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Biochemical analysis of biofilm EPS formed in different coupons immersed in two different aquatic media

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Abstract
Biofilms are composed primarily of microbial cells and EPS. Biochemical analysis of EPS extracted from different coupons was carried out the protein content was found to be more abundant than carbohydrate in both enriched and normal biofilms during the exposure period. The protein, carbohydrate, calcium and magnesium concentrations in the EPS of biofilm grown on stone coupons were higher than the EPS of biofilm grown on PVC, wood and glass coupons. Nutrient boosted the biofilm cells growing in enriched medium which resulted in more EPS production.

Keyword: biofilm, EPS, coupons, calcium, protein, carbohydrate

Introduction

Microorganisms readily adhere to all surfaces immersed in an aqueous environment and form biofilm. They are complex communities of bacteria that are enclosed in extracellular polymeric substances (EPS). Many unicellular organisms produce EPS during some phases of their life cycle. It comes from the natural secretions of bacteria, cell lysis and hydrolysis products.

Most bacteria are able to produce polysaccharides, either as wall polysaccharides (capsules) or as extracellular secretions into the surrounding environment (Donlon, 2002). It is important for the attachment of bacteria to substrata and thus develops biofilms (Costerton *et al*., 1978) [5]. Various steps are involved in the formation of bacterial biofilms including conditioning of solid substrata by adsorption of macro molecules, transport of the cells towards the substratum, physicochemical interactions between bacteria and the substratum, production of extracellular polymeric substances and multiplication of the attached cells (VanderAa and Dufrene, 1997) [31].

However, in aqueous environments adhesion is believed to be mediated by extracellular polymers (Fletcher and Floodgate, 1973 and Gessey*et al*., 1977) [12]. The production of EPS is influenced by internal and external factors including, quorum sensing, surface topography, hydrodynamic shear forces, fluid velocity and nutrient availability (Sreenivasan and Chorney, 2005) [27]. The complexity and the gel forming properties of the EPS enhance the interaction of the cells with the other components around them (Watanabe *et al*., 2000; Frank and Belfort, 2003; Olofsson*et al*., 2003 and Walker *et al*., 2005) [34, 20, 14].

EPS composition determines many important properties of biofilm such as strength, elasticity and sorption capacity for adsorbents. These properties are important for the behavior of biofilms in technical systems. The EPS matrix surrounding the biofilm prevents the cells from desiccation and the channels that formed in the EPS matrix are used to transport substrates or proteins, which would otherwise be lost in the surrounding water (Czaczyk and Myszka 2007) [6].

The proportion of EPS in biofilms can comprise between approximately 50-90% of the total organic matter (Flemming and Wingender, 2001 and Donlan, 2002) [11, 9]. It may also consist of metal ions, divalent cations, and other macro molecules (Flemming*et al.*, 2000) and helps to protect organisms in the biofilm community from environmental stresses. This matrix is composed of a mixture of components, including carbohydrate, protein, nucleic acids, lipids and other substances (Flemming, 1998 and Liu *et al*., 2004) [35]. More than 90% of the EPS volume consists of water (Sutherland, 1997 and Schmitt and Flemming, 1999) [28, 10], located in pores and a minor portion bound to the EPS molecules.

However, there is no information available regarding the use of organic fertilizer and manures for the maximum production of biofilm EPS. Hence the present study was designed to evaluate the effect of cowdung enrichment on the water quality parameters and also to determine the biochemical composition of EPS of biofilm in stone, wood, glass and PVC coupons immersed in pond water and cowdung enriched pond water.

Materials and methods

Experimental setup

The experiments were conducted in six fiber tanks (capacity 100 L). There were two experiments each had three replications. The cowdung manure were collected from local dairy farm and allowed to decompose for 10 days prior to application. In the first experiment (aquatic media I- pond water) no manure was added. In the second experiment 2 Kg cowdung manure was added in 100 litre water (aquatic media II-cowdung enriched pond water). Four different test coupons such as stone (7×7 cm), wood (6.7×6.7 cm), glass (6×6 cm), and PVC pipe (7×4 cm) were fitted in a wooden frame and immersed in the two experimental set up. Before immersion the coupons were washed thoroughly, dried and rinsed with 70% alcohol. Test coupons (wood, stone, glass, and PVC) were retrieved each week over a period of 35 days for biochemical analysis of biofilm EPS.

Extraction of EPS

The EPS was extracted by adding 10 ml of 15 mmol.L^{-1} EDTA to the biofilm sample (Platt *et al.*, 1985) [23] mixed

thoroughly and incubated at room temperature on anorbitory shaker table for 4 hours. Samples were centrifuged at 23,000g for 20 min. The supernatant was decanted and the precipitate was mixed with cold ethanol to a final concentration of 60%, and stored overnight at -10^{0}C to precipitate the high molecular mass exopolymeric substances. The mixture was then centrifuged at 23,000g for 10 min. The extracted EPS was analyzed for the estimation of protein, carbohydrate, calcium and magnesium concentration.

Protein estimation

The protein concentration of the EPS was estimated by using the method of Lowry (Lowry *et al*., 1951) [19] with bovine serum albumin as standard.

Amount of protein in the EPS =

Sample OD - Blank value × Standard concentration (µg/cm^2)

Carbohydrate estimation

Total carbohydrates were estimated by anthrone reagent method (Loewus, 1952) [18]. 1ml of the dispersed EPS was added to 10ml of anthrone reagent (0.2 g anthrone dissolved in a mixture of 8ml ethyl acetate and 30 ml distilled water).

Amount of carbohydrate in the EPS =

$$\frac{\text{Concentration of sample}}{\text{OD of the standard}} \times \text{OD of the sample (µg/ cm}^2\text{)}$$

Estimation of calcium and magnesium

For the estimation of calcium and magnesium, the methods given by Venugopalan and Paulpandian (1989) [32] were adopted. The EPS samples were completely digested with concentrated Nitric acidand Hydrogen peroxide. The digested EPS samples was diluted with known concentrations of 1N HCl and analyzed by Atomic Absorption Spectrophotometer using the hotter acetylene– nitrous oxide flame.

Statistical analysis

Each experiment was performed in triplicates. Data were expressed as mean ± standard deviation. Two-way analysis of variance was (ANOVA) used to determine significant differences between means for each surface employed and the biochemical analysis used.

Results

Quantitative analysis of protein content of EPS extract from different coupons showed progressive increase in protein concentration from 7th day to 35th day of observation in both the aquatic medium. In the aquatic medium I. Protein content was maximum in the stone coupons (224.66 ± 1.52 µg/ cm^2) followed by wood (210 ± 2.516 µg/ cm^2) glass (154.66 ± 2.081 µg/ cm^2) and was minimum in the PVC coupons (81.64 ± 1.52 µg/ cm^2) on the 35th day of extraction.

Carbohydrate concentration of EPS extracted from different coupons in aquatic media I ranged between 0.356 ± 0.015 to 2.5 ± 0.25 µg/cm^2. Maximum carbohydrate content was noticed in stone coupons on the 35th day (2.5 ± 0.25 µg/cm^2) closely followed by wood coupons (2.2 ± 0.3µg/ cm^2). Minimum carbohydrate content was observed in PVC coupons (0.356 ± 0.015 µg/ cm^2) on the 7th day.

Calcium content of the EPS extracted from different coupons suspended in pond water ranged between 0.16 ± 0.015 to 0.99 ± 0.01 µg/cm^2.Calcium was maximum in stone coupons (0.99 ± 0.01 µg/cm^2) followed by glass (0.77 ± 0.025 µg/cm^2), wood coupons (0.76 ± 0.02 µg/cm^2) and minimum in the PVC coupons (0.54 ± 0.025 µg/cm^2) on the 35th day.

Magnesium concentration was maximum in the EPS extracted from stone coupons (0.686 ± 0.025 µg/cm^2) followed by wood (0.66 ± 0.03 µg/cm^2), glass (0.63 ± 0.03µg/ cm^2) and was minimum in the PVC coupons (0.41 ± 0.02 µg/cm^2). In all coupons magnesium content of EPS progressively increased from 7th day to 35th day.

In aquatic media II, Protein concentration of the EPS extracted from different coupons ranged from 35 ± 2.6 to 235.76 ± 1.52 µg/cm^2. Protein concentration was maximum in stone coupons (235.76 ± 1.52 µg/cm^2) followed by wood coupons (223 ± 2.51 µg/cm^2), glass coupons (168 ± 1.73 µg/cm^2) and was minimum in the PVC coupons (86.61 ±1.52 µg/cm^2) on the 35th day of observation.

Carbohydrate concentration of EPS extracted from aquatic media II using different coupons ranged between 0.543 ± 0.25 to 2.65 ± 0.208 µg/cm^2. Carbohydrate concentration was maximum in stone coupons on the 35th day (2.65 ± 0.208 µg/cm^2) and was minimum in the PVC coupons on the 7th day (0.543 ± 0.25 µg/cm^2).

Calcium concentration of the EPS extracted from different coupons suspended in aquatic media II ranged between 0.51 ± 0.02 to 1.3 ± 0.2 µg/cm^2. Calcium content was maximum in the stone coupons on the 35th day (1.3 ± 0.2 µg/cm^2) and was minimum in the PVC coupons on the 7th day (0.51 ± 0.02 µg/cm^2).

Magnesium content of EPS extracted from, aquatic media II using different coupons ranged between 0.045 ± 0.025 to 1.13 ± 0.157 µg/ cm^2. Magnesium concentration was maximum in stone coupons on the 35th day (1.13 ± 0.157 µg/cm^2) and was minimum in the PVC coupons on the 7th day (0.045 ± 0.025µg/cm^2). All coupons showed progressive increase in magnesium content from 7th day to 35th day of extraction.

Table 1: Protein concentration (µg/cm^2) of EPS extracted from the biofilm of different coupons immersed in aquatic media I

Coupons	Days of observation				
	7	14	21	28	35
Wood	59 ± 1.732	78 ± 2.081	99 ± 1.52	127 ± 2	210 ± 2.516
Stone	77.33 ± 2.081	93.66 ± 2.516	105.6 ± 2.51	186 ± 3	224.66 ±1.52
Glass	44.66 ± 1.52	65.33 ± 2.08	83.33 ± 2.51	97 ± 2	154.66 ± 2.081
PVC	28 ± 4	33.33 ± 2.08	44.53 ± 2.51	62.13 ± 1.52	81.64 ± 1.52

Table 2: Carbohydrate concentration (µg/cm^2) of EPS extracted from the biofilm of different coupons immersed in aquatic media I

Coupons	Days of observation				
	7	14	21	28	35
Wood	0.65± 0.02	0.9 ± 0.02	1.13 ±0.15	1.26 ± 0.15	2.2 ± 0.3
Stone	0.86 ± 0.15	1.15 ± 0.152	1.095 ± 0.15	1.65 ± 0.20	2.5 ± 0.25
Glass	0.566 ± 0.02	0.84 ± 0.03	1.163 ± 0.21	1.23 ± 0.16	1.46 ± 0.152
PVC	0.356 ± 0.015	0.446 ± 0.025	0.546 ± 0.02	0.706 ± 0.02	0.833 ± 0.02

Table 3: Calcium concentration (µg/cm^2) of EPS extracted from the biofilm of different coupons immersed in aquatic media I

Coupons	Days of observation				
	7	14	21	28	35
Wood	0.256 ± 0.015	0.406 ± 0.025	0.54 ± 0.02	0.61 ± 0.02	0.76 ± 0.02
Stone	0.45 ± 0.02	0.66 ± 0.015	0.84 ± 0.015	0.96 ± 0.02	0.99 ± 0.01
Glass	0.23 ± 0.02	0.416 ± 0.02	0.56 ± 0.02	0.65 ± 0.015	0.77 ± 0.025
PVC	0.16 ± 0.015	0.26 ± 0.015	0.36 ± 0.02	0.396 ± 0.015	0.54 ± 0.025

Table 4: Magnesium concentration (µg/cm^2) of EPS extracted from the biofilm of different coupons immersed in aquatic media I

Coupons	Days of observation				
	7	14	21	28	35
Wood	0.093 ± 0.025	0.316 ± 0.025	0.41 ± 0.02	0.54 ± 0.01	0.66 ± 0.03
Stone	0.17 ± 0.020	0.343 ± 0.025	0.41 ± 0.02	0.61 ± 0.02	0.686 ± 0.025
Glass	0.18 ± 0.02	0.336 ± 0.015	0.41 ± 0.02	0.62 ± 0.03	0.63 ± 0.03
PVC	0.16 ± 0.152	0.243 ± 0.025	0.233 ± 0.020	0.346 ± 0.025	0.41 ± 0.02

Table 5: Protein concentration (µg/cm^2) of EPS extracted from the biofilm of different coupons immersed in aquatic media II

Coupons	Days of observation				
	7	14	21	28	35
Wood	66 ± 2	84 ± 2.51	96 ± 2.51	142 ± 2	223 ± 2.51
Stone	78.33 ± 4.04	87.43 ± 2.08	110.37 ± 2.51	186.66 ± 2.08	235.76 ± 1.52
Glass	54.66 ± 2.51	69.64 ± 1.52	89.66 ± 2.51	105.43 ± 2.08	168 ± 1.73
PVC	35 ± 2.6	39 ± 2	46.62 ± 1.52	66.67 ± 2.51	86.61 ± 1.52

Table 6: Carbohydrate concentration (µg/cm^2) of EPS extracted from the biofilm of different coupons immersed in aquatic media II

Coupons	Days of observation				
	7	14	21	28	35
Wood	0.88 ± 0.03	1.13 ± 0.15	1.09 ± 0.11	1.63 ± 0.25	2.53 ± 0.25
Stone	1.04 ± 0.21	1.045 ± 0.157	1.4 ± 0.251	2.1 ± 0.264	2.65 ± 0.208
Glass	0.79 ± 0.03	0.986 ±0.10	1.196 ± 0.20	1.33 ± 0.20	2.1 ± 0.2
PVC	0.543 ± 0.25	0.576 ± 0.035	0.66 ± 0.035	0.896 ± 0.025	1.163 ± 0.21

Table 7: Calcium concentration (µg/cm^2) of EPS extracted from the biofilm of different coupons immersed in aquatic media II

Coupons	Days of observation				
	7	14	21	28	35
Wood	0.546 ± 0.02	0.736 ± 0.02	0.756 ± 0.02	0.86 ± 0.02	0.95 ± 0.02
Stone	0.61 ± 0.02	0.73 ± 0.02	0.86 ± 0.02	0.976 ± 0.2	1.3 ± 0.2
Glass	0.556 ± 0.01	0. 64 ± 0.02	0.80 ± 0.02	0.86 ± 0.01	1.1 ± 0.15
PVC	0.51 ± 0.02	0.72 ± 0.03	0.89 ± 0.03	0.95 ± 0.03	1.16 ± 0.15

Table 8: Magnesium concentration (µg/cm^2) of EPS extracted from the biofilm of different coupons immersed in aquatic media II

Coupons	Days of observation				
	7	14	21	28	35
Wood	0.41 ± 0.02	0.663 ± 0.025	0.71 ± 0.02	0.846 ± 0.015	0.86 ± 0.02
Stone	0.456 ± 0.023	0.703 ± 0.025	0.783 ± 0.030	0.846 ± 0.015	1.13 ± 0.157
Glass	0.45 ± 0.02	0.73 ± 0.025	0.736 ± 0.011	0.87 ± 0.02	0.966 ± 0,015
PVC	0.045 ± 0.025	0.66 ± 0.020	0.663 ± 0.011	0.84 ± 0.03	0.936 ± 0.025

Discussion

Detailed biochemical analysis of an EPS is difficult. The microbial species forming biofilms vary according to the physical structure of tanks, type and amount of nutrients as well as the amount of available oxygen and pH of water (Gilbert *et al*., 1990). In the present investigation, concentration of protein and carbohydrate in both pond water and enriched pond water were increased with increased exposure time. Similar findings were reported by D'Souza and Bhosle (2003) who stated that the carbohydrate and

protein concentration increases with prolonged exposure times. Baier (1980) and Compere *et al*. (2001) reported that the carbohydrates of the conditioning biofilm showed increasing trends over a period of immersion.

The EPS of biofilm grown in cowdung enriched medium had a higher protein and carbohydrate concentration than in the EPS of biofilm grown in normal water. This indicated that the structural components of the biofilm EPS was dependent on the nutrient status in which the biofilm was grown. In the present study the EPS of extracted from stone coupon of cowdung enriched water contain high protein and carbohydrate than other coupons. These results corresponds to the work of Simoes *et al*. (2003) found that the EPS of by *P. fluorescens* biofilms under specific growth conditions produced more protein than carbohydrate.

The protein content was found to be more abundant than carbohydrate in both enriched and normal biofilms during the exposure period. This may be due to the higher absorbance of proteins on biofilm surface than carbohydrates. Rice *et al*. (2000) and D'Souza *et al*. (2005) proved that bacteria and phytoplankton populations produce lower concentrations of carbohydrate and higher amounts of protein during the early logarithmic growth phase.

In the present investigation, increasing trend was noticed in the carbohydrate content from the initial days of observation to the 35th day of observation. This increased value of carbohydrates may be due to the production of exopolysaccharides by bacteria while attached to the surfaces of the EPS. Decho (1990) [8] stated that the concentration of carbohydrate may vary with the microbial species, the nutrient availability, the growth stage and other environmental parameters.

The association of divalent cations, such as Ca^{2+} and Mg^{2+}, which have been shown to be cross-linked with EPS, provides greater stability to biofilm. In the present study, calcium concentration was higher than the magnesium concentration in both aquatic medium. Literature reviews have shown that Ca^{2+} has a significant impact on biofilm development (Koerstgens *et al*., 2001 and Lattner *et al*., 2003) and the role of Mg^{2+} is largely unknown.

Calcium is known to be important for bacterial biofilm formation and is involved in specific and non-specific interactions between cells and the substrata (Craven and Williams, 1998 and Waligora *et al*., 1999). Calcium binding proteins are often involved in bacterial adhesion to the surface and can be important for cell-cell aggregation.

The protein, carbohydrate, calcium and magnesium concentrations in the EPS of biofilm grown on stone coupons were higher than the EPS of biofilm grown on PVC, wood and glass coupons. This may be due to the surface roughness of stone coupons. Characklis *et al*. (1990) reported that the extent of microbial colonization appears to increase as the surface roughness increases. This is because shear forces are diminished and surface area is higher on rougher surfaces. Stainless steel and polyethylene support less biofilm formation than other materials (Gmbh and Coohg, 2004).

Moreover a positive correlation exists between the various physicochemical parameter of water and biochemical composition of EPS. In the cowdung enriched media there was gradual increase in nutrient content than the pond water. Correspondingly the protein, carbohydrate and calcium and magnesium ions concentration also increased in the cowdung enriched pond water.

EPS have properties that make them invaluable to the organisms that secrete them. EPS of microorganisms used in food, pharmaceutical, biomedical, bioremediation and bioleaching fields due to their wide structural diversity and their physical, rheological and other unique properties. The biofilms grown on enriched medium had more EPS than the control medium. Nutrient boosted the biofilm cells growing in enriched medium which resulted in more EPS production. It was indicated in previous studies that biofilms growing in high nutrient medium were more abundant, densely packed and thicker than biofilms growing on ordinary water.

Conclusion

Present study revealed that cowdung can be used as a cheap nutrient to produce high amount of EPS. It is therefore concluded that, the high content of protein and carbohydrate of the cow dung was directly related to the high population of bacteria.

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