6 Estimation of extracellular polysaccharides (EPS) of the CE-2 biofilms

Biofilm material developed on the SS coupons was **scraped** in 2 ml TE buffer, using nylon brush and made to a known volume (Sharma *et al*., 1990; D'Souza, 2004; Jadadish & Anil, 2005). The **scraped** biofilm material was vortexed and allowed to stand for 20 min. Cell free supernatant was obtained by centrifuging the above sample for 15 min at 10,000 g and 4 °C. A known aliquot (500 µl) of the cell free supernatant was transferred to a clean test tube and 1 ml phenol (2.5 %) followed by 2.5 ml concentrated sulphuric acid were added, and the
absorbance was read at 490 nm using a UV-VIS spectrophotometer (Shimadzu-1601) so as to estimate total carbohydrates (Dubois et al., 1956). Alternatively, biofilm material developed on SS coupon was used directly without any pre-treatment so as to estimate total carbohydrates using phenol-sulphuric acid method. These two approaches helped us to assess efficiency of the nylon brush to remove EPS from the coupons.

3b.2.7 Calcofluor staining of the CE-2 biofilms

Biofilm formed by CE-2 on the SS coupons over a period of time as described above was stained with calcofluor. Hundred micro liters of the calcofluor (250 µg ml⁻¹) were added directly on the top of the CE-2 biofilm formed on SS coupons. The coupons were incubated in dark for 30 min at room temperature (28 °C ± 2). Subsequently, the coupons were rinsed thrice with PBS so as to remove the excess stain. The dye taken up by the biofilm sample was visualized under 100X oil objective of the epifluorescence microscope using DAPI filter set (excitation 340/380 nm, emission 435/485). The images were captured using CCD camera (Evolution VF) and stored as separate digital files.

3b.2.8 Effect of calcofluor on growth of CE-2 cells

Growth of CE-2 cells was monitored in the presence and the absence of calcofluor to find out, if it was toxic to CE-2 cells. Calcofluor solution (2 mg ml⁻¹) was prepared in PBS, and the solution was sterilized by passing through 0.22 µm filter (Millipore, USA). From this, 5 ml was added to the flask containing 50 ml
BSS medium to get a final concentration of 200 µg ml\(^{-1}\). Similarly, 5 ml sterile PBS was added to other flask containing 50 ml BSS medium and was used as a control. Both the flasks were inoculated with 500 µl of pre-grown CE-2 culture (OD 540 nm = 1.6 ± 0.2), and incubated at room temperature (28 °C ± 2). Samples were removed in triplicates at regular intervals over a period of 48 h. A known amount of sample (500 µl) from both the flasks was serially diluted in 4.5 ml sterile PBS, and 50 µl from the last three highest dilutions were spread plated on the Zobell marine agar plates. The plates were incubated at room temperature for 24 h and colonies were counted manually and expressed as log CFU ml\(^{-1}\).

3b.2.9 Effect of lectins and calcoflour on CE-2 biofilm formation

Stock solutions (2 mg ml\(^{-1}\)) of Con A, WGA lectin and calcofluor were prepared in PBS. Before use, these chemicals were sterilized by filtering through 0.22 µm filter (Millipore, USA). A known amount (100 µl) of each of these chemicals was added individually to the well containing a SS coupon and 1 ml of the culture prepared as above. The microplates were incubated for 72 h at room temperature. The biofilm biomass (measured as total cell number mm\(^{-2}\)) on SS surface was evaluated as described above. The % biofilm inhibition was calculated by dividing the number of cells on the SS coupons after chemical treatment by the total cells on the control coupons. The value obtained from above was subtracted from 1 and multiplied by 100.
3b.2.10 Lectin-staining of CE-2 biofilms

CE-2 biofilms on SS coupons were stained with FITC labelled lectins using the method described in the section 3a.2.7 of the chapter 3 A.

3b.2.11 Enzyme treatment of CE-2 biofilms

In order to evaluate type of polymers involved in the development of CE-2 biofilm, it was treated with protease (*Bacillus polymyxa*), lipase (*Candida rugosa*), and cellulase (*Trichoderma reesei*). For this purpose the biofilm was allowed to form on SS coupons for 72 h as described above and then treated with the above mentioned enzymes. Biofilms containing coupons were immersed in 1ml of cellulase (60 and 120 U ml⁻¹) solution, prepared in 0.05 M sodium citrate buffer (pH 5.0), and incubated at 45 °C for 72 h (Solano *et al.*, 2002). Similarly, coupons were immersed in 1 ml of protease (2.4 and 4.8 U ml⁻¹) and lipase (175 and 350 U ml⁻¹) prepared in 10 mM PBS buffer (pH 7.2), and incubated for 3 hours at 28 °C and 37°C, respectively. In order to avoid the effect of buffer, incubation time and temperature, coupons with biofilms were immersed in 1ml buffer solution without enzyme solution. For each enzyme treatment separate sets of control coupons (in triplicates) were incubated, under similar conditions. Biofilm biomass was estimated following the procedure described above.

3b.2.12 Epifluorescence microscopy

Biofilms formed by CE-2 on SS coupons were monitored using Nikon Eclipse 80i upright microscope, as described in section 3a.2.9 of the chapter 3 A.
3b.2.13 Statistical analysis

One-way analysis of variance (ANOVA) was used to evaluate the effect of calcofluor on growth of cells. A simple linear regression analysis was carried out to assess the relationships between EPS and biofilm biomass on SS coupons. All calculations were performed using Excel software.

3B.3 Results

3b.3.1 Biofilm formation and EPS production

The culture CE-2 formed biofilms on SS coupons. Biofilm biomass measured as total bacterial counts generally increased over the period of incubation and the highest biomass on SS was achieved at 72 h following inoculation (Figure 3b.1). Biofilm biomass gradually decreased for the remaining period of incubation (Figure 3b.1). EPS concentration also increased over the period of incubation and the highest concentration was recorded at 72 h following inoculation. Thereafter, there was a gradual decrease in EPS concentration until 120 h following inoculation (Figure 3b.1). Both biofilm biomass and EPS concentration showed similar trends, and there was a significant positive correlation between these two parameters ($r = 0.979$, $p > 0.001$).
3b.3.2 Staining of the biofilms with calcofluor

Calcofluor bound to CE-2 biofilm matrix and produced blue fluorescence (Figure 3b.2). Moreover, it appears from epifluorescence photographs that fluorescence intensity of the dye was highest at 72 h following inoculation (Figure 3b.2).

Figure (3b.1). Biofilm formation ($\Delta$) and EPS production ($\square$) by CE-2 on 304-SS coupons as a function of the incubation period.
Figure (3b.2). Calcofluor stained CE-2 biofilms on SS over 120 h period of incubation. Note that, at 72h the fluorescence intensity of calcofluor stained biofilm is highest. Scale bar represents 10 μm.
3b.3.3 Effect of calcofluor on growth of CE-2 cells

There was not much variation in the growth of CE-2 cells in presence and absence of calcofluor (Figure 3b.3). Moreover, one way analysis of variation (ANOVA) suggest that there was no significant decrease in cell viability (log CFU/ml) of CE-2 cells (p = 0.8315) in the presence of calcofluor. Thus the growth and viability of CE-2 cells were not influenced to a great extent by the concentration of calcofluor (200 µg/ml) used to conduct inhibition experiments described below.

Figure (3b.3). Effect of calcofluor (200µg ml ^{-1} ) on the growth of CE-2 cells.
3b.3.4 Effect of lectin and calcofluor on the inhibition of CE-2 biofilms

When grown in the presence of calcofluor (200 μg ml\(^{-1}\)), CE-2 biofilm biomass on SS coupons decreased by \(\sim 85\%\). Conversely, when treated with Con A or WGA at the same concentration (200 μg ml\(^{-1}\)), CE-2 biofilm biomass was reduced marginally by 7% and 1%, respectively (Table 3b.1).

Table (3b.1). Effect of calcofluor, Con A and WGA on CE-2 biofilms on SS 304.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cells x 10^5 per mm^2</th>
<th>Percentage biofilm inhibition* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>65 ± 3</td>
<td>0</td>
</tr>
<tr>
<td>Calcofluor</td>
<td>9.5 ± 2</td>
<td>85 ± 15</td>
</tr>
<tr>
<td>Con A</td>
<td>61 ± 1.4</td>
<td>7 ± 3</td>
</tr>
<tr>
<td>WGA</td>
<td>64 ± 0.4</td>
<td>1 ± 0.5</td>
</tr>
</tbody>
</table>

* Mean of percentage of biofilm inhibition data from 3 replicates coupons (± standard deviation).

3b.3.5 Lectin binding to CE-2 biofilms

Con A lectin binds to the CE-2 biofilm matrix, and produced bright green cloudy appearance around fluorescent blue microcolonies (Figure 3b.4 A). However, cloudy green background in between blue cells was also observed. It may be because of weak fluorescent signals from glycoconjugates present in deeper layers of biofilm matrix.
Figure (3b.4). Photographs of CE-2 biofilm after staining with con A (A) and WGA (B). The green fluorescence represents the lectin staining and blue fluorescence represents the staining due to DAPI. Scale bar = 10 µm.
While WGA lectin showed weak green fluorescent signals and only fluorescent blue cells were predominantly seen in the photographs (Figure 3b. 4 B).

3b.3.6 Enzyme treatment of biofilms

The amount of biofilm removal by each enzyme treatment was evaluated with respect to their respective controls. Control biofilm coupons without enzyme treatments appear similar under epifluorescence microscope (Figure 3b.5). However, a small decrease in biofilm biomass (cells mm$^{-2}$) was observed for the cellulase treated control biofilms as compared to lipase and protease treated controls. About 48 % of the biofilms were removed from SS coupons when treated with cellulase enzyme (120 U ml$^{-1}$) (Table 3b.2). Furthermore, it was interesting to note that after cellulase treatment, microscopic observation revealed the presence of a central hollow in the microcolonies of CE-2 (Figure 3b.5 B). Protease and lipase treatment removed 23 % and 17% of the biofilms, respectively (Table 3b.2).

3B.4 Discussion

Removal of biofilms from test coupons is perhaps the most critical step in evaluating the biomass and other aspects of the biofilms. Removal of biofilms by brushing is one of the commonly employed techniques (Bhosle et al., 1989; Sharma et al., 1990; D’Souza & Bhosle, 2003; D’Souza et al., 2005; Jadadish & Anil, 2005). A nylon brush removed about 89 ± 2.19 % of the biofilm biomass (measured as bacterial numbers, chlorophyll-a, organic carbon and nitrogen, and
Figure (3b.5). Treatment of CE-2 biofilm using various enzymes stained with acridine orange. Biofilm not treated with enzyme (control) and after treatment with enzymes (B). Note the central hollow in microcolonies after treatment with cellulase. Scale bar represents 10 μm.
protein) from metal and non-metal surfaces (Sharma et al., 1990; D’Souza & Bhosle, 2003; Jadadish & Anil, 2005). In the present study, we evaluated the removal of EPS (as total carbohydrate) from the SS coupons. It was possible to remove 80% ± 6% of the biofilm EPS (measured as total carbohydrates) using nylon brush. A nylon brush was therefore fairly efficient to remove biofilm material from SS coupons.

Table (3b.2). Removal of CE -2 biofilms from SS-304 coupons using cellulase, protease and lipase treatment.

<table>
<thead>
<tr>
<th>Enzyme treatment</th>
<th>Cells x 10⁶ per mm²</th>
<th>Percentage biofilm removal* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cellulase</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>37 ± 0.1</td>
<td>0</td>
</tr>
<tr>
<td>60U</td>
<td>26 ± 4.9</td>
<td>22 ± 11</td>
</tr>
<tr>
<td>120U</td>
<td>19 ± 2.8</td>
<td>48 ± 8</td>
</tr>
<tr>
<td><strong>Protease</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>45 ± 2.6</td>
<td>0</td>
</tr>
<tr>
<td>2.4</td>
<td>41 ± 1.0</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>4.8</td>
<td>35 ± 6.2</td>
<td>23 ± 4</td>
</tr>
<tr>
<td><strong>Lipase</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>46.5 ± 4.9</td>
<td></td>
</tr>
<tr>
<td>175U</td>
<td>41 ± 1.4</td>
<td>12 ± 3</td>
</tr>
<tr>
<td>350U</td>
<td>38.5 ± 2.2</td>
<td>17 ± 5</td>
</tr>
</tbody>
</table>

* Mean of percentage of biofilm removal data from 3 replicates coupons (± standard deviation)
Our results indicate that CE-2 cells attached to the SS coupons, produced EPS and formed biofilms. Biofilm biomass on SS increased by at least 15 fold over the 72 h period of incubation. The observed increase in biofilm biomass was probably due to the growth of attached bacterial cells or increase in settlement of suspended cells from the bulk medium. The later seems unlikely because the biofilm containing coupons were rinsed several times with sterile PBS to remove unattached or loosely attached bacterial cells. When the incubation period was further increased to 120 h, biofilm biomass on SS decreased (Figure 3b.1). This decrease probably indicates nutrient starvation and/or production of the enzymes (polysaccharide lyases) involved in the detachment of biofilms (Allison et al., 1998). Moreover, EPS concentrations showed a trend similar to that observed for the biofilm biomass over the period of incubation. This implies strong correspondence between the biofilm biomass and the EPS production. This was also evident from the highly significant relationship \( r = 0.9797, p > 0.001 \) between the biofilm biomass and the EPS concentrations.

Epifluorescence microscopic observation revealed the binding of calcofluor to CE-2 biofilm matrix (Figure 3b.2). This implies production of cellulose like polymers containing β, 1-4 linkages by CE-2 cells (Zogaj et al., 2001). Calcofluor has a highly conjugated and planar structure, which enable it to bind cellulose type polymers (β, 1-4 linked polymers) via hydrogen bonding, dipolar interactions, and Van der Waals forces. Moreover, it is clear from the epifluorescence photographs that at 72 h calcofluor stained biofilms have highest fluorescence intensity as compared to the biofilm collected at different time.
interval (Figure 3b.2). This is in agreement with earlier reports suggesting a 7-fold increase in fluorescence intensity of calcofluor stained cellulose like polymers (Wood, 1980). The high fluorescent intensity of calcofluor also probably signifies high abundance of the β, 1-4 linked polymers (Wood, 1980). Therefore, it appears that after the initial attachment to SS coupons, CE-2 cells produced copious amounts of β, 1-4 linked polymers, especially at 72 h following inoculation. Such polymers may play important role in biofilm formation and maintenance. For example, the role of β, 1-4 linked polymers in the development of marine *Hyphomonas* sp biofilm has been well documented (Quintero & Iveyiner, 1995).

In order to further evaluate the role of β, 1-4 linked polymers, development of CE-2 biofilms on SS coupons was studied in the presence and absence of calcofluor (200 μg ml⁻¹). It was interesting to note that when grown in the presence of calcofluor (200 μg ml⁻¹), CE-2 biofilm biomass on SS was reduced by ~ 85 %. Similarly, ~ 85 % reduction in the *Hyphomonas* sp biofilm biomass on glass was recorded when it was grown in the presence calcofluor (75 μg ml⁻¹) (Quintero & Iveyiner, 1995). Nevertheless, it could be argued that the observed reduction in biofilm biomass was due to toxicity of calcofluor (200 μg ml⁻¹) to CE-2 cells. However, calcofluor did not inhibit growth and viability of the CE-2 cells implying that it was not toxic (Figure 3b.3). Moreover, Ross, (1983) reported that calcofluor is not very toxic to many microorganisms. This strongly supports the above conclusion that β, 1-4 linked polymers were involved in CE-2 biofilm development and maintenance.
The observed reduction in CE-2 biofilm formation on SS coupons was probably due to the binding of the calcofluor with β, 1-4 linked polymers. Such interaction might have changed the polymer conformation, probably resulting in the production of weak biofilms (Haigler et al., 1980; Roberts et al., 1982). These weak biofilms could not withstand the shear exerted during washing that ultimately lead to the removal of the biofilms. Our data strongly indicate the possible role of β 1-4 linked polymers (may be cellulose type) in maintenance of the CE-2 biofilm.

Biofilm biomass did not decrease in the presence of both Con A and WGA lectins, (Table 3b.1). Epifluorescence microscopic observation revealed the binding of Con A but not of WGA to the CE-2 biofilms (Figure 3b.4). These results indicate the presence of terminal α-D-mannose and/or α-D-glucose residues and the absence of N-acetyl glucosamine residues in the CE-2 biofilm matrix. However, from our results, it was evident that these molecules did not play any major role in the formation and maintenance of the CE-2 biofilms on SS coupons.

In order to further assess the possible role of β, 1-4 linked polymers in CE-2 biofilm structure, it was treated individually with protease, lipase and cellulase. It appears from total cell mm$^{-2}$ of control biofilm that buffer, incubation time and temperature did not affect biofilms significantly during each enzyme treatment. This suggests that the effect seen on biofilm after enzyme treatment was purely because of enzyme activity. Protease and lipase treatment removed small amounts (~ 20 %) of CE-2 biofilms from SS coupons (Table 3b.2). In contrast,
of the CE-2 biofilms from the SS coupons (Table 3b.2). After cellulose treatment, a central hollow was recorded in the microcolonies of CE-2 biofilms when observed under epifluorescence microscope. This observation suggests that cellulase hydrolyzed calcofluor binding β, 1-4 linked polymers present in the CE-2 biofilms (Figure 3b.5 B). Role of calcofluor binding and cellulase sensitive polymers in biofilms of *Pseudomonas fluorescens*, *Escherichia coli*, *Salmonella enteritidis*, *Salmonella typhimurium*, and *Yersinia pestis* has been reported (Zogaj *et al.*, 2001; Darby *et al.*, 2002; Spiers *et al.*, 2002; Solano *et al.*, 2002). Moreover, our data are in accordance with the above observations indicating that calcofluor binding and cellulase sensitive β, 1-4 linked polymers were involved in formation and maintenance of CE-2 biofilm on SS coupons. Thus, understanding the types of linkages in EPS and their role in maintenance of marine biofilm structure are of particular interest to develop techniques to control the problem of biofilms on marine structure. However, biofilm development on solid surfaces has been studied only for a few bacteria. Therefore, further studies using different bacteria and growth media are needed to better understand the mechanism of bacterial adhesion.