3.0 RESULTS AND DISCUSSION

3.1 (A) Study on bioadhesive

3.1.1 Screening of bioadhesive producing bacteria

3.1.1.1 Primary screening based on mucoidal nature of the colony
The mucoid characteristic of a strain developed on solid media is the only practically feasible method for screening of exopolysaccharide (EPS) producers (Fusconi and Godinho, 2002). When number of biofilm samples from different environmental/ecological niche (as enlisted in Table 3A), were plated on high C:N ratio medium, sixty bacterial isolates producing highly mucoid colony were obtained.

3.1.1.2 Secondary screening based on adhesive property
Initially using whole cell biomass from culture plate of the sixty isolates, when “paper peel test” was undertaken eight isolates showed comparatively better adhesive ability. The EPS of the eight isolates was recovered from culture filtrate, and again checked for its adhesive property by “paper peel test”. In the case of some of the EPS samples, when one paper was pulled apart, it came out with some part of the counter-paper due to stronger bonding between two adhered papers. Out of this, one of the isolate designated here as ADE-0-1 from mar:nc source which showed good amount of EPS production as well as the strongest adhesive ability and was selected for further studies. Later as this isolate was identified as Bacillus megaterium ADE-0-1, henceforth this EPS was termed as “EPS-BM adhesive”.

45
<table>
<thead>
<tr>
<th>Sample source</th>
<th>Adhesive ability</th>
<th>Gram-reactions</th>
<th>Colony characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhizosperic soil (sample 1)</td>
<td>+</td>
<td>-Ve, Short rods</td>
<td>raised, mucoid, opaque, off-white</td>
</tr>
<tr>
<td>Rhizosperic soil (sample 2)</td>
<td>+</td>
<td>+Ve, Short rods</td>
<td>raised, mucoid, translucent, smooth margin</td>
</tr>
<tr>
<td>Rhizospheric sea-shore sample (ADF-0-1)</td>
<td>+++</td>
<td>+Ve, Large rods</td>
<td>raised, round, smooth, colourless, opaque with the entire edge and mucoid in nature</td>
</tr>
<tr>
<td>sea-shore sample (1-3)</td>
<td>++</td>
<td>+Ve, Cocci</td>
<td>raised mucoid, irregular margin, light brown pigment, opaque</td>
</tr>
<tr>
<td>sea-shore sample (3-5)</td>
<td>++</td>
<td>+Ve, Large rods</td>
<td>raised, mucoid, off-white, opaque, serrated margin</td>
</tr>
<tr>
<td>sea-shore sample (5-7)</td>
<td>++</td>
<td>-Ve, Cocci</td>
<td>irregular margin, mucoid, raised, opaque, off-white</td>
</tr>
<tr>
<td>Garden soil</td>
<td>+</td>
<td>+Ve, Cocci</td>
<td>highly irregular margin, flat, dry, opaque, off-white</td>
</tr>
<tr>
<td>Air-condition duct</td>
<td>+</td>
<td>-Ve, Large rods</td>
<td>flat, irregular margin, opaque, off-white</td>
</tr>
</tbody>
</table>
3.1.2 Characterization of BM adhesive

3.1.2.1 Gross chemical composition of BM adhesive

Chemical analysis of the polysaccharide by Dubois et al. (1956), revealed that the total sugar present in the BM adhesive was found to be 75 % (w/w), indicating that the polymer was mainly a polysaccharide. Further, in the BM adhesive, no protein and acetyl content were detected by Bracford et al. (1976) and Hestrin et al. (1949), methods, respectively.

A modified carbazole method for determination of uronic acid in the EPS as described by Bitter and Muir (1962) was used due to its increased sensitivity for the detection of uronic acids such as mannuronic acid and glucuronic acid (Sutherland, 1970). Uronic acid content in the EPS adhesive was found to be 17 % (w/w), which was comparatively high for an EPS. Although EPS of Sphingomonas paucimobilis GS-1 and Rhizobium radiobacter BE-1 have been reported to contain 18 % and 22 % uronic acid, respectively, they were without adhesive property and unlike BM adhesive, produced highly viscous solution (Ashtaputre and Shah, 1995; Iyer, 2008). Since, ionised carboxyl groups of uronic acid confer a net negative charge to the polymer, cells can interact with the variety of cationic surfaces bearing Ca\(^{12}\), Mg\(^{12}\) and Sr\(^{12}\) and can showed adherence to such surfaces.

3.1.2.1.1 Estimation of pyruvate

Pyruvyl content in EPS adhesive was estimated by the method described by Slonekar and Orentas (1962) and it was found to be 0.00125 % (w/w). In addition to uronic acids, ketal-linked pyruvate imparts anionic nature to the EPS and can influence its ordered structure too (Sutherland, 2001).

3.1.2.1.2 Determination of monomeric composition of BM adhesive

3.1.2.1.2.1 Hydrolysis of EPS

In order to determine monosaccharide compositions, polysaccharides are usually hydrolysed using strong acids such as H\(_2\)SO\(_4\) or HCl (Kennedy and Sutherland, 1987; van Casteren, et al., 1998; Shanta et al., 2001). Use of such strong acids for hydrolysis of EPS has disadvantages such as the degradation of aldopentoses, deoxy sugars, uronic acids (through decarboxylation) and loss of acetyl groups in N-
acetylated sugars. Hence in the present study, trifluoroacetic acid (TFA) was used for hydrolysis of EPS adhesive, as it is easily evaporated from sample (i.e. no need to neutralization after hydrolysis).

Initially, to determine the time required for complete hydrolysis of EPS adhesive, 2 M trifluoroacetetic acid (TFA) was used at 100 °C for different time intervals (0, 0.5, 1.0 1.5, 2.0, 2.5 and 3 h). In an earlier report, for determination of monomeric composition, an EPS of *Pseudomonas* *spp.* strain ATCC 53923 was hydrolysed using 2 M TFA for 4 h at 120 °C (Dasinger, 1994).

### 3.1.2.1.2.1 Paper chromatographic analysis

For obtain preliminary information on the likely composition of the EPS, paper chromatography of the EPS hydrolysates has been carried out in several previous studies (Linker and Jones, 1966; Kennedy and Sutherland, 1987; Shanta *et al.*, 2001). Hence, when the extent of hydrolysis of EPS adhesive was followed by paper chromatography i.e. from 0.5 to 3 h, a faint spot with smear was observed in the hydrolysate obtained after 0.5 h, appeared progressively distinctive and after 2 h almost complete hydrolysis of EPS adhesive was observed. The partially hydrolysed EPS would contain reducing ends compared to native EPS (Aspinall, 1982). Upon estimation no further increase in reducing sugar content in the EPS hydrolysate was observed beyond 2 h of hydrolysis, supporting the results obtained from paper chromatography (Figure 3.1 A and B).

In paper chromatograms in case of EPS hydrolysates, the Spots corresponding to $R_f$ 0.44, 0.18 and 0.2 indicated the presence of glucose, glucuronic acid and galacturonic acid, respectively (Figure 3.1 A and B). Similarly in a different paper chromatogram, mannose and arabinose ($R_f$ 0.5 and 0.46, respectively) were also detected in comparison to the authentic sugars. In a previous report, using a similar solvent system employed in the present study, the monomer composition of *Klebsiella aerogenes* Type 8 polysaccharide was determined (Sutherland, 1970).
Figure 3.1 Paper chromatogram of BM adhesive hydrolysate

EPS was hydrolysed using 2 M TFA at 100 °C for 0.5 to 3 h. 2 μl of authentic standards (1 mg/ml) and lyophilized hydrolysates (10 mg/ml) were applied on Whatman No. 1 filter paper and the chromatograms were developed using solvent system:n-butanol:pyridine:water (6:4:3). Silver reagent was used for spots visualization. Chromatogram (A) Spot numbers 1=glucose, 2= galactose, 3=fructose, and spots of hydrolysates of EPS for different time intervals (h) were as follows: spots 4 = 0.5 h, 5=1 h, 6=1.5 h, 7=2 h, 8=2.5 h and 9=3 h. Chromatogram (B) spot 10=glucuronic acid, 11=galacturonic acid. In both the chromatograms (A) and (B), spots a, c and b corresponds to R<sub>f</sub> for glucose = 0.44, glucuronic acid= 0.18, galacturonic acid= 0.2, respectively.

EPS was hydrolysed using 2 M TFA at 100 °C for 2 h. Chromatogram (C) Spot numbers 1- Mannose, 2- EPS hydrolysate, 3- Xylose, 4-Arabinose, 5- EPS hydrolysate, 6-Galactose, 7- Glucosamine, 8- EPS hydrolysate + Xylose and 9- glucose + EPS hydrolysate, In both the chromatograms (C) spots d and e corresponds to R<sub>f</sub> for mannose= 0.5, Arabinose = 0.46, respectively.

3.1.2.1.2 High performance liquid chromatography

The monosaccharide composition of BM adhesive was analyzed using HPLC. A comparison of retention times (R<sub>t</sub>) of the peaks obtained for components in hydrolysate (2 h) with those of authentic compounds, revealed the presence of galacturonic acid, glucuronic acid, glucose, mannose and arabinose in the hydrolysate (Figure 3.2), indicating that the above EPS (BM adhesive) was an acidic heteropolysaccharide. In the chromatogram, at R<sub>t</sub> = 5.8 a large and steep peak was observed, which could be a region in polysaccharide which was resistant to hydrolysis by TFA.
Earlier reports suggested that the presence of monomer sugar like arabinose in EPS helps in cells aggregation in bacteria and acidic nature of polysaccharides helps in biofilm formation. Moreover, EPS that are rich in acidic moieties are refractile and hence take longer time for decomposition by bacteria and digestion by feeders (Fletcher and Floodgate, 1973; Decho, 1990; Efrat et al., 2004).

**Figure 3.2 HPLC analysis of EPS hydrolysate.**

HPLC analysis of the EPS hydrolysate (2 M TFA, 100 °C, 2 h) was performed using a Waters 2410 HPLC system. Water containing EDTA (40 mg/l) and CaCl₂ (15 mg/l) was used as a mobile phase at a flow rate of 0.4 ml/min and the peaks were detected using RI detector. 30 µl of either authentic standards (20 mg/ml) or hydrolysate (20 mg/ml) was injected. Peak at Rₜ = 5.8 observed might be attributed to partially hydrolyzed part of EPS.

Compared to BM adhesive (present study), MB (Montana Biotech) EPS adhesive contained 95 % carbohydrates, 1 to 3 % protein and only 1 % uronic acid (Combie et al., 2004). While EPS of *Bacillus megaterium* Strain 98TH11316 isolated from sea water which was capable of biofilm formation on glass surface was composed of glucose, mannose, galactose and glucuronic acid (Kwon et al., 2002). However the above EPS was not characterized for its adhesive property. Capsular material of *Bacillus megaterium* WH320, chemically characterized as poly-γ-glutamate (PGA), has been reported as a biodegradable ‘bionylon’ (Shimizu et al., 2007).
3.1.2.2 Fourier transform-infrared (FT-IR) analysis

Polysaccharides contain a significant number of hydroxyl groups. An intense broad stretching peak at 3445 cm\(^{-1}\) was typical of hydroxyl groups (Figure 3.3). Two weak C-H stretching peaks at 2929 and 2857 cm\(^{-1}\) corresponded to methyl as well as methylene group while strong absorption at 1638 cm\(^{-1}\) and very weak absorption at 1350 cm\(^{-1}\) indicated C=O stretch of the COO\(^{-}\) group. A broad but weak peak at 1159 cm\(^{-1}\) suggested the presence of ketal (pyruvate).

**Figure 3.3 FT-IR spectrum of the EPS**

![FT-IR spectrum of the EPS](image)

The FT-IR spectrum of EPS was recorded as KBr pellet in the region of 4000–250 cm\(^{-1}\), by using Perkin-Elmer FT-IR spectrometer.

The polar hydroxyl groups in the adhesive promote adhesion to polar surfaces like aluminium (Al) but they are also hydrophilic and lead to low water resistance. The carboxyl groups present in EPS revealed may serve as a binding site for divalent cations thereby facilitating adhesion on to aluminium and iron substrates/surfaces (Bramhachari *et al*., 2007).
3.1.2.3 Determination of ionic nature

A large number of native polysaccharides carry anionic (carboxyl, phosphate and sulphate) or cationic functions. Compared to cationic EPS, anionic polysaccharides are more predominant. Chitosan bearing amine functions is one of the few such cationic polysaccharides. EPS can also be substituted, normally ester or N-linked, with pyruvate, acetate, formate, sulfate, phosphate and other side groups (Jann and Westphal, 1975) adding to their heterogeneity. The anionic nature of BM adhesive was determined by measuring its efficiency of binding towards cation (Dowex 50) exchange resins. Almost 91.6% of the EPS remained bound to anion exchange resin. Further, the quartenary ammonium compound, cetyl pyridinium chloride (1 % w/v), could precipitate EPS. The above observations suggested that the EPS was **anionic and acidic in nature** (Danishefsky, 1970; Kumar et al., 2004).

3.1.2.4 Molecular weight determination

High molecular weight is one of the desirable structural features useful in adhesive as mechanical properties generally improve with high molecular weight (Lazaridou et al., 2003). Using sepharose-4B gel filtration column, molecular weight of BM adhesive was found to be **0.5 X 10^6 Da**. The molecular weight of exopolysaccharides isolated from eight biofilm forming strains from glass surface ranged from 0.38 to 25.19 x 10^4 Da and *Bacillus megaterium* strain (98TH11316) had molecular weight 2.1 x 10^4 Da (Kwon et al., 2002). While molecular weight of EPS-MB adhesive was 4x 10^4 Da (Haag et al., 2004).

3.1.3 Evaluation of adhesive property

3.1.3.1 Adhesive ability

Out of 28 combinations of specimens analysed, maximum lap shear strength was 5.46 ± 0.04 MPa in the case of wood-wood specimen (Table 3.1). Among the metals, lap shear strength was found in order of Al-Iron (2.79 ±0.08 MPa) > Steel-Steel (2.26 ±0.12 MPa) > Iron-Iron (1.59 ±0.01 MPa) and Al-Al (1.59 ±0.02 MPa) > Tin-Iron (1.33 ±0.01 MPa). With the exception of steel, it was always noticed that when specimen was prepared using two metal surfaces, higher adhesive ability was observed than that prepared using one metal surface and one non-metal surface.
Table 3.1 Shear strengths (MPa) of different specimens bonded with adhesive

<table>
<thead>
<tr>
<th>Samples</th>
<th>Wood</th>
<th>Smc</th>
<th>Acrylic</th>
<th>Iron</th>
<th>Steel</th>
<th>Al</th>
<th>Tin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wood</td>
<td>5.46±.04</td>
<td>1.99*</td>
<td>0.79±.05</td>
<td>0.26±.01</td>
<td>0.79±.02</td>
<td>0.53±.01</td>
<td>0.66±.02</td>
</tr>
<tr>
<td>Smc</td>
<td>1.99*</td>
<td>0.93±.04</td>
<td>0.39±.05</td>
<td>1.06±.04</td>
<td>0.66±.02</td>
<td>0.79±.05</td>
<td>0.39±.02</td>
</tr>
<tr>
<td>Acrylic</td>
<td>0.79±.05</td>
<td>0.39±.05</td>
<td>0.93±.07</td>
<td>0.39±.05</td>
<td>1.06±.06</td>
<td>0.26±.04</td>
<td>0.26±.01</td>
</tr>
<tr>
<td>Iron</td>
<td>0.26±.01</td>
<td>1.06±.04</td>
<td>0.39±.05</td>
<td>1.59±.01</td>
<td>0.53±.03</td>
<td>2.79±.08</td>
<td>1.33±.01</td>
</tr>
<tr>
<td>Steel</td>
<td>0.79±.02</td>
<td>0.66±.02</td>
<td>1.06±.06</td>
<td>0.53±.03</td>
<td>2.26±.12</td>
<td>0.53±.03</td>
<td>0.39±.02</td>
</tr>
<tr>
<td>Al</td>
<td>0.53±.01</td>
<td>0.79±.02</td>
<td>0.26±.02</td>
<td>2.79±.08</td>
<td>0.53±.03</td>
<td>1.59±.02</td>
<td>1.06±.04</td>
</tr>
<tr>
<td>Tin</td>
<td>0.66±.02</td>
<td>0.39±.02</td>
<td>0.26±.01</td>
<td>1.33±.01</td>
<td>0.39±.02</td>
<td>1.06±.04</td>
<td>0.79±.02</td>
</tr>
</tbody>
</table>

An adhesive solution of 10 % (w/v) and having pH 7 was used. Specimen dimensions were 30×2.5×0.3 cm for wood (Shorea robusta) and 30×2.5×0.1/0.2 cm for metals and plastics. Surfaces of adherends were roughed using sand paper, wiped with ethanol and adhesive (13.33 mg/cm²) was applied to 2.5x3 cm corner area of one of the adherend and the second adherend overlapped that area. Both adherends were clamped tightly together for 1 h and were pressed under 7.5 kg weights for further 18 h to allow the adhesive to set up. Finally bonded specimens were cured for 7 days at 30 °C ±1 and 50 ±5 % relative humidity and lap shear strength was determined using UTM.

Each individual determination was carried out in triplicate and each complete experiment was carried out twice.

*In this case, substrate failure in Sunmika (Smc) had occurred.
Table 3.2 Effect of environmental factors on shear strength (MPa) of different specimens bonded with adhesive

<table>
<thead>
<tr>
<th>Samples</th>
<th>pH 4&lt;sup&gt;a&lt;/sup&gt;</th>
<th>pH 8&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Salinity&lt;sup&gt;b&lt;/sup&gt; (5 %)</th>
<th>Salinity&lt;sup&gt;b&lt;/sup&gt; (20 %)</th>
<th>Control&lt;sup&gt;c&lt;/sup&gt;</th>
<th>4 °C&lt;sup&gt;d&lt;/sup&gt;</th>
<th>50 °C&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wood-Wood</td>
<td>3.3±.4</td>
<td>5.79±.03</td>
<td>4.92±.10</td>
<td>5.85±.02</td>
<td>5.46±.04</td>
<td>NA</td>
<td>6.12±.03</td>
</tr>
<tr>
<td>Iron-Al</td>
<td>2.13±.15</td>
<td>2.92±.03</td>
<td>2.26±.10</td>
<td>2.92±.23</td>
<td>2.79±.08</td>
<td>NA</td>
<td>3.06±.04</td>
</tr>
<tr>
<td>Smc-Wood</td>
<td>1.46±.10</td>
<td>1.99*</td>
<td>1.73 ±.10</td>
<td>1.99*</td>
<td>1.99*</td>
<td>NA</td>
<td>1.99*</td>
</tr>
<tr>
<td>Steel-Steel</td>
<td>1.86±.07</td>
<td>2.53±.02</td>
<td>2.26±.10</td>
<td>2.53±.03</td>
<td>2.26±.12</td>
<td>NA</td>
<td>2.66±.04</td>
</tr>
<tr>
<td>Iron-Iron</td>
<td>1.33±.07</td>
<td>1.73±.01</td>
<td>1.46±.10</td>
<td>1.86±.05</td>
<td>1.59±.01</td>
<td>NA</td>
<td>1.99±.06</td>
</tr>
<tr>
<td>Al-Al</td>
<td>1.2±.11</td>
<td>1.73±.01</td>
<td>1.46±.10</td>
<td>1.86±.08</td>
<td>1.59±.02</td>
<td>NA</td>
<td>1.99±.05</td>
</tr>
</tbody>
</table>

<sup>a</sup> Adhesive used had pH 4 or 7 (control) or 8. <sup>b</sup> Adhesive used contained 5 % or 20 % (w/v) sodium chloride. <sup>c</sup> Adhesive without sodium chloride having pH 7 was used. An adhesive solution of 10 % (w/v) was used. Specimens were prepared and cured as described in the footnote under Table 1. <sup>d</sup> Bonded specimens prepared as described previously were cured at 4 °C, 30 °C (control- relative humidity 50 ± 5 %) and 50 °C.

NA- Not attempted as adherends got separated during curing at 4 °C.

Each individual determination was carried out in triplicate and each complete experiment was carried out twice.

*In this case, substrate failure in Sunmika (Smc) had occurred.
It has been reported that the porous nature of the wood surface facilitate good penetration of the adhesive into the wood enabling very intimate wood adhesive interaction which results into high bond strength. Further high wood surface roughness due to its cellular structure and multi-polymer (cellulose, hemicellulose and lignin) composition is an additional factor which enhances good wood bonding compared to other surfaces (Frihart, 2005). Also the polar and H-bonding functional groups of polysaccharides such as ethers, hydroxyls and carboxylates impart good adhesion to high energy surfaces such as wood and metal and also strong inter-chain interaction for cohesive strength (Haag, 2006).

Compared to temperature and salinity, pH significantly influenced adhesive ability (Table 3.2). Compared to pH 4, adhesive ability was higher at pH 8 and this increase was in the range of 30 to 72 % depending upon the combinations of surfaces in the specimens. This is understandable because ionization of carboxylate anions (uronic acids) increases under alkaline condition and consequently increased ionic interaction involved in adhesion makes bond stronger. However non-ionic interactions are also involved in adhesion process as even at pH 4 considerable adhesive strength (3.3 MPa for wood specimen) was observed.

In the case of wood-wood combination, even at 20 % (w/v) NaCl and 50 °C, higher adhesive ability was observed than that of control at pH 7. High salt and high temperature lead to more decrease in water solvent in bond area and this in turn increased bond strength under such conditions. Maximum lap sheer strength observed was 6.12 ±0.03 MPa with the wood to wood specimen at 50 °C, pH 7 and without NaCl.

BM adhesive showed a low moisture resistance and the integrity of adhesive bond was compromised by rehydration at 4 °C due to prevailing high humidity. Like BM adhesive, sensitivity of adhesive bond to moisture has been also reported in the case of Montana Biotech (MB) as well as Speciality Biopolymers (SB) adhesive and less hydrophilic chemically modified derivatives of both showed improvement in shear
strength during submersion in water and under humid atmospheric conditions (Haag et al., 2004; Haag, 2006).

Previously evaluation of “polysaccharide adhesive viscous exopolysaccharide” (PAVE) material isolated from nine marine strains of *Alyromonas coloelliana* LST for seven different substrates using lap shear specimens and 6.25 cm² bond area showed maximum shear strength up to 0.5 MPa (with Aluminium-0.8 MPa, stainless steel-0.24 MPa, Acrylic- 0.17 MPa and wood-0.57 MPa) (Labare et al., 1989).

Testing of an adhesive preparation containing at 33% (w/v) solid residues, residual cellulose, bacterial cells and associated adhesions from a *Ruminococcus* cellulose fermentation using three-ply aspen panels (Lap shear specimen) has shown shear strength of 1.69 MPa and 0.67 MPa under dry and wet conditions respectively. However panels prepared by incorporating fermentation residues into phenol-formaldehyde adhesive formulation to a tune of 73% by weight of total adhesive exhibited shear strength similar to that obtained by phenol-formaldehyde adhesive alone which was 3.37 MPa (Weimer et al., 2003).

Evaluation of two well investigated bacterially derived MB and SB adhesives, composed of exopolysaccharides, primarily as a wood adhesive using maple wood specimen, have shown 12.5 and 14.5 MPa shear strength (bond area 19.4 cm²) respectively (Haag et al., 2004; Haag, 2006). Further MB adhesive showed shear strength of 5.6 to 6.2 MPa with bare aluminium (3.22 cm² bond areas) and epoxy glass. Of the six samples tested, di-allyl phthalate and acrylonitrile butadiene styrene were the only plastic that exhibited shear strength above 0.69 MPa against 0.93 MPa shear strength observed with summika and acrylic plastic glued with BM adhesive.

While comparing shear strengths of MB/SB adhesive with BM adhesive (present work), it should be noted that in above cases adhesive preparations used had 31% (w/v) solids (MB adhesive) and 33% (w/v) solids (SB adhesive) against only 10% (w/v) solids used in the case of BM adhesive. Further application of MB and SB adhesive was 13-20 mg/cm² bond area of both the adherends compared to 13.33 mg/cm² bond area of only one adherend as in the case of BM adhesive. Also with wood specimen total bond area was 19.4 cm² (MB/SB adhesive) against 7.5 cm² in the case of BM adhesive. Taking above mentioned differences into consideration, MB/SB
adhesives were tested at 5 to 8 times higher concentration than that of BM adhesive and hence BM adhesive appeared superior than MB and SB adhesive.

In the case of *Bacillus megaterium* ADE-0-1, capsular material based adhesive ability resulted in maximum biofilm formation at around 24 h (Section 3.2.1, figure 3.9 b) and was found amylase and protease producer (Table 3.4). Like solid residues of *Ruminococcus* cellulose fermentation, amylase and protease activity based solid state fermentation residues of appropriate stage (growth) can be exploited for a cheaper capsular material based water resistant adhesive.

### 3.1.3.2 Adhesion study under moist condition

Like the use of solid residues from *Ruminococcus* cellulose fermentation, a qualitative kind of preliminary experiment for testing of whole cell biomass (scrapped from culture plates) as such for adhesive property under moist/water condition was also carried out where cell biomass grown for different growth period (24, 48, 72, 96 and 120 h) was used to adhere wood-wood and glass-glass specimens and cured at 30 ±1 °C and relative humidity 50 ±5 % for seven days. After curing, the bonded specimens were kept at 4 °C (under moist condition) for 48 h and then adhesive nature of biomass was checked by pulling apart the two adherends.

**Figure 3.4 Whole cell biomass as water resistant adhesive**

Wood to wood specimen bonded with the whole cell biomass (48 h grown) culture isolate ADE-0-1 was kept into a beaker containing distilled water for several hours.
Interestingly in contrast to the previously reported results at 4 °C (table 3.2), wood-wood (and also glass-glass) adherends did not separate in the case of 48, 72 and 96 h grown biomass. Further, on immersing the specimens in the water body, adherends did not separate at least up to 8 h, particularly only in the case of 48 h grown biomass (Figure 3.4). These observations revealed maximum water resistant adhesive property of cells at 48 h of growth stage. These findings suggested that as ADE-0-1 is an amylase and protease producer, biomass grown for optimum stage (in terms of adhesiveness) on cheap starchy/proteinaceous substrates could be a potential cheap source of adhesive with water resistant nature and could be exploited for binding of ply-wood panels like Ruminococcus cellulose fermentation residues.

3.1.4 Yield improvement of BM adhesive

3.1.4.1 Box-Behnken design

The values of variables and the estimated effect of each variable on BM adhesive yield and biomass production are as shown in table 3.3. Here, runs 4, 7 and 10 represent “un-optimized medium” in which EPS and biomass average yield of 1.12 g/l and 0.82 g/l respectively, were obtained. Comparison of yield obtained from un-optimized medium with that from “response surface methodology” revealed that no improvement in the EPS production was achieved by the approach in the concentration range tested. Drop in pH from initial 7.2 to less than 4.0 was observed in all the sets. Lack of improvement in yield was surprising as composition of the medium referred above as “un-optimized” was reasonably satisfactory for Sphingomonas paucimobilis GS-1 where 6 to 7 (g/l) EPS was produced (Ashtaputre and Shah, 1995).
### Table 3.3 Optimization of nutrients for EPS production by Box-Behnken design

<table>
<thead>
<tr>
<th>Tests</th>
<th>Sucrose (g/l)</th>
<th>KNO₃ (g/l)</th>
<th>K₂HPO₄ (g/l)</th>
<th>EPS Yield (g/l)</th>
<th>Biomass (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>1.0</td>
<td>0.25</td>
<td>0.40</td>
<td>0.34</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>1.0</td>
<td>0.75</td>
<td>0.80</td>
<td>0.25</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>0.5</td>
<td>0.50</td>
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<td>0.5</td>
<td>0.50</td>
<td>1.34</td>
<td>0.25</td>
</tr>
</tbody>
</table>

According to the combinations of concentrations of three nutrients given in the table, media flask (50 ml in 250 ml Erlenmeyer flask) were prepared and other nutrients present in the growth medium were also added, pH was adjusted to 7.2 ±0.2 and autoclaved at 10 psi for 20 minutes, *Bacillus megaterium* ADE-0-1 was inoculated at (OD₆₀₀nm = 0.4; 10 % v/v) level and flasks were incubated on orbital shaker at 180 rpm at 30 ±1°C for 72 h. Biomass of the *B. megaterium* ADE-0-1 was estimated gravimetrically. EPS from the culture supernatant (72 h) was precipitated using acetone, dried and estimated gravimetrically. The un-optimized media contained (g/l): Sucrose, 40; KNO₃, 1; K₂HPO₄, 0.5; MgSO₄, 0.2; NaCl, 0.1; CaCl₂, 0.1; yeast extract, 0.1 at a pH 7.2 ±0.2.
3.1.4.2 Effect of maintenance of pH and supplementation of phosphate on the growth and EPS production

During previous kinetic studies, a considerable drop in pH (from 7.2 to 4.5 or less) was always observed after exponential phase i.e. 24 h in the growth medium which contained 0.003 M $K_2HPO_4$. Drop in pH may affect growth and bioadhesin production. Hence, experiments were conducted using strongly buffered media (tris-buffer and phosphate buffer, pH 7.2 ±0.2) to control drop in pH during the experiment. Use of phosphate buffered media prevented the drop in pH during the experiment and pH was maintained throughout the experiment, while drop in pH was observed in control. There was an increase in the EPS production gradually with the increase in the concentration of phosphate buffer. EPS yield (g/l) was increased to about ten times (11.85 g/l) with 0.1 M phosphate buffer as compared to that of control (1.12 g/l) (Figure 3.5 A).

The growth was also increased significantly in the phosphate buffered media as compared to the control medium. In the case of a biofilm forming marine *Bacillus megaterium* strain having a different polymer composition, EPS yield of 1.329 g/l has been reported (Kwon et al., 2002). In a previous report, xanthan production by *Xanthomonas compestris* was shown to be increased by control of the pH (Moraine and Rogovin, 1971). A considerable increase in EPS yield observed in phosphate buffered media compared to the control medium could be due to favourable effect of (i) maintenance of pH, and (ii) more phosphate supplementation on growth and thereby on EPS production.

Also in the case of different concentrations of tris-buffered media (where phosphate present in the medium was similar to that of control medium i.e. 0.003 M), drop in pH was indeed prevented like phosphate buffered media. However, biomass obtained was nearly same to that of control medium. While negligible amount of powdery mass precipitated on solvent addition to cell-free supernatant of culture broth.

Although comparison of yield of biomass and EPS from phosphate buffered media with those from tris-buffered media suggested that stimulatory effect on growth and
Shake flask cultivation was carried out in phosphate buffered media (0.02 to 0.1 M, pH 7 ±0.2) and control media (where phosphate concentration was 0.003 M) at 180 rpm and 30 ±1 °C for 72 h. a) Biomass of the Bacillus megaterium ADE-0-1 was estimated gravimetrically. EPS from the culture supernatant (72 h) was precipitated using acetone, dried and estimated gravimetrically. pH was measured through pH meter. b) “Phosphate content” and “carbohydrate content” in the EPS were estimated as described by Fiske-subbaRow (1925) and Dubois et al., (1956), respectively.
EPS production observed in phosphate buffered media could be primarily due to more phosphate supplementation rather than due to maintenance of pH, stimulatory effect seen in phosphate buffered media, because of both i.e. maintenance of pH as well as higher phosphate supplementation should not be ruled out.

Instead of supplying phosphate in the form of phosphate buffer, when only K$_2$HPO$_4$ was provided at the same concentration, like buffered media, pH was found to be controlled and EPS yield increased to 5.5 to 6.5 times and growth yield higher than control was observed (Figure 3.6 A).
Shake flask cultivation at (30 ±1 °C, 180 rpm, for 72 h) was carried out in liquid non-buffered media (where K$_2$HPO$_4$ concentration was varied from 0.02 to 0.1 M, pH 7.2 ±0.2) and control media (where phosphate concentration was 0.003 M). a) Biomass of the Bacillus megatertum ADE-0-1 was estimated gravimetrically. EPS from the culture supernatant (72 h) was precipitated using acetone, dried and estimated gravimetrically. pH was measured through pH meter. b) “Phosphate content” and “carbohydrate content” in the EPS were estimated by Fiske-Subbarow and phenol-sulphuric acid methods respectively.
Association of phosphate with EPS through ionic interaction or esterification has been reported in the case of several EPS. Further a drastic increase in EPS yield (from 1.12 to 11.65 g/l) observed in phosphate buffered (0.1 M Phosphate) media (Figure 3.5 A) and increase (5.5 to 6.5 times) in EPS yield observed in phosphate non-buffered media (Figure 3.6 A) could be partly due to complexing of phosphate with EPS [referred here as EPS(p)] and thereby contributing to increase in weight of precipitated polymer. Increase in phosphate concentration in production media upto 25 mM improved the yield of EPS of Sphingomonas paucimobilis GS-1 (Ashtaputre and Shah, 1995). However the EPS so obtained was contaminated with phosphate which ultimately decreased the quality (viscosity) of the polymer.

Hence, estimation of “phosphate content” (as impurity) and “carbohydrate content” (as purity) of the precipitated EPS(p) was carried out to know quality (percent impurity/purity) of the recovered EPS(p). Along with the increase in phosphate concentration in the buffered and non-buffered media (from 0.02 to 0.1 M), phosphate impurity in the harvested polymer was found to increase up to 60 % and 9.25 % in 0.1 M of phosphate buffered media and non-buffered media, respectively (Figure 3.5 B and 3.6 B).

In conclusion, supplementation of K$_2$HPO$_4$ in the medium at 0.02 and 0.04 M could be a better choice for EPS production as only 3.47 % and 5.53 % phosphate contamination was found respectively, and adhesive property was found similar to that of control.

3.1.4.3 Effect of aeration on polymer production

When degree of aeration varied by incubating different culture broth volumes in 250 ml Erlenmeyer flask, compared to more aerated condition under less aerated condition (60 ml and 100 ml broth per 250 ml flask volume), more polymer production was obtained (Figure 3.7) while the biomass production did not change significantly.

In the case of Staphylococcus epidermidis, “polysaccharide intercellular adhesin” (PIA) involved in biofilm formation was found to be increased under conditions of
Alow oxygen availability along with the inhibition of tricarboxylic acid cycle activity (Cuong et al., 2005).

**Figure 3.7 Effect of aeration on EPS production**

![Graph showing effect of aeration on EPS production](image)

Shake flask cultivation at (30 ±1 °C, 180 rpm for 72 h) was carried out in a liquid medium by varying the volume of liquid medium (20, 40, 50, 60 and 100 ml) in 250 ml Erlenmeyer flasks. Biomass of the *Bacillus megaterium* ADE-0-1 was estimated gravimetrically. EPS from the culture supernatant was precipitated using acetone, dried and estimated gravimetrically.

### 3.1.5 Characterization of the strain ADE-0-1

#### 3.1.5.1 Morphological, cultural and biochemical characterization

For identification of the isolate, various characteristics of the isolate ADE-0-1 were studied. The strain ADE-0-1 was Gram positive, rod shaped, capsulated, sporulating and motile bacterium. Moreover the above isolate exhibited biochemical characteristics such as aerobic growth, catalase positive, β-galactosidase negative and esculin hydrolysis negative. Colonies of ADE-0-1 were found to be raised, medium sized, round, smooth, colourless and opaque with the entire edge and mucoid in nature. The culture characteristics of the isolate ADE-0-1 are shown in **table 3.4**.
Table 3.4 Culture and biochemical characteristics of isolate ADE-0-1

<table>
<thead>
<tr>
<th>Cultural characteristics</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram reaction</td>
<td>Gram positive</td>
</tr>
<tr>
<td>Rod size</td>
<td>≥1 μm</td>
</tr>
<tr>
<td>Voges-Proskauer test (V-P test)</td>
<td>-</td>
</tr>
<tr>
<td>Growth in anaerobic jar</td>
<td>-</td>
</tr>
<tr>
<td>Growth at 50 °C</td>
<td>-</td>
</tr>
<tr>
<td>Growth in 7 % NaCl</td>
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</tr>
<tr>
<td>Acid and gas in glucose</td>
<td>-</td>
</tr>
<tr>
<td>Acid in glucose</td>
<td>+</td>
</tr>
<tr>
<td>Starch hydrolysis</td>
<td>+</td>
</tr>
<tr>
<td>Casein hydrolysis</td>
<td>+</td>
</tr>
<tr>
<td>Growth at 65 °C</td>
<td>-</td>
</tr>
<tr>
<td>Parasporal body detection</td>
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</tr>
</tbody>
</table>

The culture characteristics were observed after performing the tests as described in Bergey’s Manual of Bacteriology.
Table 3.4 cont... Biochemical characteristics of the isolate ADE-0-1

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inulin fermentation</td>
<td>+</td>
</tr>
<tr>
<td>Sodium gluconate fermentation</td>
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</tr>
<tr>
<td>Glycerol fermentation</td>
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</tr>
<tr>
<td>Salicin utilization</td>
<td>+</td>
</tr>
<tr>
<td>Glucosamine fermentation</td>
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</tr>
<tr>
<td>Dulcitol fermentation</td>
<td>+</td>
</tr>
<tr>
<td>Inositol fermentation</td>
<td>+</td>
</tr>
<tr>
<td>Sorbitol fermentation</td>
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</tr>
<tr>
<td>Mannitol fermentation</td>
<td>+</td>
</tr>
<tr>
<td>Adonitol fermentation</td>
<td>-</td>
</tr>
<tr>
<td>α-methyl-D-glucosamine fermentation</td>
<td>+</td>
</tr>
<tr>
<td>Rhamanose fermentation</td>
<td>+</td>
</tr>
<tr>
<td>Cellobiose fermentation</td>
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</tr>
<tr>
<td>Melezitose fermentation</td>
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</tr>
<tr>
<td>α- methyl-D-Mannoside fermentation</td>
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</tr>
<tr>
<td>Xylitol fermentation</td>
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</tr>
<tr>
<td>D-arabinose fermentation</td>
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</tr>
<tr>
<td>Citrate utilization</td>
<td>+</td>
</tr>
<tr>
<td>Malonate utilization</td>
<td>-</td>
</tr>
<tr>
<td>Sorbose fermentation</td>
<td>+</td>
</tr>
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</table>

The biochemical tests were performed according to the methods described in information provided in HiCarbohydrate™ Kit (Himedia, India).
3.1.5.2 Phylogenetic characterization

3.1.5.2.1 16S rDNA sequence analyses

Molecular methods involving the comparison of 16S rDNA sequences provide new and more precise means in delineating genera as well as in establishing phylogenetic relationships among microorganisms. The isolated strain ADE-0-1 was further confirmed by 16S rDNA analysis in order to determine its taxonomic position.

A comparison of 1484 base pairs from the 16s rDNA sequence of isolate ADE-0-1 with the 16s rDNA sequences type strains extracted from the GenBank database was carried out. Based on nucleotide homology and phylogenetic analysis by 16S rDNA analysis, strain was identified as *Bacillus megaterium* (Gene Bank Accession number-KF280264). *Bacillus megaterium* isolate TS IW 36 having Gene Bank accession number FM164631 showed 98 % homology with *Bacillus megaterium ADE-0-1* (Figure 3.8 and table 3.5).
Figure 3.8 Phylogenetic tree of isolate ADE-0-1

Phylogenetic Tree made in MEGA 3.1 software using Neighbor Joining method:

The phylogenetic relationship of *Dactilus megaterium* species and the isolate ADE-0-1 based on 16S rDNA sequences. The phylogenetic tree was created using maximum-likelihood distanced clustered by the neighbour-joining method. Numbers in parentheses are NCBI GenBank and RDP database nucleotide accession numbers.
Table 3.5 Alignment view using combination of NCBI GenBank and RDP database

<table>
<thead>
<tr>
<th>Alignment View</th>
<th>ID</th>
<th>Alignment results</th>
<th>Sequence description</th>
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<td>Studied sample</td>
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Distance Matrix based on Nucleotide Sequence Homology (Using Kimura-2 Parameter):

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</tbody>
</table>

The nucleotide similarity (above diagonal) and distance (below diagonal) identities between the studied strain ‘ADE-0-1’ and ten other closest homologues of bacterial strains are shown.
3.1.6 References: Part A


Chapter 3


Preamble: Attachment (Biofilm formation) and detachment (dispersal of biofilm) in *Bacillus megaterium* ADE-0-1

Microbial cells are able to adhere to surfaces and through an exopolymeric matrix they establish microbial community known as biofilm. This form of immobilized biomass can be responsible for heat and mass transfer limitation in industrial processes and be a source of contamination and proliferation of infections in water supply systems and medical devices. Of course, biofilms are advantageous and required in biological waste water treatment and bioremediation systems.

The increasing understanding of how a biofilm is formed and the role of each mechanism involved in cell adhesion is providing precious information to the development of sound strategies either to combat or encourage cell colonization as the case may be.

Interferences i) in the initially cell-to-surface and cell-to-cell contact, responsible for the formation of the first microcolonies at the surfaces, ii) with the molecules responsible for cell-to-cell communication or quorum sensing and iii) with the formation of EPS, responsible for the structure of the biofilm, can disrupt the process of biofilm formation and proliferation.

Although application of chemical biocides and disinfectants is practised widely for the control of biofilms, often they are not eco-friendly and also lead to the development and transfer of resistance in microbial population. Against this, use of enzyme capable of destroying the physical integrity of the matrix, interfering with bacterial adhesion or initiating cell detachment from surfaces are good alternatives.

Among the groups of bacteria, reported to cause metal corrosion during biofilm formation, one of the group of bacteria is able to excrete organic acids (Beech and Coutinho, 2003). As *Bacillus megaterium* ADE-0-1 studied here has both these characteristics i.e. biofilm forming ability and organic acid secretion ability, study on its biofilm is of significance.
Previously one/two factors affecting cell attachment have been studied in different organisms (van Loosdrecht et al., 1987a and b; Rijnaarts et al., 1993; Moller et al., 1997; Del et al., 2000; Parkar et al., 2001; Kos et al., 2003; Walker et al., 2005; Wijman et al., 2007; Srinandan et al., 2010).

Unlike previous studies, here time course experiments were conducted on variety of physicochemical/physiological/biochemical parameters in relation with growth and biofilm formation. This approach has enabled us to correlate them and to get a comprehensive picture about sequential events involved in cell attachment and detachment in *Bacillus megaterium* ADE-0-1.
3.0 RESULTS AND DISCUSSION

3.2 (B) Study on biofilm

3.2.1 Cell attachment study

3.2.1.1 Kinetics of growth in relation with biofilm forming ability

Growth kinetics as shown in figure (3.9 a) revealed active growth upto 15-16 h and after 24 h it entered into stationary phase. Biofilm forming ability estimated by “crystal violet staining procedure” was also increased along with active growth up to 24 h and then declined gradually during stationary phase (Figure 3.9 b). Light microscopic (Figure 3.10), Scanning Electron Microscopic (SEM) (Figure 3.11) and Confocal Laser Scanning Microscopic (CLSM) (Figure 3.12 A, B and C) observations were also found in accordance with the above mentioned observations. SEM enabled the study of surface topology of the biofilms and particularly at 24 h stage, dense population of cells embedded in the thick mucilaginous matrix was observed. While at 48 h stage, biofilm cell’s detachment scars were seen clearly. In CLSM observation also maximum biofilm intensity was observed at 24 h and it decreased gradually during 48 and 72 h. The intensity profile was generated to create a plot of pixels along rectangular selection in the image on Z-stack mode. Here the X-axis represents the horizontal distance through the selection and the Y-axis the gray value i.e. vertically averaged pixel intensity. This is the sum of the gray values of all the pixels in the selection divided by the number of pixels.

Variation in literature with respect to biofilm forming ability/activity in relation with growth phase has been observed. Some studies have described the adhesive nature of cells at mid-exponential phase as in the case of Pseudomonas (Bruinsma et al., 2001) and E. coli (Ong et al., 1999; Jones et al., 2003; Walker et al., 2004), while stationary growth phase in the case of Bacillus subtilis (Daughney et al., 2001; Bruinsma et al., 2001). In contrast to reports on E. coli just described above, Walker et al. (2005) observed that cells in stationary phase were notably more adhesive than those in mid-exponential phase in the case of E. coli D21g.
Similar to our observations, in the case of *Bacillus cereus* (Wijman *et al.*, 2007) and *Vibrio vulnificus* (Joseph and Wright, 2004), significant biofilm formation within 24 h, followed by dispersion resulting in the absence of biofilm after 48 h was observed suggesting that nutrient limiting cells were detaching. However, mechanisms involved in dispersion of *Bacillus cereus* and spores from biofilm were not elucidated.

### 3.2.1.2 Cell surface hydrophobicity and electrochemical potential on cell surface in relation with growth and biofilm forming ability

Reciprocal kind of kinetics i.e. **increase of cell surface hydrophobicity and decrease in electrochemical potential on cell surface** were observed during exponential growth phase (i.e. upto 24 h) and parallel to this, an increase in biofilm forming activity reaching to peak level by 24 h was observed (**Figure 3.9 c and d**). After 24 h, decrease in hydrophobicity paralleled with decrease in biofilm forming ability (**Figure 3.9 b and c**).

Similar kind of observations have been reported by Walker *et al.* (2005) in the case of *E. coli* D21g and van Loosdrecht *et al.* (1987a and b) in the case of several bacteria, where decrease in acidity/surface titrated charges (with reference to acidic hydrophilic outer membrane proteins) and corresponding increase in hydrophobicity resulted in more adhesive ability of stationary phase cells. However, higher sensitivity of adhesion ability of log phase *E. coli* cells to ionic strength suggested that electrostatic forces dominated and resulted into repulsion between cells and quartz surface.

Using charge defined fluor-conjugated dextran, Moller *et al.* (1997) have demonstrated presence of both positive and negative charges in the basal layer of cells and polymers and predominantly negative charge on the cells and EPS of the mound structure of the biofilm. Role of charged groups in electrostatic repulsion at secondary minimum (~15 nm) during process of adhesion of cells has been discussed (Walker *et al.*, 2005; Weiner *et al.*, 1995). Involvement of hydrophobicity of cells in the process of adhesion at primary minimum (~1 nm) is well documented (van Loosdrecht 1987a, and b; Rijnaarts *et al.*, 1993).
Figure 3.9 Kinetics of change in various parameters in relation with biofilm forming ability and growth of the organism

The culture was cultivated into 250 ml Erlenmeyer flask containing 50 ml growth medium with glass slides (two per flask), on a rotary shaker (180 rpm) at 30 ± 1°C for 72 h. a) Biomass was estimated gravimetrically. b) Biofilm density on glass slides was estimated by “crystal violet staining procedure”. Glass slides were removed, washed, stained, washed and absorbance of eluted dye was measured at 595nm. c) Cell surface hydrophobicity was determined using Microorganism Adhesion to Hydrocarbon (MATH) Test. d) Cell surface charge of washed cell pellet suspended in buffer was measured with an electrometer. e) Cell surface polysaccharide was released by treatment with mixture of KCl and EDTA and total carbohydrate content present in the supernatant was estimated by phenol sulphuric acid method. ▲ EPS production from culture filtrate was estimated gravimetrically. f) pH was measured through pH meter.
Chapter 3

a) Growth

b) Biofilm density

c) Cell surface Hydrophobicity

d) Cell surface charge

e) Cell surface polysaccharide and EPS production

f) pH change

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CPS (g/g)
EPS (g/l)
Figure 3.10 Light microscopic observations of biofilm developed on glass slides during growth of *Bacillus megaterium* ADE-0-1

The culture was cultivated into 250 ml Erlenmeyer flask containing 50 ml growth medium with glass slides (two per flask) on a rotary shaker (180 rpm) at 30 ±1 °C for 72 h. Glass slides were removed at regular interval of time, washed with PBS (pH 7), biofilms were stained by crystal violet and observed under light microscope at 1000 X magnification.
Figure 3.11 Scanning electron microscopic observations of biofilm at different stage of growth of *Bacillus megaterium* ADE-0-1

The culture was cultivated with glass slides as described previously in the case of light microscopic observations. Glass slides were removed, biofilms were fixed using glutaraldehyde and osmium tetroxide, dehydrated, gold plated and viewed at 2000X magnification under scanning electron microscope.
Figure 3.12 (A) Confocal Laser Scanning microscopic (CLSM) observations of biofilm at 24 h

a) 24 h biofilm

b) 24h Intensity Profile

Culture was cultivated with glass slides as described previously in the case of light microscopic observations. Glass slides were removed at regular interval of time, stained with nucleic acid stain (syto 9) and observed at 400 X magnification under Confocal Laser Scanning Microscope (CLSM). The intensity profile was generated along rectangular selection in the image on Z-stack mode. Biofilms observed after (A) 24 h, (B) 48 h, and (C) 72 h. In all the CLSM micrographs (Figure 3.12 A, B and C), a= Biofilm image at a particular stage and b= Intensity profile of that stage.
Figure 3.12 (B) Confocal Laser Scanning microscopic (CLSM) observations of biofilm at 48 h

a) 48 h biofilm

b) 48 h Intensity Profile
Figure 3.12 (C) Confocal Laser Scanning microscopic (CLSM) observations of biofilm at 72 h

a) 72 h biofilm

b) 72 h Intensity Profile
A unique behaviour of the culture Bacillus megaterium ADE-0-1 in terms of ‘auto-aggregation’ of cells was also observed during early growth period (6-10 h) for a transient duration (Figure 3.13). Cell-cell aggregates size was in the range of 100-150 μM and consisted of more than 100 cell numbers. Direct relationship of auto-aggregation and hydrophobicity of cells with adhesive ability has been also reported in the case of Lactobacillus (Kos et al., 2003) and bifidobacteria (Del Re et al., 2000).

Figure 3.13 Auto-aggregation behaviour of Bacillus megaterium ADE-0-1 cells

The culture was cultivated into 250 ml Erlenmeyer flask containing 50 ml growth medium, on a rotary shaker (180 rpm) at 30 ±1 °C. At 6-10 h growth stage, auto-aggregation of cells was observed.

3.2.1.3 Surface polysaccharide/capsular polysaccharide (CPS) content of cells in relation with biofilm forming ability

There is evidence that EPS is involved in the development of surface film (Allison and Sutherland, 1987), adhesion of cells (Fletcher and Floodgate, 1973) and the formation of complex three dimensional biofilm structure (Danese et al., 2000).

By immunoelectron microscopy of a marine pseudomonad, using antiserum as probe, Fletcher and Floodgate (1973), suggested that shorter EPS molecule are integrally bound to the outer membrane (integral capsule) while the longer polymers are loosely (peripherally) associated. Further it was shown that
integral EPS was constitutively produced, while the peripheral EPS was synthesized as response of starvation. It was speculated that integral EPS was involved in adhesion, while the peripheral EPS aided in detachment from the surface.

Cell surface bound polymeric carbohydrate (CPS) was found around 25 % (w/w) on dry weight basis during active growth phase and during this period biofilm forming ability increased and reached to its peak level. However sharp decline (reduction by 40 % of original) in cell surface polymeric carbohydrate was observed along with decline in biofilm forming ability after 24 h growth period (Figure 3.9 e). Along with the decrease in biofilm forming ability of cells, considerable decrease (6.8 to 4.5) in pH of the broth was observed after 24 h (Figure 3.9 f).

Reciprocal to decline of cell surface bound polymeric carbohydrate, EPS found to be accumulated extracellularly in culture broth which noticeably increased after 24 h growth period and which showed adhesive ability (Figure 3.9 e). This suggested that surface polysaccharide was peeled off/shredded away leading to gradual decrease in biofilm activity. In the case of Sphingomonas, planktonic Vs sessile dimorphism has been reported. The sessile state (biofilm forming ability) is marked by the presence of an exopolysaccharide capsule and non-motile cells and has been proposed that Sphingomonas must shed a large fraction of its capsule in order to move (detachment of cells, poor biofilm forming ability) (Pollock and Armentrout, 1999).

It has been reported (Sutherland, 2001) that EPS present in biofilm almost certainly resemble closely the corresponding polymers synthesized by planktonic cells. This has been demonstrated by the use of antibodies prepared against EPS from planktonic cells and also by comparison of the enzymatic products following digestion of planktonic and biofilm EPS using highly specific polysaccharases.
Alginate has been shown to play a role in colonization of *Pseudomonas aeruginosa* by increasing adherence of the bacteria to solid surfaces (Chitnis and Ohman, 1990; Martin et al., 1993; Mathee et al., 1997; Nivens et al., 2001). The rugose colony variant of *Vibrio cholera* 01, biotype El Tor has been shown to produce an EPS (cell bound/capsular) that confers biofilm forming capacity to the cells (Yildiz and Schoolnike, 1999). Mutations to smooth colony variants were found unable to produce EPS and biofilm deficient.

Genetic studies and polysaccharide analysis has indicated that cell bound polysaccharide (capsular polysaccharide), chemically polymeric β-1,6-N-acetyl-D-glucosamine, of *E. coli* and *Staphylococcus epidermidis*, is required for biofilm formation (Gotz 2002; Wang et al., 2004).
3.2.2 Cell detachment study

3.2.2.1 pH change in relation with biofilm forming ability

As described previously in “cell attachment study” considerable decrease in pH of the broth was observed after 24 h (Figure 3.9 f) and this change in pH accompanied a tendency of decrease in biofilm forming ability.

A decrease in pH could be responsible for the loss of adherence capacity of cells after 24 h. Uronic acid has been detected as one of the major chemical component of “adhesive polymer”. By virtue of ionised carboxyl groups of uronic acid of cell bound adhesive polymer, cells will be able to interact with the surfaces bearing Ca$^{2+}$, Mg$^{2+}$, Si$^{4+}$ etc and can adhere to such surfaces if ionic interaction is involved in adhesion. However, such ionic interaction will be reduced to a considerable extent when the pH decreases as ionization of COO$^-$ group of uronic acid residues decreases under such condition.

Generally, ionic interactions in adhesion process are implicated in repulsion rather than in attraction between cells and substratum. However in the case E. coli D21g (Walker et al., 2005), sensitivity of adhesion to ionic interaction has been reported.

Unlike non-buffered media, when pH was maintained (using buffered media), “biofilm forming ability” was considerably maintained (Figure 3.14a and b). This observation indicated that possibly ionic interaction was involved in adhesion process and pH change after 24 h could be a reason for the detachment of biofilm cells.
Figure 3.14 Kinetics of biofilm forming ability in buffered and non-buffered medium

(a) Biofilm density

![Graph showing biofilm density over time with buffered and non-buffered medium]

Figure 3.14 b) Kinetics of pH change

![Graph showing pH change over time with buffered and non-buffered medium]

The culture was cultivated in the phosphate buffered (0.02 M, pH 7) and non-buffered (Control- only K₂HPO₄ at 0.003 M concentration) medium. Glass slides were removed and A) biofilm density on glass slides was estimated by “crystal violet staining procedure”. B) The pH was measured through pH meter.
3.2.2.2 Extracellular EPS-depolymerase activity in relation with detachment of cells from the biofilm

Extrinsic factors such as shear, sloughing, erosion (Rupp et al., 2005), change in nutrient (Sauer et al., 2004) and oxygen availability (Thormann et al., 2005) have been shown to influence the cellular detachment from biofilm. In *Pseudomonas aeruginosa*, production of rhamnolipids has been shown to influence the cellular detachment of cells (Boles et al., 2005). For *Shewanella oneidensis* and other bacteria, the level of cyclic di-GMP has been reported to regulate the attachment and detachment of cells from a biofilm (Romling et al., 2005; Thormann et al., 2006). There are some precedents on the use of commercial enzyme preparations for pre-formed biofilm removal (Hahn et al., 2001; Johansen et al., 1997). Treatment of preformed biofilms of *E. coli* K-12 and *Staphylococcus epidermidis*, with the enzyme β-hexosaminidase/DSP B/dispersin, for its removal was also reported during in-vitro studies (Itoh et al., 2005).

However largely there is no clarity with respect to mechanisms involved in detachment of cells from biofilm particularly at cell surface level from biochemical point of view in relation with the physiology during growth of the organism.

In non-polysaccharide producing bacterial species, polysaccharases/polysaccharide lyases have been detected which enable the bacteria synthesizing them to utilize varicus polysaccharides as a nutrient (Preiss and Ashwell, 1963). Hence we looked for presence of EPS depolymerase kind of activity in *Bacillus megaterium* ADE-0-1 by using EPS (extracellular, recovered from culture filtrate and showing adhesive ability) and EPS(p) (with bound phosphate) as a sole source of carbon and carbon and phosphate respectively in the synthetic solid medium. As shown in the figure (3.15), the dense growth of the organism and zone of clearance due to the hydrolysis of EPS/EPS(p) indicated that the organism is being able to utilize its own EPS as nutrient. Such EPS-depolymerase have also been characterized from several other genera such as *Pseudomonas*, *Brevibacterium*, *Streptococcus*, and *Bacteroides*. Depolymerase produced by these organisms release glucose
as final product of hydrolysis that supports growth of the organism (Khalikova et al., 2005). Depolymerase known as polysaccharide lyase have also been characterized in *Sphingomonas Paucimobilis* (Sutherland and Kennedy, 1996).

**Figure 3.15 Growth of Bacillus megaterium ADE-0-1 on EPS containing medium**

![Zone of clearance](image)

The organism was spot inoculated on a solid synthetic medium containing EPS (1 % w/v) as a sole source of carbon and energy. Plates were incubated at 30 ±1 °C for 72 h. Medium without EPS served as a control.

### 3.2.2.3 Kinetics of production of extracellular EPS-depolymerase and its activity at different pH

Subsequently a time-course experiment, using buffers having different pH, revealed kinetics of production of extracellular EPS-depolymerase as shown in figure (3.16). Interestingly **EPS-depolymerase showed maximum activity at pH 5.6** (Acetate buffer). At this stage, it should be noted that a considerable drop in pH after 24 h growth period has been described previously (See figure 3.9 f) which is a favourable event for the action of EPS-depolymerase. After initial appearance of the enzyme activity (~ 25 %), a lag in production was observed between 18 to 24 h and then it increased considerably (~ rest 75 %) to its maximum level. This kind of kinetics of production of EPS-depolymerase suggested its possible **physiological role in the detachment of cells from biofilm** after 24 h onwards of growth period.
Figure 3.16 Kinetics of production of extracellular EPS-depolymerase

Culture flasks were harvested at regular interval of time and proteins present in culture filtrate were precipitated by 100% (w/v) ammonium sulphate saturation, dissolved in water and used as a source of enzyme. Enzyme was assayed using 1% (w/v) EPS as a substrate and acetate buffer (0.2 M pH 5.6), Phosphate buffer (0.2 M pH 7) and tris buffer (0.2 M pH 8.5). Released sugar was estimated by DNSA method.

In *in-vitro* experiment time-course treatment of preformed biofilm of 24 h growth stage with concentrated (ammonium sulphate precipitated) EPS-depolymerase preparation showed decrease in biofilm due to detachment of cells and at the end of 5 h only little quantity of attached cells (8%) left (Figure 3.17a and b).

Thus the observations reported here on ‘EPS-depolymerase’ in terms of (i) stage (time of production) i.e. after 24 h, (ii) association of decrease in pH to 5.5 with optimum activity of the enzyme at pH 5.5 and (iii) its ability to detach cells from preformed biofilms suggested clearly its physiological role/involvement in the detachment of cells from biofilm. This is one of the rarest report where an endogenous (from organism itself) enzyme has
been shown to be involved in the detachment of cells from biofilm. So far
 detachment of *Actinobacillus actinomycetemcomitans* cells from biofilm by β-
 hexosaminidase is an exceptional report available in literature where
 endogenously produced enzyme disrupts the biofilm (Kaplan *et al.*, 2003).

**Figure 3.17 Kinetics of removal of preformed biofilm cells on treatment
with EPS-depolymerase**

(a) Biofilm density

![Graph showing biofilm density over time](image)

Proteins present in the culture filtrate, obtained after 72 h growth period, were
recovered by 100 % (w/v) ammonium sulfate saturation, dissolved in acetate buffer
(0.2 M, pH 5.6) and used as a source of enzyme EPS-depolymerase. Biofilms
developed on glass slides, withdrawn at 24 h growth period, were treated with the
enzyme for different period of time at 30 ±1 °C. a) Biofilm density on enzyme
treated slides was estimated by “crystal violet staining procedure”. b) From separate
set of experiment, such slides were also observed by light microscopy at 1000 X
magnification.
(b) Microscopic observations

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Polysaccharide lyases have been found from same bacteria which produce polysaccharide substrate. *Pseudomonas aeruginosa*, other alginate synthesizing species, bacteria of genus *Sphingomonas* synthesizing gellan and structurally related polysaccharide, none can utilize EPS produced by them as sole carbon and energy source.

Polysaccharide lyases described above and enzyme active on alginate (alginate lyase) have been shown to be strongly inhibited by the presence of O-acetyl or other acyl group present on native polymer substrate (Davidson *et al*., 1977, Kennedy *et al*., 1992). O-acetyl group can greatly affect the ordered structure adopted by polysaccharide in solution.

Crescenzi *et al*. (1987) observed that in the conformation adopted in aqueous solution, the short side chains of L-mannose or L-rhamnose mask the uronic acid residues through H-bonding. Lee and Chandrasekaran (1991) used X-ray and computer modelling of gellan and three structurally related polysaccharides to conclude that the side chains shielded the carboxylate group to varying degree. Complete removal of the side chains would be required to cleave completely exposed carboxylate groups, as is found in gellan. This might allow the enzyme to cleave at its recognition sites.

This raises the question of a possible role for EPS-degrading enzymes in bacterial strains which excrete EPS. Are these lyases therefore, essentially connected with polysaccharide synthesis and excretion? (Sutherland and Kennedy, 1996). Occurrence of corresponding depolymerases also has been reported from *Rhizobium* species synthesizing EPS succinoglycan (Glucksman *et al*., 1993) and CM-cellulase from cellulose-synthesizing bacteria (Standal *et al*., 1994; Matthysse *et al*., 1995.)

These might suggest that the role of enzymes is in cleavage of polysaccharide chain at the surface of the cell. At such a location the enzyme might have released polysaccharide or cleaves material covalently link to other surface macromolecules. Some bacteria secretes esterase with wide specificities, this
can remove acyl-group from bacterial polymers (Cui et al., 1999). Time specific activity of such esterase can facilitate subsequently the action of polysaccharide lyase.

Polymeric β-1,6-N-acetyl-D- glucosamine (Poly-β-1,6-Glnc NAc) serves as a biofilm adhesin in phylogenetic diverse species and results of blast analysis has revealed presence of pgaABCD loci in *E.coli*, *S. epidrmidis*, *Yersinia pestis* and *Pseudomonas fluorescens* (Itoh et al., 2005). However surprisingly blast analysis of NCBI microbial-genome database revealed presence of Dsp B (Dispersin B/β hexoseaminidase-biofilm dispersing enzyme for Poly-β-1,6-Glnc NAc) homologues only in *Actinobacillus actomyocetemcomitans* and not in the above mentioned organisms. Hence detachment of biofilm cells by endogenously produced enzyme is only reported in *Actinobacillus actomyocetemcomitans*.

### 3.2.3 Conclusion and correlation of study on i) attachment (biofilm formation) and ii) detachment (dispersal of biofilm) of cells during growth of the organism

Bacteria are inherently dynamic organisms and their protein coverage (Huisman et al., 1996; Nikaido 1996) and Lipopolysaccharide (LPS) conformation evolve as a function of growth phase (Ivanov and Fomchenkov, 1989; Huisman et al., 1996). The extent to which these modifications alter the adhesive nature of cells has not been much addressed (Druinsma et al., 2001; Manas and Mackey, 2004). In context of this, all events taking place during growth of the *Bacillus megaterium* ADE-0-1 can be sequentially correlated as follows:

During active growth phase, as growth increases, cell surface charge decreases and reciprocal to this, auto-aggregation behaviour of cells followed by increase in hydrophobicity up to 24 h growth period facilitate biofilm formation up to 24 h. At this stage, culture enters in the stationary phase and the level of EPS depolymerase enzyme increases along with considerable decrease in pH which creates a favourable environment for activity of enzyme
depolymerase. Action of enzyme on cell bound surface polysaccharide (CPS) results into detachment of cells of biofilm accompanied with decrease in CPS and increase in appearance of adhesive EPS extracellularly.

The findings reported here has given greater insight into the mechanisms involved in bacterial adhesion and it can be hypothesized that the evolution of cell with the growth phase, as manifested by subtle alterations in cell surface characteristics dramatically alters the adhesive nature of the organism.
3.2.4 References: Part B


Chapter 3


