

**Role of β , 1-4 linked polymers in biofilm structure of marine *Pseudomonas* sp CE-
2 on 304 - stainless steel coupons**

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Abstract

Pseudomonas sp CE-2 cells attach and form biofilms on 304- stainless steel (SS) coupons. A series of experiments were carried out in order to understand the role of exopolymers (EPS) in the formation and maintenance of CE-2 biofilms on SS coupons. Biofilm density and EPS concentration increased over the period of incubation and the highest values for both were recorded at 72h following incubation. Calcofluor and concavalin A (Con A) lectin showed positive interaction with 72 h old biofilms indicating the presence of β 1-4 linked polymers, and α -D-glucose and α -D-mannose in the CE-2 biofilm matrix. When the CE-2 cells were grown in the presence of calcofluor (200 μ g/ml), biofilm formation was significantly reduced (~ 85 %). Conversely, Con A, or *Triticum vulgare* lectin (WGA), did not influence the CE-2 biofilms on SS coupons. Furthermore, treatment with cellulase, an enzyme specific for the degradation of β 1- 4 linked polymers, removed substantial amounts of CE-2 biofilms from SS coupons. These results strongly suggest the involvement of β 1- 4 linked polymers in the formation and maintenance of *Pseudomonas* sp CE-2 biofilms on SS coupons.

Keywords: Pseudomonas sp, biofilms, calcofluor, β 1-4 linked polymer, cellulase

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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 and Neihof, 1975; Bhosle et al. 2005). Microorganisms then colonize 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 (β 1-3 linkage), alginate (β 1-4 linkage), and polysaccharide intercellular adhesin (PIA) (β 1-6 linkage) produced by *Escherichia coli*, *Pseudomonas aeruginosa*, 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 (β 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, 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. Therefore, the main objectives of the present study were, 1) to study the biofilm formation by the culture CE-2, 2) to evaluate the EPS

production by the CE-2 biofilms, and 3) to identify the involvement of linkages in biofilm formation.

Materials and methods

Chemicals and reagents

Calcofluor, 4,6-diamidino-2-phenylindole (DAPI) and FITC labelled lectins such as Concanavalin A (Con A) and wheat germ agglutinin (WGA) were purchased from Sigma-Aldrich, USA. All the three enzymes, protease (*Bacillus polymyxa*), lipase (*Candida rugosa*), and cellulase (*Trichoderma reesei*) were also purchased from Sigma-Aldrich, USA. Zobell marine agar (ZMA), Zobell marine broth (ZMB), peptone, yeast extract, and other chemicals were purchased from Hi-media, INDIA.

coupon preparation

Coupons of 304-stainless steel (SS) (1.2 mm diameter) were purchased from **BIOSURFACE TECHNOLOGY COOPERATION, USA**. Coupons were prepared following the method described in Jain et al. (2007). Briefly, coupons were treated with liquid detergent (Labolene; Qualigens Fine Chemicals, INDIA), washed thoroughly with distilled water and air-dried. The coupons were then covered with aluminum foil and sterilized in autoclave at 121 °C and 15 lb for 20 min.

Growth media, bacteria and inoculum preparation

Throughout the course of this study a modified basal salt solution (BSS) medium was used. The medium contained (g l⁻¹): 5.0 Peptone, 1.0 Yeast Extract, 25.0 NaCl, 0.75 KCl, 7.0 MgSO₄, 0.9 NH₄Cl, 0.2 CaCl₂, 7.0 K₂HPO₄, 3.0 KH₂PO₄, 1.0 glucose and 1.0 ml l⁻¹ trace metal solution (Bhosle. 1981). All media were sterilized as **described** above. The bacterial culture used in the present study was obtained from the culture collection of the Marine Corrosion and Materials Research Division, NIO, Goa (India). The culture was isolated from the biofilm developed on copper panels immersed in the surface

coastal waters of Dona Paula Bay, west coast of India. For routine use the culture was maintained over Zobell marine agar slant and preserved at $-4\text{ }^{\circ}\text{C}$ in a refrigerator. Inoculum was prepared by transferring **loop full** culture from slant to flask containing 10 ml of the BSS medium. The flask was incubated at room temperature ($28 \pm 2\text{ }^{\circ}\text{C}$) for 24 h. This step was repeated once more, and the culture was used as a source of inoculum for the experiments described below.

Biofilm formation

Biofilm was formed on the SS coupons (1.2 mm diameter) using 24 well polystyrene microplates (Corning, USA). In brief, 25 ml of **the** BSS medium was inoculated with **250 μl** of the pregrown culture ($\text{OD}_{540} = 1.6 \pm 0.2$; cell density = 4.5×10^8 cells ml^{-1}). One ml of this suspension was dispensed into each well of the micro-plate. Subsequently, one coupon was placed flat into each well using a sterile forcep under laminar flow (Micro filter, India), and incubated at room temperature ($28\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$) over a period of 120 h.

Quantification of biofilm formation

Biofilm formed on the SS coupons was quantified by estimating total bacterial counts. Briefly, coupons were removed in triplicates at fixed intervals over a period of 120 h and then rinsed thrice with phosphate buffer saline (10 mM PBS). The coupon was placed in a clean Petri plate with 5 ml TE buffer (10 mM Tris – HCl, pH 8, 10 mM EDTA and 1.5 M NaCl). Coupon was held with sterile forcep and firmly scraped with nylon brush for about one minute (Sonak and Bhosle 1995; D'Souza, 2003; D'Souza et al., 2005). The entire content was transferred to the clean test tube and 150 μl formaldehyde (40%) was added as fixative. Two ml aliquot of this sample was sonicated for 30 seconds and vortexed for 1 min, **in order to get individual cells**. Two hundred microliters of 0.1% acridine orange (Sigma-Aldrich, UAS) was added and the sample was incubated for 10 min in dark. The sample was filtered onto 0.22 μm black polycarbonate filter paper (Millipore, USA). After filtration the paper was placed over immersion oil on glass slide. The number of bacteria was counted in 10 randomly chosen fields under 100 X oil

immersion objective of epifluorescence microscope (Nikon 80 i, Japan) using FITC filter (excitation 465/465nm, emission 515/555nm).

Estimation of extracellular polysaccharides (EPS) of the CE-2 biofilms

Biofilm material developed on the SS coupons was scrapped 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 scrapped 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 at 4 °C. A known aliquot (500 µl) of the cell free supernatant was transferred to a clean test tube and 1ml 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 so as to estimate total carbohydrates using phenol-sulphuric acid method. These two approaches helped us to asses efficiency of the nylon brush to remove EPS from the coupons.**

Calcofluor staining of the CE-2 biofilms

Biofilm formed on the SS coupons over a period of time as described above, was stained with 100 µl of the calcofluor (250 µg/ml). The dye was added directly on the top of biofilm on SS coupons, and the coupons were incubated in dark at room temperature for 30 min. The coupons were rinsed thrice with PBS and air- dried. The dye taken up by the biofilm sample was visualized under 100 X oil immersion objective of the epifluorescence microscope using DAPI filter set (excitation 340/380nm, emission 435/485 nm). The images were captured using CCD camera (Evolution VF) and stored as digital files.

Effect of calcofluor on growth of CE-2 cells

Growth of CE-2 cells was monitored in the presence and absence of calcofluor to find out if it was toxic to CE-2 cells. Calcofluor solution (2 mg/ml) 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. Similarly, 5 ml sterile PBS was added to other flask containing 50 ml BSS medium and was used as a control. Both the flask were inoculated with 500 µl of pregrown CE-2 culture ($OD_{540} = 1.6 \pm 0.2$), and incubated at room temperature ($28\text{ }^{\circ}\text{C} \pm 2$). 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.

Effect of lectins and calcofluor on biofilm formation.

Stock solutions (2 mg/ml) 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 72h 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.

Lectin-binding

Biofilms of CE-2 were stained with 4,6–diamidino–2- phenylindole (DAPI) specific for nucleic acid to demonstrate the location of glycoconjugates relative to cells and microcolonies in the biofilms. FITC labelled lectins such as concanavalin A (Con A)

from the jackbean *Canavalia ensiformis* and the wheat germ agglutinin (WGA) from *Triticum vulgare* were used to stain glycoconjugates in CE-2 biofilm formed on SS coupons. The Con A from the jackbean *Canavalia ensiformis* is specific for α -D-mannose or α -D-glucose while WGA from *Triticum vulgare* is specific for N-acetyl-D-glucosamine (Goldstein and Hayes, 1978). Stock solutions of the above lectins (1 mg/ml) were prepared in 10 mM PBS (pH 7.5) and stored frozen. Before use, the lectins were thawed and diluted using PBS to get a concentration of 10 μ g/ml (Strathmann et al. 2002). Biofilms on the SS coupons was stained following the procedure of Hamilton (2003). Briefly, 100 μ l of the lectin solution was added on the top of the biofilms on SS coupons. The samples were kept in dark for 30 min at room temperature, excess of lectin was removed by washing with PBS. The samples were air-dried and counter stained with 100 μ l of DAPI (0.01mg/ml). The samples were again incubated in dark for 30 min followed by washing with PBS. Immediately, after staining the samples were visualized under epifluorescence microscope. Signals for lectins were recorded using FITC filter (excitation 465/465nm, emission 515/555nm) and DAPI was recorded using DAPI filter (excitation 340/380nm, emission 435/485 nm). The images were captured through 100 X oil immersion objective, using CCD camera (Evolution VF) and images were stored as separate digital files.

Enzyme treatment of CE-2 biofilms

In order to evaluate type of polymer in CE-2 biofilm development, it was treated with protease (*Bacillus polymyxa*), lipase (*Candida rugosa*), and cellulase (*Trichoderma reesei*). The biofilm was allowed to form on SS coupons for 72h as described above and then treated with enzymes. In brief, biofilms containing coupons were immersed in 1ml of cellulase (60 and 120 U/ml) solution, prepared in 0.05 M sodium citrate buffer (pH 5.0), and incubated at 45 °C for 72 hours (Solano et al. 2002). Similarly, coupons were immersed in 1 ml of protease (2.4 and 4.8 U/ml) and lipase (175 and 350 U/ml) 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.

Epifluorescence microscopy

Biofilms formed on SS coupons were viewed by transmitted light generated from fluorescence illuminator (EXFO, Canada) passed through FITC and DAPI (Nikon, Japan) filters fitted in Nikon Eclipse 80i upright microscope using plan-fluro objectives. The CCD camera (Evolution VF, Media-Cybernetics, USA), and the Image Pro-Plus 6.1 software (Media-Cybernetics, USA) were used to capture the images.

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.

Results

Biofilm formation and EPS production

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 1). Biofilm biomass gradually decreased for the remaining period of incubation (Figure 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 1). Both biofilm biomass and EPS concentration showed similar trends, and there was a significant positive correlation between these two parameters ($r = 0.979$, $p > 0.001$).

Staining of the biofilms with calcofluor

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

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 3). Moreover, one way analysis of variation (ANOVA) suggest that there was no significant decrease in cell viability ($\log \text{CFU ml}^{-1}$) of CE-2 cells ($p = 0.8315$) in presence of calcofluor. Thus the growth and viability of CE-2 cells were not influenced to a great extent by the concentration of calcofluor ($200\mu\text{g/ml}$) used to conduct inhibition experiment described below.

Effect of lectin and calcofluor on the inhibition of CE-2 biofilms

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

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 4A). 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. While WGA lectin showed weak green fluorescent signals and only fluorescent blue cells were predominantly seen in photographs (Figure 4B).

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 (picture not shown). 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) (Table II). 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 5 B). Protease and lipase treatment removed 23 % and 17% of the biofilms, respectively (Table II).

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, and is routinely used in our laboratory (Bhosle et al. 1989; Sharma et al.1990; Jadadish and Anil, 2005 D'Souza and Bhosle, 2003; D'Souza et al. 2005). A nylon brush removed about 89 ± 2.19 % of the biofilm biomass (measured as bacterial numbers, chlorophyll-a, organic carbon and nitrogen, and protein) from metal and non-metal surfaces (Sharma et al.1990; D'Souza, 2003; Jadadish and Anil, 2005). In the present study, we evaluated the removal of EPS (as total carbohydrate) from the SS coupons. Using nylon brush we could remove $80\% \pm 6\%$ of the EPS (measured as total carbohydrates) from the coupons used in the present study. A nylon brush is therefore fairly efficient to remove biofilm material from SS coupons.

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 1). This decrease probably indicates nutrient starvation and/or production of polymer degrading enzymes (polysaccharide lyases) in the biofilms (Allison et al 1998). Similarly, over the period of incubation (up to 72h), EPS concentrations showed a trend similar to that observed for the biofilm biomass. This implies strong correspondence between the biofilm biomass and 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 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 have highest fluorescence as compared to the other time intervals (Figure 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 signifies high concentration of 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 polymer in the development of marine *Hyphomonas* sp biofilm has been well documented (Quintero, 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 ($200 \mu\text{g ml}^{-1}$). It was interesting to note that when grown in the presence of calcofluor ($200 \mu\text{g ml}^{-1}$), CE-2 biofilm biomass on SS was reduced by $\sim 85\%$. Similarly, $\sim 85\%$ reduction in the *Hyphomonas* sp biofilm biomass on glass was recorded when it was grown in the

presence calcofluor ($75 \mu\text{g ml}^{-1}$) (Quintero, 1995). Nevertheless, it could be argued that the observed reduction in biofilm biomass was due to toxicity of calcofluor ($200 \mu\text{g ml}^{-1}$) to CE-2 cells. However, calcofluor did not inhibit significant growth and viability of the CE-2 cells implying that it was not toxic (Figure 3). Moreover, Ross (1983) reported that calcofluor was 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 changed polymer conformation (Haigler et al. 1980; Roberts et al. 1982) probably resulting in the production of weak biofilms. 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 I). Epifluorescence microscopic observation revealed the binding of Con A but not of WGA to the CE-2 biofilms (Figure 4). These results indicate the presence of terminal α -D-mannose and/or α -D-glucose residues and 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 ($\sim 20\%$) of CE-2 biofilms from SS coupons (Table II). In contrast, cellulase (120U/ml) treatment removed relatively large amounts ($\sim 48\% \pm 8\%$) of the CE-2 biofilms from the SS coupons (Table II). After cellulase treatment, a central hollow was recorded in the

microcolonies of CE-2 biofilms when observed under epifluorescence microscope. This observation suggest that cellulase hydrolyzed calcofluor binding β 1-4 linked polymers present in the CE-2 biofilms (Figure 5B). Role of calcofluor binding and cellulase sensitive polymers in biofilms of *P. fluorescense*, *E. coli*, *S. enteritidis*, *S. typhimurium*, and *Y. 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 are needed to better understand the mechanism of bacterial adhesion.

Summary

In summary, CE-2 cells of *Pseudomonas sp* formed biofilms, and produced EPS on SS coupons. Interaction of calcofluor with 72 h old biofilms suggests the presence of β 1-4 linked polymers. Moreover, in the presence of calcofluor, formation of the CE-2 biofilms reduced. Similarly, cellulase treatment removed biofilms, and produced a central hollow in micro-colonies on SS coupons. These results indicate the role of β 1-4 linked polymers in the formation and maintenance of CE-2 biofilms on SS coupons.

Acknowledgements

The authors thank Dr Satish R Shetye, the Director NIO, for his support and encouragement. The first author thanks CSIR (India) for granting senior research fellowship to him. The authors also thank Mr. Arpan Bhachi and Miss P.Prathipa, dissertation students for their help. We are grateful to two anonymous referees and Dr Paul Stoodley, the handling editor for critical but very useful comments on the earlier version of the manuscript.

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Table I. The effect of calcofluor, con A and WGA on biofilm formation of CE-2 on SS-304 coupons.

Treatment	Cells x 10 ⁵ per mm ²	Percentage biofilm inhibition* (%)
Control	65 ± 3	0
Calcofluor	9.5 ± 2	85 ± 15
Con A	61 ± 1.4	7 ± 3
WGA	64 ± 0.4	1 ± 0.5

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

Table II. Biofilm removal of CE-2 from SS-304 coupons by cellulase, protease and lipase treatment.

Enzyme treatment	Cells x 10 ⁵ per mm ²	Percentage biofilm removal* (%)
Cellulase		
Control	37 ± 0.1	0
60U	26 ± 4.9	22 ± 11
120U	19 ± 2.8	48 ± 8
Protease		
Control	45 ± 2.6	0
2.4	41 ± 1.0	9 ± 2
4.8	35 ± 6.2	23 ± 4
Lipase		
Control	46.5 ± 4.9	
175U	41 ± 1.4	12 ± 3
350U	38.5 ± 2.2	17 ± 5

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

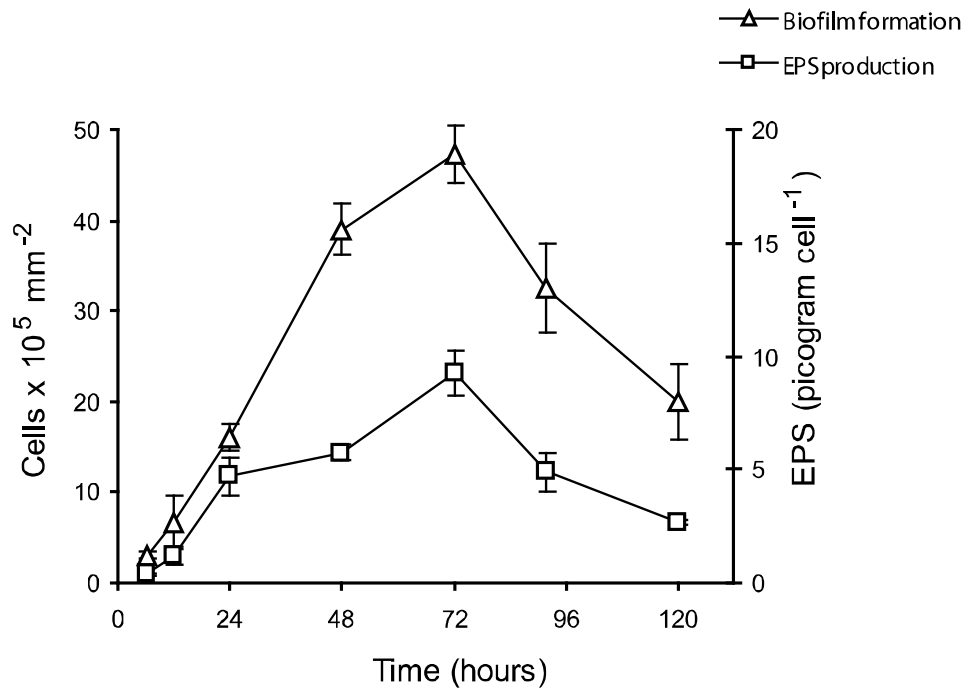


Figure 1. Biofilm formation and EPS production by CE-2 on 304 –SS coupons as a function of the incubation period.

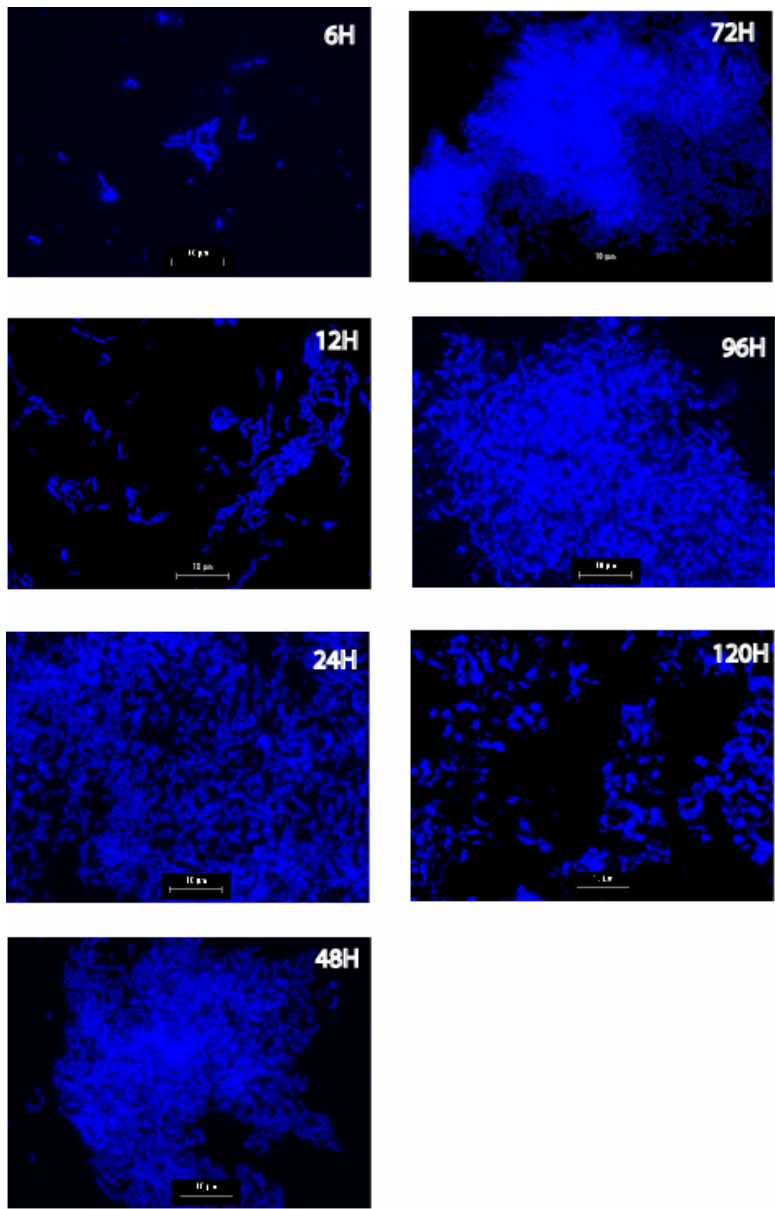


Figure 2. Calcofluor stained CE-2 biofilms on SS over the period of incubation. Note that, at 72h the fluorescence of calcofluor is highest as compare to other time intervals. Scale bar represents 10 µm

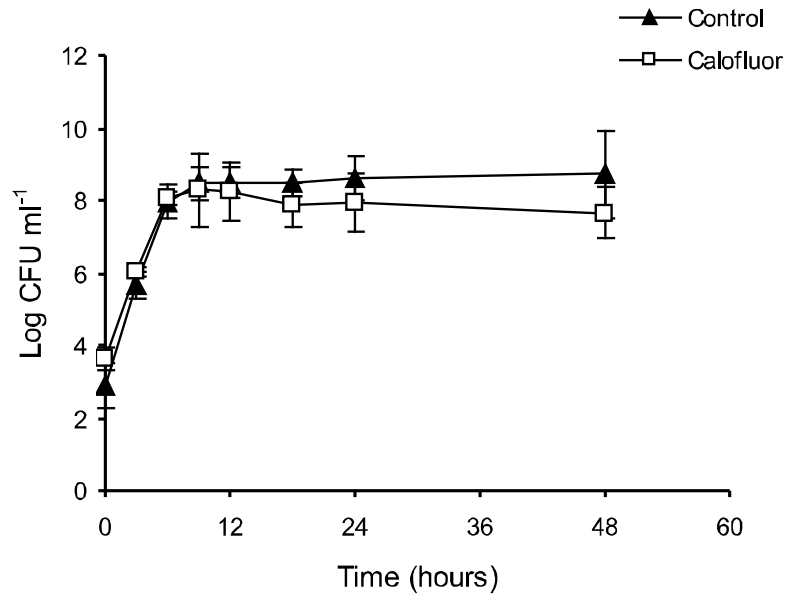


Figure 3. Effect of calofluor (200µg ml⁻¹) on the growth of CE-2 cells.

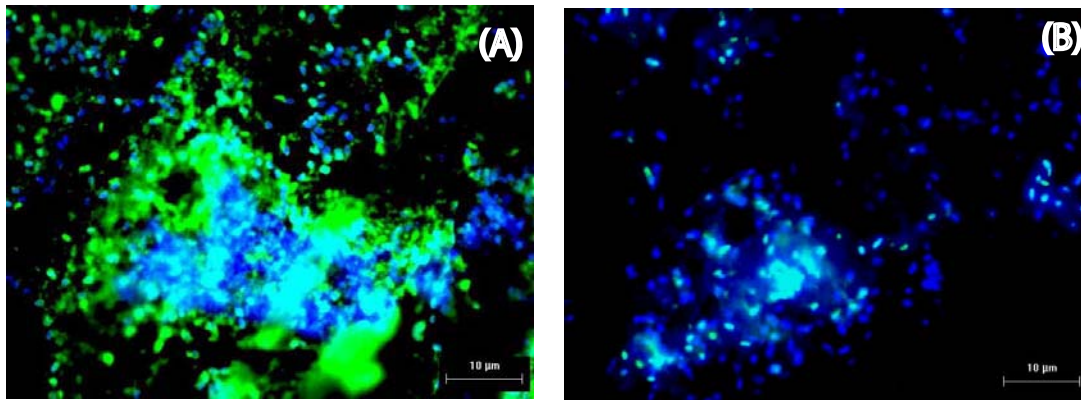


Figure 4. Photographs of the 72 h old CE-2 biofilms after staining with Con A (A) and WGA (B). The green fluorescence represents the lectin staining and blue fluorescence represents DAPI. Scale bar represents 10 µm.

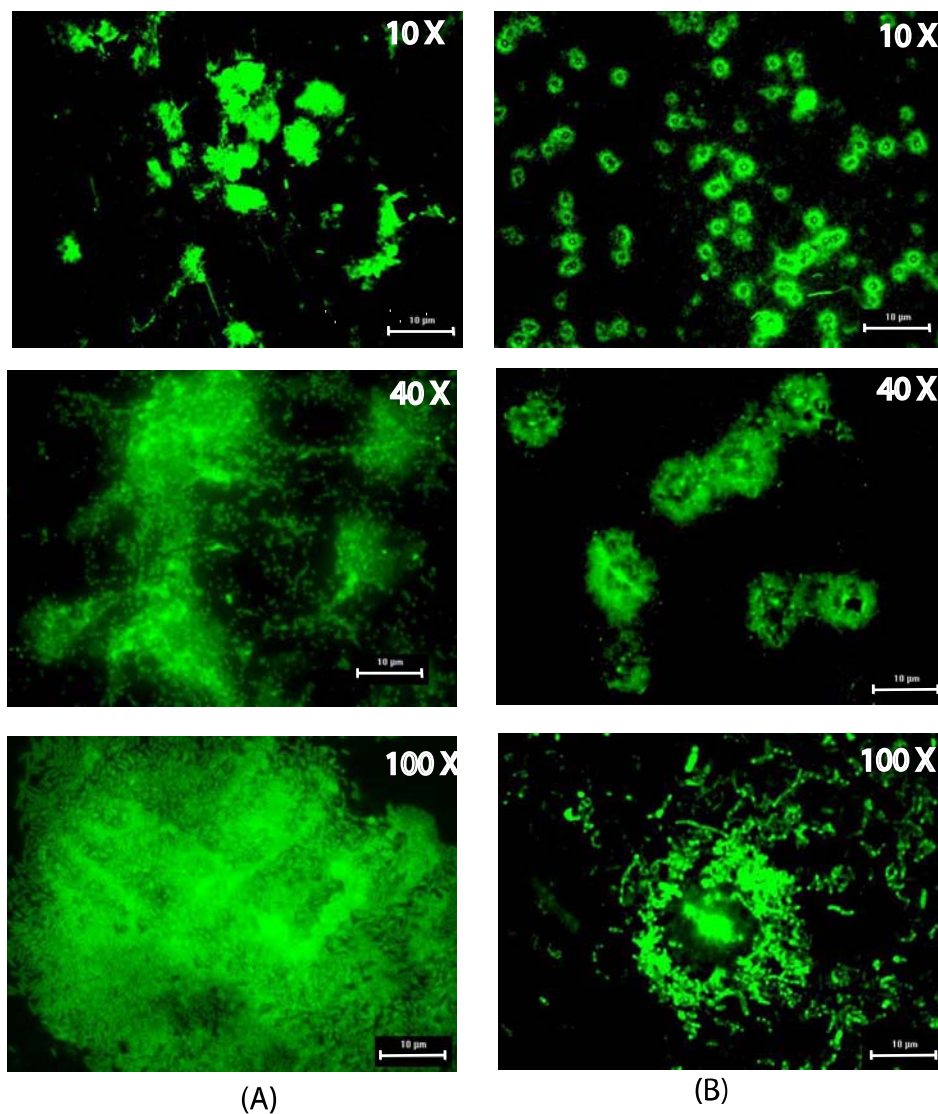


Figure 5. Cellulase treatment of CE-2 biofilm stained with acridine orange. Biofilm under control condition (A) and after treatment with cellulase (B). Note the central hollow in microcolonies after treatment with cellulase. Scale bar represents 10 µm.