

Differences in carbohydrate profiles in batch culture grown planktonic and biofilm cells of *Amphora rostrata* Wm.Sm.

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Abstract:

Diatoms are abundant in biofilms developed on surfaces immersed in sunlit waters. In both planktonic and biofilm mode of growth, diatoms produce carbohydrate polymers which perform several functions including motility, protection, maintenance, macro aggregates production, and detoxification. However, little is known about the differences, if any, in the production and molecular level characterization of carbohydrates in planktonic and biofilm cells of diatoms. In order to identify the differences in these two modes of growth, we measured concentrations of total carbohydrates, carbohydrate fractions, neutral carbohydrates, uronic acids, and amino sugars in planktonic and biofilm cells of *Amphora rostrata*. Distribution of carbohydrate fractions, uronic acids and amino sugars was different between biofilm and planktonic cells of the diatom. Cell normalized concentrations of these biochemical components were 2 to 5 times greater in the planktonic as compared to the biofilm cells of the diatom. Concentrations of glucose and glucosamine decreased, whereas fucose increased in planktonic cells over the period of cultivation. Conversely, concentrations of glucose and glucosamine increased while that of fucose decreased in attached cells. Our study suggests that there exist marked differences in carbohydrates of planktonic and biofilm cultures of *A. rostrata*.

Keywords: carbohydrate; glucose; fucose; glucosamine; protein; planktonic cells; biofilm cells; *Amphora rostrata*

Introduction

Solid surfaces immersed in aquatic environments adsorb dissolved organic matter to conditioning its surface, for colonization by various microorganisms such as bacteria, diatoms, and fungi. Attached microorganisms enmeshed in exopolymeric substances are known as biofilms. Diatoms are amongst the earliest autotrophic colonizers of surfaces immersed in sunlit environments (D'Souza and Bhosle 2003; Mitbavkar and Anil 2008; Molino et al. 2009). Diatoms are also known to release extracellular organic compounds in the growth medium and in biofilms that may serve as carbon sources to heterotrophic bacteria (Bahulikar and Kroth 2008; Bellinger et al. 2009). Therefore the quality and quantity of extracellular organic compounds released by diatoms may influence the microfouling community structure (Decho 1990; Hoagland et al. 1993; Molino and Wetherbee 2008).

While attaching to surfaces, diatoms produce extracellular polymeric substances which are rich in carbohydrates. Carbohydrates are common structural and storage products of both biofilm and planktonic diatoms (Cooksey and Cooksey 1995; Smith and Underwood 1998, 2000; Callow and Callow 2002; Bellinger et al. 2009). The quality, quantity, and composition of these polymers produced by diatoms are influenced by nutrient status and growth phase (Myklestad 1977; D'Souza and Bhosle 2001). Several functions including maintenance, protection and detoxification have been ascribed to diatom polymers. Carbohydrate polysaccharides bind to other organic molecules by different linkages which can be hydrolyzed using treatment with heat or alkali or both (Handa 1969; Haug and Myklestad 1976; Hitchcock 1977). Therefore, polysaccharides can be isolated using a selective sequential extraction technique (Handa 1969; Haug and Myklestad 1976; Hitchcock 1977; Bhosle et al. 1993).

When grown under normal experimental conditions, some diatom cells attach to culture flask and form biofilms while others remain suspended in culture broth. The diatom cells living in attached mode (biofilm) may have different carbohydrate concentration and composition than those of free-floating (planktonic) forms. Understanding the biochemistry of the polymers produced by diatom under each growth condition is important in enhancing the knowledge of what leads to biofilm development onto surfaces or why organisms maintain a planktonic lifestyle. However, not much is known about the differences, if any, of cellular carbohydrates in attached and planktonic cultures of diatoms. This is because earlier studies on diatoms have mostly focused their attention on characterization of exo or extracellular polysaccharides of planktonic and biofilm cultures of diatoms (Khandeparker and Bhosle 2001; Faraloni et al. 2003). In contrast, numerous studies have been carried out to evaluate the differences in biochemical metabolites in planktonic and biofilm cells of bacteria (Sauer et al. 2001, 2002; Whiteley et al. 2001; Davey and O'Toole 2000; Gjersing et al. 2007 and references therein). These studies suggest that there are differences in metabolism of biochemical components including carbohydrates and proteins in biofilm and planktonic cells of bacteria (Sauer et al. 2001, 2002; Whiteley

et al. 2001; Davey and O'Toole 2000; Gjersing et al. 2007 and references therein). Therefore, in the present research, our aims were to evaluate the total concentrations of cell carbohydrate (TCHO), TCHO fractions, neutral carbohydrate (TNCHO), uronic acid (UA), amino sugar (AS), and proteins (PP) along with the TNCHO and AS composition of planktonic and biofilm cultures of *A. rostrata* over the period of cultivation, and 2) to identify differences, if any, in the abundances and relative distribution of these compounds in planktonic relative to biofilm cultures.

Materials and methods

Culture conditions and source of algal material

Amphora rostrata Wm. Sm., a pennate diatom was used for this study. The culture was maintained at the Marine Corrosion and Materials Research Division, National Institute of Oceanography, Goa, India. *A. rostrata* was originally isolated from biofilms developed on a glass slide immersed in the marine waters of Dona Paula Bay, west coast of India (15°.31'N, 73°.59'E) (Bhosle et al. 1995). A stock culture was routinely maintained at room temperature (28 °C ±2 °C) in the laboratory by weekly re-inoculation into fresh f/2 medium, prepared in autoclaved seawater (~35 psu) enriched with nutrients (Guillard and Ryther 1962). Cells were grown on a shaker (30 rpm) in Erlenmeyer flasks (100 ml) containing 10 ml of culture medium and were maintained at 28 °C ± 2 °C under a light photcycle of 12h:12h light:dark (L:D) condition. Illumination was provided by two 40 W white fluorescent tubes.

Before use, the diatom cultures were made bacteria free by treating diatom cells with penicillin G (1000 mg L⁻¹), streptomycin sulphate (500 mg L⁻¹) and chloramphenicol (100 mg L⁻¹) for 24 h and repeating the treatment for another 24 h. The culture was then transferred to antibiotic-free medium (f/2) and cultivated as above.

Monitoring of growth and chemical and biochemical constituents of the diatom cells

The experiments to assess diatom growth and the production of various chemical and biochemical constituents were carried out in 1 L Erlenmeyer flasks under a light photo-cycle of 12L:12D condition as above. Experimental flasks containing 200 ml of f/2 medium were inoculated with 20 ml of the 5 day old culture (1.8 X 10⁵ cells ml⁻¹). The flasks were incubated at room temperature as above to grow planktonic and biofilm cells. The cells which attached to the culture flask were defined as biofilms, and those remained suspended as planktonic. In order to sample planktonic and biofilm cells, the flasks were removed at 2 d intervals over the period of 10 d. Their contents were decanted to collect the planktonic cells and spent growth medium. The flask containing attached cells was rinsed twice with 15 ml of filtered (0.22 µm mixed cellulose ester filter paper, Millipore) seawater to remove unattached cells.

Rinsed seawater containing unattached cells was then transferred to the flask containing planktonic cells and made to a known volume.

The known aliquots (5 to 10 ml) of planktonic cells were individually filtered on pre-combusted (400 °C for 4 h) GF/F glass fiber filter (0.7 µm, Whatman) for the analysis of dry weight (DW), chlorophyll *a* (Chl *a*), organic carbon (OC), PP, TCHO, dilute acid soluble (Acid-TCHO), dilute alkali soluble (Alkali-TCHO) and residual (Res-TCHO) carbohydrate fractions, UA, TNCHO and AS concentration and composition. A small aliquot (1 ml) of planktonic cells were fixed with Lugol's iodine and stored at 4 °C for determination of cell number.

Removal of attached cells

Diatom cells attached to the culture flask were removed using a soft nylon brush and filtered (0.22 µm) seawater (Bhosle et al. 1989; Khandeparker and Bhosle 2001; Jain and Bhosle 2009). The scrapped material was made to a known volume (100 ml) using filtered (0.22 µm) seawater. The known aliquots (5 to 10 ml) of biofilm cells were individually filtered on pre-combusted (400 °C for 4 h) GF/F glass fiber filter (0.7 µm, Whatman) for the analysis of DW, Chl *a*, OC, PP, TCHO, Acid-TCHO, Alkali-TCHO, Res-TCHO, UA, TNCHO and AS. A small aliquot (1 ml) was preserved as above to determine cell count of biofilms.

Analytical methods

DW of the diatom cells retained on the GF/F filter was determined after drying the filter in an oven at 50 °C for 24 h. The filter was cooled and weighed on a microbalance. Diatom cells retained on glass filters were treated with 90% acetone in dark for 24 h at 4 °C to extract Chl *a*. Samples were centrifuged (3000 *g* for 10 min), and the absorbance of the supernatant was measured at 665 nm and 750 nm on a UV-Vis spectrophotometer (Shimadzu Model UV-1700) (Parsons et al. 1984). Cell numbers were counted using a haemocytometer and Nikon microscope (magnification 100X). OC was determined by NCS analyzer (CE Instruments, Model-2500), and 2, 5, Bis-(5-tert-butyl-benzoxazol-2-yl)-thiophen (C₂₆H₂₆N₂O₂S) was used as a standard. Protein was extracted from the cells using 0.1 N NaOH, and was analyzed by the bicinchoninic acid method (Smith et al. 1985). UAs were analyzed following the method of Filisetti-Cozzi and Carpita (1991) as described in Khodse et al. (2007). The precision of the analytical methods based on 3 replicates was better than 8%.

TCHOs were estimated by the phenol-sulphuric acid method (Dubois et al. 1956) as described in Khodse et al. (2007, 2008). Acid-TCHO, Alkali-TCHO, and Res-TCHO carbohydrates were extracted as described earlier (Hitchcock 1977; Bhosle and Dhople 1988; Bhosle et al. 1993). In brief, the filter paper containing planktonic or biofilm cells of *A. rostrata* were extracted twice in 4 ml of 0.1 N H₂SO₄ for

2 h. Samples were centrifuged after each acid extraction step, and supernatants were pooled. This was followed by two times extraction with 4 ml of 0.1 N NaOH as described above. Acid and alkali supernatants were concentrated individually and made to a known volume and were analyzed for Acid-TCHO and Alkali-TCHO. Res-TCHO concentration was calculated by subtracting the sum of the concentrations of Acid-TCHO and Alkali-TCHO carbohydrates from the TCHO concentration.

TNCHO concentration and composition of the planktonic and biofilm *A. rostrata* cells were determined using the capillary gas chromatographic method (D'Souza et al. 2005; Khodse et al. 2007). Briefly, a sample of planktonic or biofilm cells was treated with 12 M H₂SO₄ for 2 h, then diluted to 1.2 M H₂SO₄ and hydrolyzed for 3 h at 100 °C. Samples were cooled, neutralized, treated with NaBH₄, acetylated, and analyzed using a Shimadzu capillary gas chromatograph (GC) (model-2010) equipped with flame ionization detector (FID). Both detector and injector were maintained at 300 °C. The oven temperature was programmed as follows: 70 °C to 150 °C at 10 °C/min and then at 3 °C/min to 230 °C, and maintained for 10 min. Identification of monosaccharides was done by comparing to the retention time of unknown monosaccharides with that of saccharide standards. Quantification was done using the data handling software (GC-solution) installed in the instrument. In this study, TNCHO has been defined as the sum of all the identified monosaccharides. The contribution of individual monosaccharide to the TNCHO is expressed as mol %. Analytical reproducibility of the GC method was ± 6%, whereas it was < 11% for the GC plus sample preparation method. TNCHO concentration for d 2 of the cultivation period is based on a single observation because enough cells were not available for replicate GC analysis.

ASs were determined following the method of Zhang and Amelung (1996). Briefly, samples were hydrolyzed with 6 M HCl for 5 h at 105 °C. The samples were cooled, neutralized with 0.4 M KOH, and ASs were converted to aldononitrile derivatives (Guerrant and Moss 1984), and analysed by the GC-mass spectrometer (MS). A GC-MS system (Shimadzu Model-GC-MS QP-2010) with electron impact ionization mode (70 ev) and a capillary column (30 m, 0.32 mm i.d. 0.25 µm df) was used. The injector temperature was 250 °C, and the oven temperature was programmed from 120 °C to 250 °C at 10 °C/min and held for 2.5 min and again increased to 270 °C at 20 °C/min and maintained for 2 min. The interface and ion source temperatures of the MS were maintained at 280 °C and 250 °C, respectively. Sum of all the indentified amino sugars has been defined as total AS. Analytical reproducibility of total amino sugar by GC-MS method was ± 7%.

Nutrient analysis

The decanted spent medium was filtered through GF/F filter, and was used for the analysis of nutrients phosphate, nitrate, and silicates following the methods of Parson et al. (1984).

Statistical analysis

Analysis of variance (ANOVA) was carried out to understand the variations of OC, Chl *a*, TCHO, TNCHO, AS, UA and PP in biofilm and planktonic cultures of *A. rostrata*. Data of these components of the biofilm and planktonic cultures collected over the 10 d period of cultivation were used to perform ANOVA analysis. The ANOVA analysis was performed using Microsoft excel software loaded on personal computer.

Results

Changes in DW, Chl *a*, and diatom cell counts

After few hours following inoculation, *A. rostrata* cells started attaching to the culture flask. The Chl *a* concentration of both the planktonic and biofilm *A. rostrata* cells increased over the cultivation period, and reached a maximal value on d 6 and d 8, respectively, following inoculation (Figure 1a). Thereafter, a small decrease in Chl *a* concentration was observed for both cell types. A similar trend was observed for the cell count of the planktonic and biofilm cells of the diatom (Figure 1b). Growth rate was relatively higher for biofilm cells than planktonic cells (Figure 1c). For the planktonic cells, DW increased with the cultivation period, and reached a maximal value on d 6 (Figure 1d), and then decreased for the remaining period of cultivation. In contrast, DW of the biofilm cells increased over the period of cultivation (Figure 1d). Concentrations of nutrients in the culture medium declined rapidly over the period of cultivation (Figure 2).

OC, carbohydrates and protein profile

In order to compare OC, carbohydrates and PP profile of the planktonic and biofilm cells, concentrations of OC, PP, TCHO, TNCHO, UA and AS were normalized to cell numbers (Figure 3). Concentrations of OC, PP and TCHO generally increased over the period of cultivation in both planktonic and biofilm cultures of *A. rostrata* (Figure 3). Concentrations of OC, PP, and TCHO were significantly different (Table 1), and were 2 to 5 times greater in planktonic as compared to the biofilm cultures (Figure 3a, b, c). For the planktonic diatom cells, concentrations of PP showed some decrease after 6 d period of cultivation (Figure 3b).

TCHO/Chl *a*, PP/Chl *a* and PP/TCHO ratios

For both the planktonic and biofilm cells of *A. rostrata*, TCHO/Chl *a*, and PP/Chl *a* ratios generally increased over the period of cultivation, with the ratios being higher for planktonic cells (Table 2). PP/TCHO ratio was higher in biofilm cells, and generally decreased in both planktonic and biofilm

cultures over the period of cultivation (Table 2). A higher UA/TCHO ratio was noted in planktonic cells, which generally decreased in both cell types.

Carbohydrate fractions

Concentrations of Acid-TCHO, Alkali-TCHO and Res-TCHO generally increased over the period of cultivation in both planktonic and biofilm cells, and were higher in the former cells (Table 3). When expressed as a percentage of TCHO, Acid-TCHO did not vary much in planktonic cells. In contrast, for the biofilm cells, the contribution of Acid-TCHO to the TCHO increased from 43% at d 2 to 70% at d 10 following inoculation (Table 3). For both cell types, Alkali-TCHO contribution to TCHO decreased over the period of cultivation. Res-TCHO contribution to TCHO increased in planktonic cells and decreased in the biofilm cells (Table 3).

TNCHO concentration and composition

Capillary gas chromatographic analysis detected the presence of arabinose, fucose, galactose, glucose, mannose, rhamnose, ribose and xylose in the planktonic and biofilm cells (Table 4). For the planktonic cells, concentrations of TNCHO increased over the 8 d, and then decreased (Figure 3d). In contrast, TNCHO concentrations in biofilm cells showed consistent increase over the period of cultivation, however TNCHO concentrations were not significantly different in these two cell types (Table 1).

For planktonic cells, the abundance (as mol %) of glucose, rhamnose, ribose, xylose decreased while that of fucose, mannose and galactose increased through time (Table 4). In contrast, abundance of glucose increased, whereas that of fucose, ribose, arabinose, and xylose decreased in the biofilm cells over the period of cultivation. Overall, glucose and fucose distribution in planktonic and biofilm cells showed contrasting trends (Table 4). When monosaccharides were classified into hexoses (glucose, galactose and mannose), pentoses (ribose, xylose and arabinose) and deoxyhexoses (rhamnose and fucose), hexoses decreased while pentoses and deoxyhexoses increased in planktonic cells with the cultivation period (Table 4). In the biofilm cells, concentrations of hexoses increased while that of pentoses and deoxyhexoses decreased over the cultivation period (Table 4).

UA concentration

As observed for the most of the other components, concentrations of UA were higher in the planktonic cells and lower in the biofilm cells of *A. rostrata* (Figure 3e). For the planktonic cells, the UA concentration was highest at d 6, and then decreased. In contrast, biofilm UA concentration showed small but consistent increase over the period of cultivation. However, the observed differences in the

UA concentration in the planktonic and biofilm cells were not significant ($p > 0.05$) (Table 1). The UA/TCHO ratio varied from 0.13 to 0.48 and from 0.11 to 0.26 in planktonic and biofilm cells, respectively (Table 2).

AS concentration and composition

Glucosamine (GlcN), galactosamine (GalN) and mannosamine (ManN) were detected in both planktonic and biofilm cells of *A. rostrata*. Total AS concentrations increased over the period of cultivation for the planktonic cells (Figure 3f). Whereas for the biofilm cells, AS concentration was greater at d 2 which decreased at d 4 following inoculation. Thereafter, concentration of AS in biofilm cells increased over the period of cultivation. For the planktonic cells, the mol % GlcN increased from 2 mol % at d 2 to 23 mol % at d 10 (Table 5). GalN mol % values decreased in planktonic cells over the period of cultivation (Table 5). ManN mol % increased over the period of cultivation. For the biofilm cells, GlcN contribution to total AS was very high (36 to 74 mol %). Distribution of GlcN, GalN and ManN did not depict consistent trend for the biofilm cells over the entire period of cultivation.

Discussion

Cell growth and concentrations of carbohydrates and proteins

The observed increase in DW, Chl *a*, and cell numbers indicated growth of both planktonic and biofilm cells of *A. rostrata* (Figure 1). Concentrations of Chl *a*, OC, PP, and TCHO, in planktonic and biofilm cells showed significant differences ($p < 0.001$). Diatoms are known to produce greater amounts of TCHO in culture (Handa 1969; Myklestad 1989), and in natural waters, particularly during the stationary growth phase and/or under nutrient deficient conditions (Myklestad 1977; Ittekkot et al. 1981; Tanoue and Handa 1987; Bhosle et al. 1998). This was also the case with planktonic and biofilm cells of *A. rostrata*. However, the production of TCHO and PP by the planktonic cells was higher than the biofilm cells (Figure 3). This was also evident from the higher TCHO/Chl *a* and PP/Chl *a* ratios for the planktonic cells. Production of TCHO by diatoms is influenced by nutrient concentrations in the growth medium (Myklestad 1977; D'Souza and Bhosle 2001; Underwood et al. 2004). Concentrations of nitrate, phosphate and silicate in the culture medium decreased over the period of cultivation (Figure 2). Nutrient deficient condition may be responsible for the higher TCHO/Chl *a* ratio in planktonic cells. In contrast, biofilm cells of *A. rostrata* had low TCHO/Chl *a* ratio suggesting relatively low production of TCHO by the biofilm cells probably due to nutrient sufficient condition (Sekar et al. 2002). The PP/TCHO ratio generally decreased with incubation period for both cell types (Table 2). However, PP/TCHO ratio was generally higher (0.50 to 0.81) for the biofilm cells as compared to the planktonic cells (0.33 to 0.62) (Table 2), implying that biofilm cells produced greater amounts of PP relative to

TCHO. During the growth of diatoms, protein synthesis appears to dominate during the initial exponential growth phase, while amounts of cellular protein decrease with increasing nutrient reduction (Myklestad 1977; Mayzaud et al. 1990; Underwood et al. 2004; Diekmann et al. 2009). As compared to the planktonic cells PP/TCHO ratio was higher in biofilms. Higher PP/TCHO ratio suggests that nutrients were not depleted, and protein production was still dominant in biofilms of *A. rostrata*.

Carbohydrate fractions

In diatoms, carbohydrate polymers bind to organic molecules by different linkages. Hydrogen and van der Waals bonds that bind organic macromolecules with each other and with cell-surfaces can be dissociated by heating (Handa 1969; Haug and Myklestad 1976; Hitchcock 1977). Ester linkages are involved in binding of polysaccharides to other organic molecules while carbohydrates and proteins are bound to each other by *O*-glycosidic bonds. Both ester and *O*-glycosidic bonds are easily destroyed by alkaline treatment (Collins and Ferrier 1995). Therefore, selective sequential extraction technique often has been applied to extract carbohydrates with specific linkages from diatoms (Handa and Tominaga 1969; Handa and Yanagi 1969; Hitchcock 1977). When biofilm and planktonic cells were extracted with dilute acid and alkali, there were apparent differences in the relative distribution of TCHO fractions in these two types of cells (Table 3). Relatively greater abundance of Alkali-TCHO indicates higher concentration of TCHO with *O*-glycosidic and ester bonds in the biofilm cells of the diatom (Table 3). This suggests that polysaccharides with ester and *O*-glycosidic bonds probably were involved in the development of biofilms of *A. rostrata* on glass surface. Carbohydrate polymers play important role in attachment of diatoms to surfaces (Bahulikar and Kroth 2008). It appears that alkali extracted polysaccharides were responsible for the cell to cell attachment (Wustman et al. 1997; Wigglesworth-Cooksey et al. 2001; Bahulikar and Kroth 2008). Furthermore, it has been observed that firmly attached diatoms on glass slides can be removed only after addition of hot bicarbonate solution (Bellinger et al. 2005).

It was also interesting to note that the concentrations of dilute acid extractable TCHO for the first 6 d following inoculation were lower for the biofilm cells as compared to the planktonic cells (Table 3). This suggests that polysaccharides that bind to other organic molecules by hydrogen and/or van der Waals bonds were not produced in large quantities by the biofilms of *A. rostrata*. This probably was expected because *A. rostrata* cells need to produce carbohydrates that bind to other organic molecules with stronger ester and *O*-glycosidic bonds so as to form biofilms.

TNCHO concentration and composition

Production of TNCHO by both the planktonic cells of *A. rostrata* increased over the period of cultivation (Figure 3d). Glucose was the most abundant monosaccharide in both the planktonic and biofilm cells (Table 4). Glucose can account for 20 to 80 mol % of TNCHO during diatom blooms in natural environments and in pure culture grown in laboratory (Handa and Yanagi 1969; Mykalestad 1974; Ittekkot et al. 1982; Tanoue and Handa 1987; Bhosle et al. 1990; D'Souza and Bhosle 2001). Many diatom species are also known to produce glucose rich storage polysaccharide such as β -1,3 glucan (Handa and Tominaga 1969; Bhosle et al. 1995; Granum and Myklestad 1999; Storseth et al. 2004; Diekmann et al. 2009). However, it was interesting to note that the relative distribution of glucose and fucose in planktonic and biofilm cells was very different. Abundance of glucose (as mol %) decreased rapidly in planktonic cells over the period of cultivation probably suggesting its utilization by the respiring cells of *A. rostrata*. Decrease in glucose mol % has been recorded for the respiring cells of diatoms (Handa and Yanagi 1969; Hitchcock 1977; Tanoue and Handa 1987). The consistent depletion of glucose was accompanied by an increase in fucose during the stationary phase of growth of the planktonic cells of *A. rostrata* (Table 4). This suggests that fucose substitutes for glucose during stationary phase of growth of the planktonic cells of *A. rostrata*. It appears that fucose content increased with age or growth or estuarine conditions in some diatoms (Hecky et al. 1973; Bahulikar and Kroth 2008). Moreover, in some diatoms, glucose rich polysaccharides are replaced by fucose rich polysaccharides during the stationary phase of growth of some diatoms (Percival 1970). Roszkowski et al. (1989) have reported that fucose rich polysaccharide inhibits the adhesion process of metastatic sarcoma L-1 cells in lungs of mice. In view of this, it is possible that fucose rich carbohydrates are produced by the planktonic cells of *A. rostrata* perhaps to prevent their adhesion to glass surface.

Concentrations of TNCHO, especially glucose, increased in *A. rostrata* biofilms over the period of cultivation, indicating the production of glucose rich polysaccharides by the biofilms of *A. rostrata*. The pads, capsules and stalk are enriched in glucose rich adhesive polysaccharides. These polysaccharides are involved in keeping cells together and attachment of cells to surface substratum (Underwood et al. 1995; Decho 2000; Molino and Wetherbee 2008; Bahulikar and Kroth 2008). Moreover, attached cells spend less energy for locomotion and metabolic requirements. As a result, glucose exhibits different trends in planktonic and biofilm cells of *A. rostrata*.

When grouped together as hexoses, pentoses and deoxy hexoses, abundance of hexoses decreased in planktonic and increased in biofilm cell of *A. rostrata* (Table 4). Temporal variation in hexoses in both cell types was mostly controlled by the changes in glucose concentration. As discussed above glucose abundance decreased in planktonic cells and increased in biofilm cells of *A. rostrata* indicating different metabolic pattern for this monosaccharide. Similarly, contrasting trend was

also recorded for the distribution of deoxy sugars (rhamnose plus fucose) in biofilms and planktonic cells of the diatom. This again demonstrates the metabolic differences in the distribution of these sugars in planktonic and biofilm cells of the diatom.

UA concentration

UAs are carboxylated sugars which may influence the adhesion of microorganisms to glass coupons (Daniel et al. 1987; Decho 1990; Hoagland et al. 1993; Garg et al. 2009; Jain and Bhosle 2009). Moreover, these acidic carbohydrates are also involved in immobilization, diatom cell aggregation, sedimentation, cell adhesion to surfaces, detoxification of toxic compounds, adsorption of dissolved organic matter, and removal of metal ions (Decho 1990; Mopper et al. 1995; Tang et al. 2001; Jain and Bhosle 2009). Distribution of UA in planktonic and biofilm cells of *A. rostrata* over the period of cultivation yet again points to the fact that these two cell types followed different metabolic trends.

UA/TCHO ratio was higher in planktonic cells as compared to the biofilm cells suggesting that UA accounted for a greater proportion of carbohydrates in planktonic cells (Table 2). Higher UA/TCHO ratio in biofilms at d 4 following inoculation suggests that UAs were produced during the initial stages of adhesion of *A. rostrata* cells to glass surface. High UA/TCHO ratio in planktonic cells at d 6 and 8 coincide with decrease in cell numbers probably indicating their involvement in cell aggregation and sedimentation.

AS concentration and composition

GlcN and GalN are important constituents of diatom cells (Benner and Kaiser 2003) and adhesive structures (pads, stalk and capsules) formed by diatoms during attachment (Bahulikar and Kroth 2008). GlcN concentrations were 2 to 3 times greater in biofilm cells than the planktonic cells of *A. rostrata* (Table 5). This implies that more GlcN was produced by the biofilm cells of the diatom (Table 5). Storage polymers are preferentially degraded as compared to the structural polymers (Ittekkot et al. 1982; Hernes et al. 1996; Unger et al. 2005). Preferential removal of GlcN may be responsible for the relatively lower concentrations in planktonic cells. It is hypothesized that nutrients, especially nitrate influence the relative distribution of GlcN and GalN. Under nitrate depleted condition, abundance of GlcN decreased while that of GalN increased in two marine bacteria when they were grown under nitrate depleted condition (Khodse and Bhosle unpublished results). It is possible that some of these factors may be responsible for the observed differences in AS concentrations and composition in planktonic and biofilm cells of *A. rostrata*.

In summary, *A. rostrata* cells when grown under batch culture, showed clear differences in concentrations of TCHO, PP, Acid-TCHO and Alkali-TCHO Res-TCHO, TNCHO, and AS concentration

and composition. However, further studies using different diatom cultures are needed to better understand the production of biochemical compounds in planktonic and biofilm cells. Such studies may be of value in developing techniques to control diatom biofilms on industrially useful structures.

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Table 1. One way analysis of variance (ANOVA) to assess changes in chlorophyll *a* (Chl *a*), organic carbon (OC), total carbohydrate (TCHO), protein (PP), total neutral carbohydrate (TNCHO), amino sugars (AS) and uronic acids (UA) concentration in planktonic and biofilm cells of *Amphora rostrata*.

Parameter	Chl <i>a</i>	OC	TCHO	PP	TNCHO	AS	UA
MS	3.38	474.31	153.86	28.00	38.64	0.02	7.09
F	27.57	48.30	13.89	27.63	4.36	6.56	4.38
<i>p</i> -value	0.001	0.001	0.006	0.001	0.070	0.034	0.069

Values shown in bold indicate significant differences.

Table 2. Changes in TCHO/Chl *a*, PP/Chl *a*, PP/TCHO, and UA/TCHO ratios in planktonic and biofilm cells of *Amphora rostrata*.

Cultivation period (d)	TCHO/Chl <i>a</i> ratio	PP/Chl <i>a</i> ratio	PP/TCHO ratio	UA/TCHO ratio
Planktonic cells				
2	24.46	14.29	0.58	0.13
4	24.32	15.18	0.62	0.16
6	37.73	20.90	0.55	0.48
8	51.40	27.01	0.53	0.33
10	65.75	21.95	0.33	0.20
Biofilm cells				
2	13.27	6.94	0.52	0.11
4	11.87	9.61	0.81	0.26
6	11.79	9.21	0.78	0.24
8	15.44	11.85	0.77	0.15
10	23.37	11.60	0.50	0.13

TCHO = total carbohydrates; PP = protein; Chl *a* = chlorophyll *a*; UA = uronic acid.

Table 3. Dilute acid soluble (Acid-TCHO), dilute alkali soluble (Alkali-TCHO) and residual (Res-TCHO) carbohydrates and their contribution to total carbohydrates (TCHO) in planktonic and biofilm cells of *Amphora rostrata*.

Cultivation period (d)	Acid-TCHO		Alkali-TCHO		Res-TCHO	
	a	b	a	b	a	b
Planktonic cells						
2	3.93 ±0.00	64.20	1.11 ±0.00	18.00	1.08	18.00
4	4.80 ±0.55	70.12	1.09 ±0.13	12.91	0.96	16.94
6	6.49 ±0.93	74.54	1.24 ±0.10	14.28	0.97	11.18
8	9.12 ±0.64	67.78	1.09 ±0.21	8.13	3.24	24.09
10	10.90 ±1.00	63.89	1.14 ±0.20	6.68	5.02	29.44
Biofilm cells						
2	0.85 ±0.00	43.23	0.51 ±0.00	25.90	0.60	30.92
4	1.16 ±0.09	47.89	0.51 ±0.10	20.91	0.75	31.22
6	1.17 ±0.16	51.80	0.55 ±0.02	24.29	0.79	23.91
8	1.68 ±0.31	61.38	0.50 ±0.03	18.06	0.56	20.55
10	2.33 ±0.40	69.86	0.63 ±0.03	18.81	0.38	11.32

a = concentration expressed as $\mu\text{g}/10^5$ cells;

b = percentage of TCHO.

Table 4. Monosaccharide composition (Mol %) of planktonic and biofilm cells of *Amphora rostrata*

Cultivation period (d)	Monosaccharides (Mol %)								Group composition (Mol %)		
	Rhamnose	Fucose	Ribose	Arabinose	Xylose	Mannose	Galactose	Glucose	Hexo	Pento	Deoxy-Hexo
Planktonic cells											
2	7.32 ^a	9.61 ^a	1.05 ^a	3.47 ^a	6.52 ^a	8.14 ^a	10.15 ^a	53.75 ^a	72.03	11.04	16.93
4	3.46 ±0.01	9.79 ±0.02	3.13 ±0.00	2.65 ±0.00	5.67 ±0.01	5.86 ±0.01	27.42 ±0.04	42.12 ±0.07	75.40	11.45	13.25
6	1.80 ±0.30	31.52 ±3.06	0.58 ±0.09	0.75 ±0.08	5.13 ±1.01	12.87 ±0.01	22.20 ±0.37	25.03 ±4.18	60.10	6.46	33.32
8	1.46 ±0.11	37.10 ±5.49	0.73 ±0.03	5.09 ±0.45	3.62 ±0.97	13.50 ±0.45	21.49 ±2.82	17.00 ±3.60	52.00	9.44	38.56
10	1.90 ±0.13	38.44 ±5.00	0.56 ±0.09	7.77 ±1.17	2.63 ±0.52	13.89 ±2.10	24.36 ±1.97	10.45 ±1.20	48.70	10.95	40.34
Biofilm cells											
2	4.34 ^a	16.00 ^a	3.06 ^a	2.22 ^a	6.60 ^a	9.95 ^a	22.51 ^a	35.88 ^a	68.34	11.89	20.33
4	3.75 ±0.99	11.42 ±1.30	4.95 ±0.91	0.66 ±0.14	4.11 ±0.66	6.81 ±1.52	31.72 ±4.04	36.57 ±5.56	75.10	9.72	15.17
6	2.47 ±0.07	6.27 ±0.16	1.82 ±0.56	3.01 ±0.90	2.63 ±0.21	3.60 ±0.09	22.57 ±1.13	57.53 ±4.38	83.69	7.47	8.75
8	2.46 ±0.24	7.56 ±1.22	1.59 ±0.13	0.48 ±0.09	4.01 ±0.70	3.36 ±0.01	19.40 ±1.15	61.14 ±2.12	83.91	6.08	10.01
10	3.84 ±0.34	6.56 ±0.19	1.26 ±0.16	0.38 ±0.05	3.26 ±0.50	3.36 ±0.17	21.25 ±1.89	60.75 ±3.81	85.36	4.91	10.41

Hexo = Hexoses (mannose + galactose + glucose); Pento = Pentoses (ribose + arabinose + xylose); Deoxy-Hexo = Deoxy hexoses (rhamnose + fucose).

^a = Single observation as cells were very less.

Table 5. Amino sugar composition of planktonic and biofilm cells of *Amphora rostrata*.

Cultivation Period (d)	AS composition (Mol %)			GlcN/GalN ratio
	GlcN	GalN	ManN	
Planktonic cells				
2	2.21	61.58	36.22	0.04
4	11.96	59.81	28.23	0.20
6	ND	ND	ND	ND
8	18.72	33.99	47.29	0.55
10	22.84	26.10	51.05	0.86
Biofilm cells				
2	72.73	18.80	8.47	3.87
4	52.15	32.59	15.26	1.60
6	35.83	56.75	7.42	0.63
8	44.88	30.53	24.59	1.47
10	74.21	12.61	13.18	5.89

AS = amino sugars; GlcN = glucosamine; GalN = galactosamine; ManN = mannosamine; ND = no data.

Figure captions:

Figure 1. Changes in Chlorophyll *a* (a), cell numbers (b) growth rate (c), and cell dry weight (d) for planktonic and biofilm cells of *Amphora rostrata* over the period of cultivation.

Figure 2. Changes in concentrations of nitrate, phosphate and silicates in culture medium during the growth of *Amphora rostrata*.

Figure 3. Concentration of organic carbon (OC) (a), protein (PP) (b), total carbohydrate (TCHO) (c), total neutral sugars (TNCHO) (d), uronic acids (UA) (e), and amino sugars (AS) (f) in planktonic (—◆—) and biofilm (—○—) cells of *Amphora rostrata*.

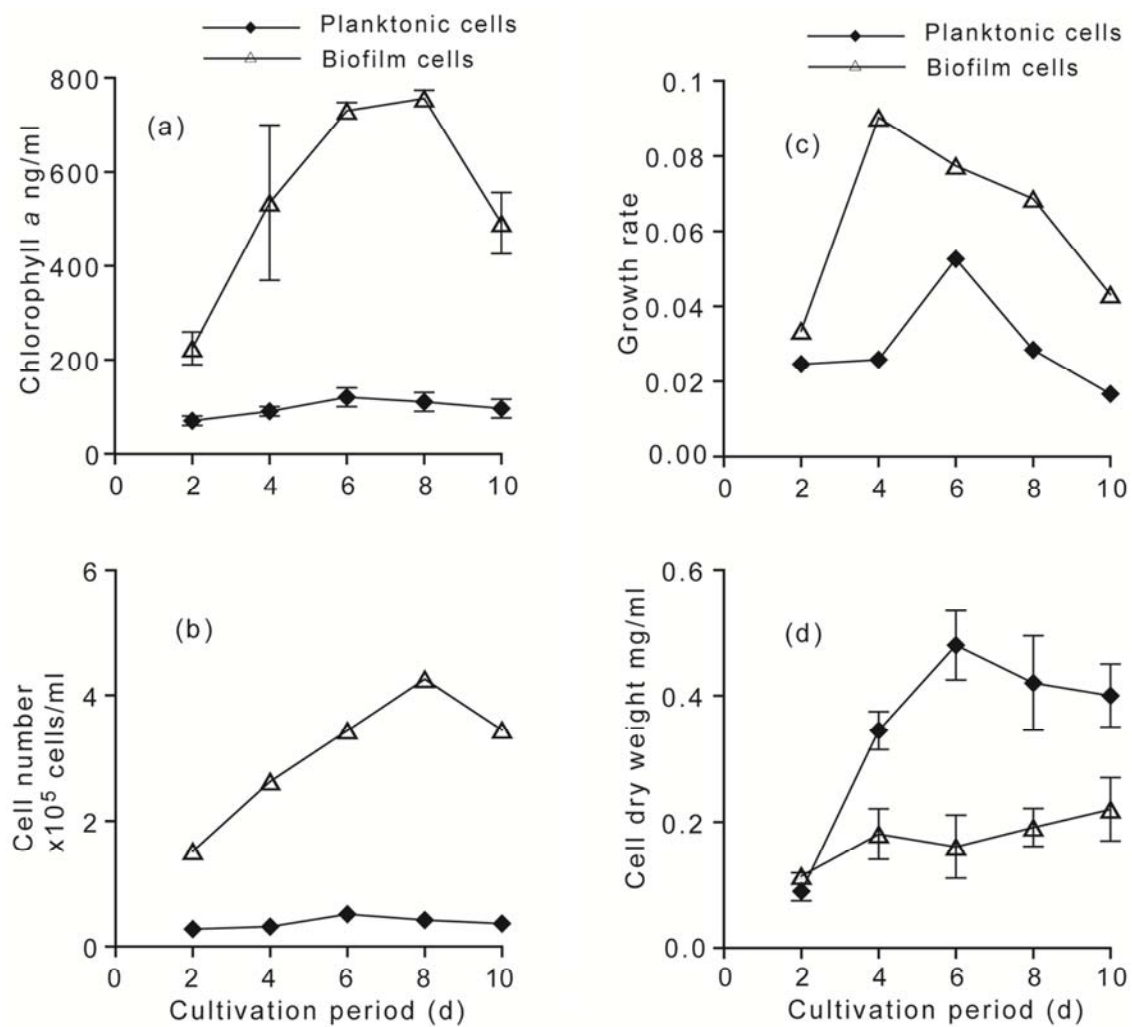


Figure 1.

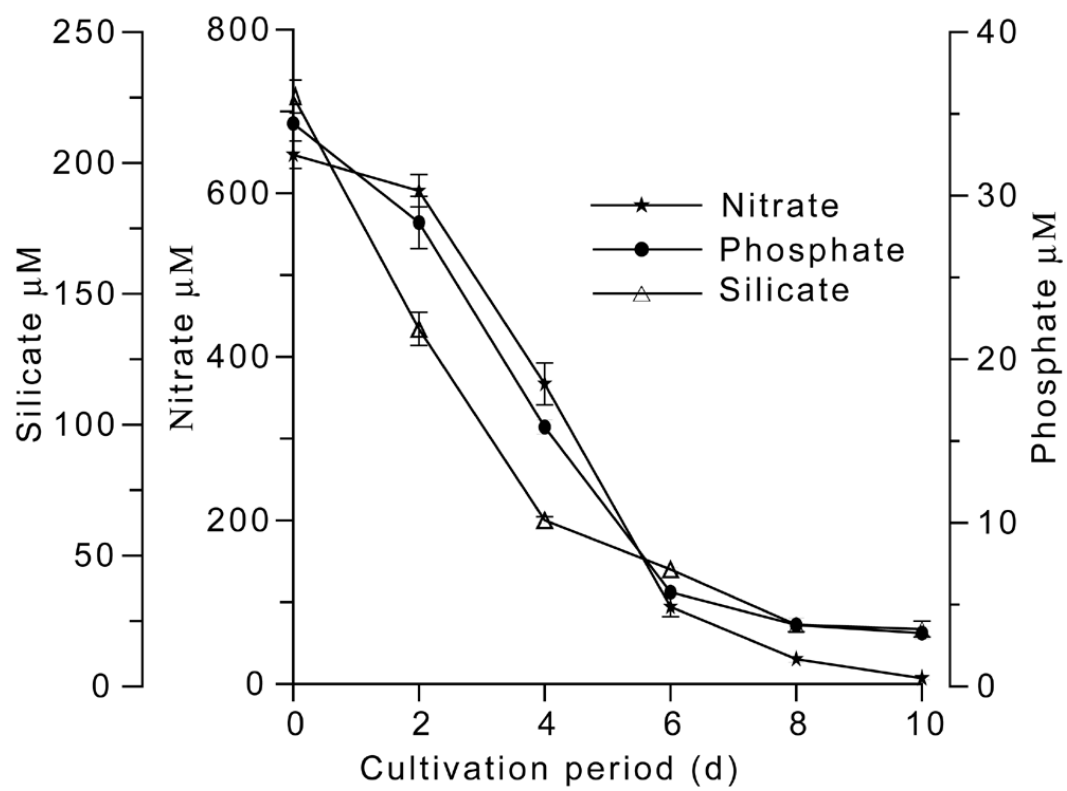


Figure 2.

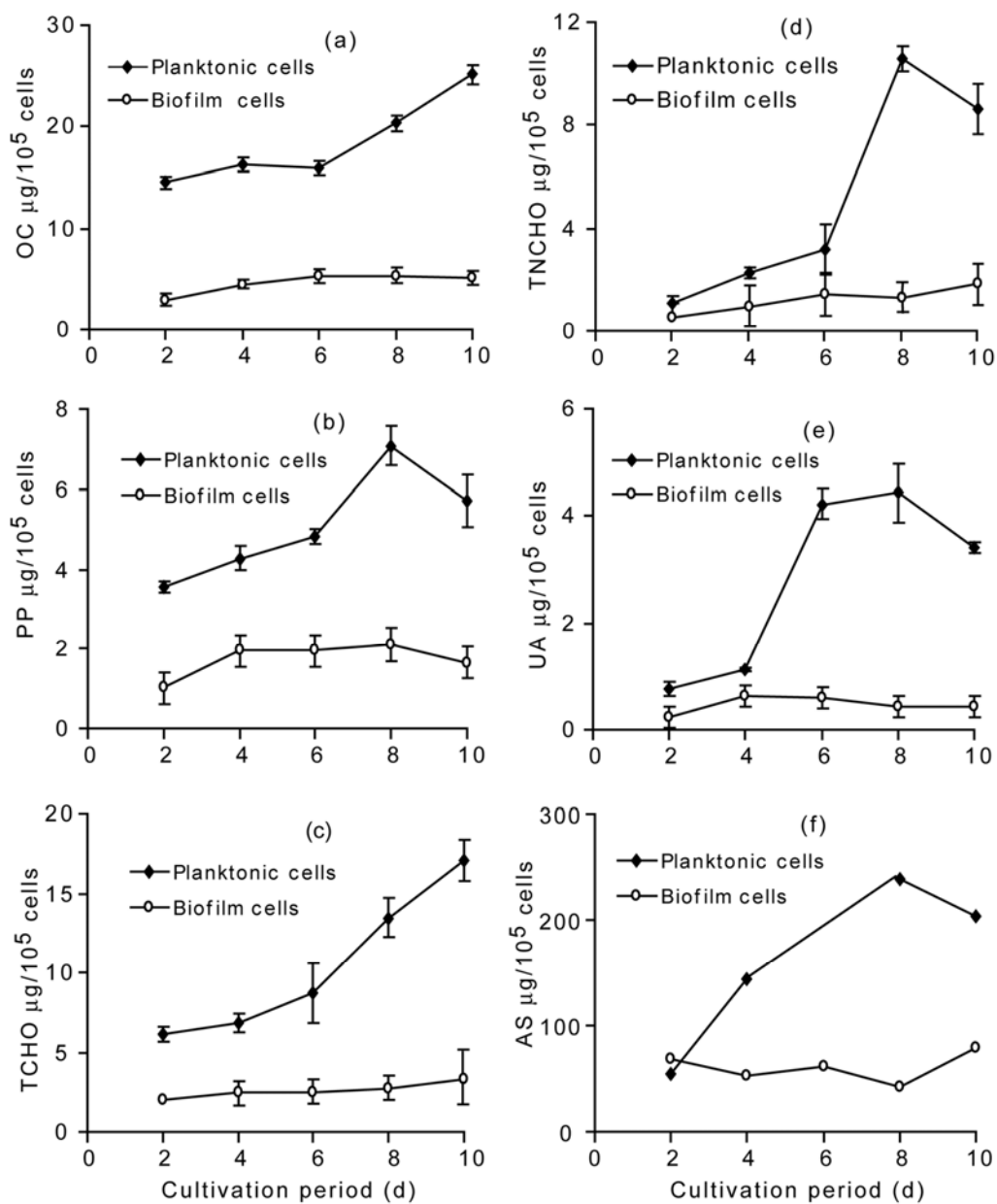


Figure 3.