Characterization, production and application oriented studies on bioadhesin and biofilm of *Bacillus megaterium* ADE-0-1

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By

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DECLARATION

Statement under O.Ph.D 8 / iii of M. S. University of Baroda,

Baroda, India.

The work presented in this thesis has been carried out by me under the guidance of **Dr. A. K. Shah,** Department of Microbiology and Biotechnology Centre, M. S. University of Baroda, India. The data reported herein is original and has been derived from studies carried out by me.

Place: Baroda

Date: **Santosh Kumar**

This is to certify that the above declaration is true.

Place: Baroda

Date: **Dr. A. K. Shah**

(Research Guide)

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Santosh kumar

DEDICATED TO

My

Parents

CONTENTS

1.0 INTRODUCTION

1.1 Biofilms: Main mode of existence of microorganisms in nature

Microorganisms are traditionally thought to exist and therefore studied, characterized and identified as planktonic single cells. However, detailed studies of sessile communities in different environments have lead to the conclusion that planktonic microbial growth rarely exists in nature. A basic question which needs answer is why the different members of the microbial communities should come together and exist as biofilms? Although it has been envisaged that it could be for mutual benefit leading to better existence/survival of individual members of the community constituting the biofilm, however **scarce information is available** in this direction and aspects of physiological interaction between microbial cells and substratum and between the members of community are poorly understood (Liu *et al*., 2004).

Biofilms have been found to protect the microbial community from environmental stresses like resistance to bacteriophage, amoebae, chemically diverse biocides, host immune responses and antibiotics (Singh *et al*., 2006; Ahimou *et al*., 2007). Cells in biofilms have higher resistance to antibiotics and biocides than planktonic cells due to (i) horizontal gene transfer between resistant and non-resistant strains, and (ii) slow growth rate observed in biofilms and/or transport limitations of nutrients, metabolites and oxygen between the surface and the interior of the biofilm (Stewart and Costerton, 2001; Donlan and Costerton, 2002**)**. The above importance of biofilms has led to the development of a new branch of microbiology known as biofilmology or "biofilm science" and "biofilm engineering".

Biofilms of environmental bacteria can develop biofilms on a number of different surfaces and thus constitutes a serious problem in many industrial processes (e.g. paper, food, cosmetics and pharmaceutical industries). They can colonize living tissues, medical devices (contact lenses, catheters, endotracheal tubes, mechanical cardiac valves and surgical sutures) and lead to secondary infections. Biofilms of

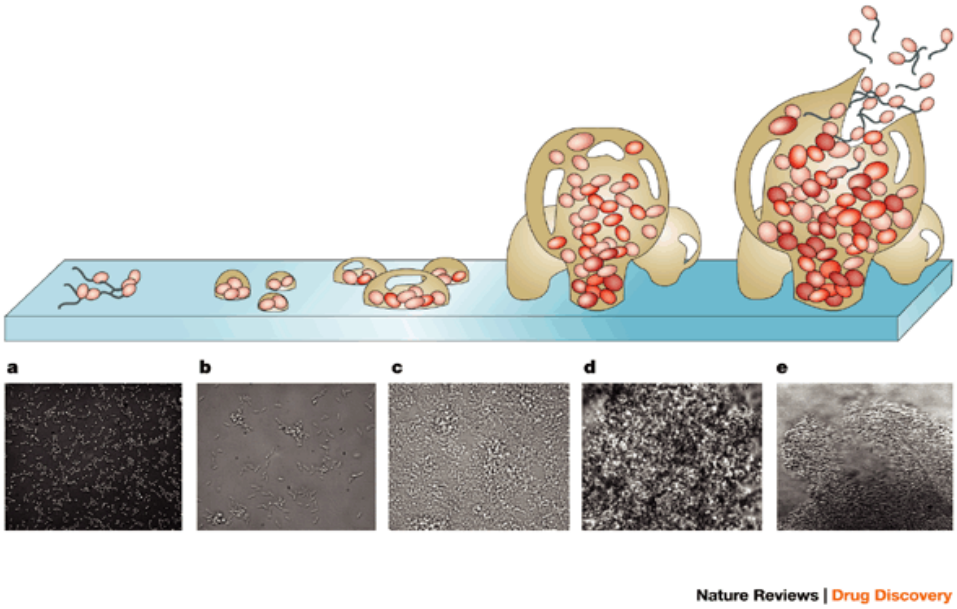
environmental bacteria are the major cause of biofouling leading to serious problems in ships and other marine establishments (Donlan, 2002).

1.1.1 Biofilm formation

Movement of microorganisms towards surfaces may result due to either (i) water flow (e.g. sessile form) or (ii) the organism's motility (planktonic) or (iii) combination of the above processes. When organisms are in the vicinity of a surface, they are subjected to various attractions governed by Van der Waals' forces, Brownian motions, gravitational forces and repulsion forces due to overlap of electrical double layers associated with charged groups. These yield two distance scales at which a particle could be attracted to the substratum. At a "primary minimum" (~1 nm), attractive forces are strong and at the "secondary minimum" (~15 nm) the existing forces are weaker. These two distances are separated by an intermediate repulsion barrier. Microorganisms usually gather at secondary minimum and much of the strategy in surface colonization is concerned with remaining at the secondary minimum along with overcoming the repulsive barrier to reach the primary minimum (Oliveira, 1992). For this purpose microorganisms produce a range of tethers. All the tethers have narrow diameter and sufficient length to minimize and 'breakthrough' the repulsive layer. Such structures include cell surface polysaccharides/capsular polysaccharides (CPS), pili, and flagella. Once bacteria are attached to the surface, several events occurred to carry it to the primary minimum at which various bonds of a more permanent nature (hydrophobic bonds, polymer bridging by polymers i.e. cementing) are formed between the organism and substratum. When the cells remain thus attached for a little time, they start to secrete extracellular materials forming a matrix gel that embeds several layers of cells, as the biofilm matures.

Thus biofilm development phenomena essentially involves five stages viz (a) reversible attachment of the bacterial cells to the surface (Secondary minimum), (b) irreversible attachment of cells (Primary minimum), (c) first maturation phase (d) fully mature biofilms, and (e) dispersion of motile cells from mature colony to new locations as shown in **figure 1.1** .

Figure 1.1 The development of a biofilm



The development of a biofilm depicted as a five-stage process. (a) bacterial cells attach reversibly to the surface (Secondary minimum), (b) cells attach irreversibly (Primary minimum), (c) first maturation phase, (d) fully mature biofilms, and (e) dispersion of motile cells from mature colony to new locations.

Adapted from: Davies, 2003.

1.1.2 Biofilm dispersal

The dispersal of biofilm cells occurs by processes including shedding of daughter cells from actively growing cell in response to either nutrient starvation, quorum sensing, or shearing of biofilm aggregates due to flow effects. The mechanisms involved in the **process of shedding by actively growing cells in a biofilm are not yet well understood**. According to Brading *et al*. (1995), three main physical forces found to favour detachment are erosion or shearing through continuous removal of small portions of the biofilm**,** sloughing, and abrasion resulting from detachment due to collision of particles from the bulk fluid with the biofilm. Among the above physical forces sloughing is more random than erosion which is implicated to

involve nutrient or oxygen depletion within the biofilm structure. Usually, biofilms in fluidized beds, filters, and particle-loaded environments (surface waters) undergo abrasion mediated biofilm dispersal. In *Escherichia coli* and *Pseudomonas aeruginosa* biofilms surface hydrophobicity of newly divided daughter cells differ substantially from those of matured biofilms and these differences cause detachment of newly divided daughter cells from biofilm (Gilbert *et al*., 1993).

Pseudomonas aeruginosa produces alginate and also alginate lyase (encoded by *alg L*). Higher expression of *alg L* resulted into low molecular size alginate having decreased adherence capacity and facilitated biofilm dispersal (Boyd and Chakrabarty, 1994).

Interestingly, detachment of biofilms seems to be species specific. For example, *Pseudomonas fluorescens* disperses and recolonizes a surface of a flow cell after approximately 5 h. Similarly, *Vibrio harveyi and Vibrio parahaemolyticus* colonizes the surface after 2 and 4 h, respectively. This process probably provides a mechanism for cells to migrate from heavily colonized areas which have been depleted of surface associated nutrients to areas more supportive of growth (Korber *et al*., 1995).

1.2 Important factors affecting biofilm establishment and stability

The attachment of microorganisms to surfaces is a very complex process with several variables affecting the outcome. In general, attachment occurs most readily on surfaces that are rougher, more hydrophobic, and coated by surface "conditioning" films. In addition to that an increase in flow velocity, water temperature, or nutrient concentration may also facilitate increased attachment provided all are within critical levels. However, when a mixed community is involved, features such as properties of the cell surface, specifically the presence of fimbriae, flagella, and surface-associated polysaccharides or proteins and could provide a competitive advantage for one organism.

1.2.1 Substratum

Microorganisms attach more rapidly to hydrophobic, non-polar and rough surfaces such as teflon and other plastics than to hydrophilic materials such as glass or metals (Barbara *et al*., 2009). Due to lack of standardized methods for determining surface hydrophobicity of substrates, the above studies have been sometimes contradictory. Essentially, some kind of hydrophobic interaction occurs between the cell surface and the substratum that enables the cell to overcome the repulsive forces active within a certain distance from the substratum surface and ultimately facilitate irreversible attachment.

1.2.2 Conditioning of films

A material surface exposed in an aqueous medium instantly becomes conditioned or coated by polymers from the medium and the resulting chemical modification on material surface then influences the rate and extent of microbial attachment. In an earlier study, Loeb and Neihof (1975) observed that films that were formed within minutes of exposure were organic in nature and continued to grow for several hours. The nature of conditioning films may be quite different for surfaces exposed in different environments. A prime example could be the proteinaceous conditioning film called "acquired pellicle," which develops on tooth enamel surfaces in the oral cavity. Bacteria from the oral cavity colonize pellicle-conditioned surfaces within hours of exposure to these surfaces. A number of host-produced conditioning films have been reported such as blood, tears, urine, saliva, intravascular fluid, and respiratory secretions which influence the attachment of bacteria to biomaterials (Donlan, 2002).

1.2.3 Characteristics of the aqueous medium

Characteristics of the aqueous medium, such as pH, nutrient levels, ionic strength and temperature may influence the rate of microbial attachment to a substratum. Several studies have revealed a seasonal effect on bacterial attachment and biofilm formation in different aqueous systems. The above effect could be attributed to the temperature of aqueous system (water) or to other unmeasured seasonally affected parameters (Donlan *et al*., 1994). The attachment of *Pseudomonas fluorescens* to

glass surfaces was found to increase with increase in the concentration of several cations such as sodium, calcium and ferric iron, most likely by way of reduction of the repulsive forces between the negatively charged bacterial cells and the glass surfaces (Fletcher, 1988a and b). In a laboratory study, Cowan *et al*. (1991) showed the correlation between the nutrient concentration and number of attached bacterial cells and found that with an increase in concentration of nutrients, the number of attached bacterial cells also increased.

1.2.4 Properties of the cell

Cell surface polymers with non-polar sites such as fimbriae, other proteins and components of certain gram-positive bacteria (mycolic acids) are more important in attachment of cells to hydrophobic substrata; while exopolysaccharides (EPS) and lipopolysaccharides appear to contribute in attachment of cells to hydrophilic materials. Flagella are important in attachment also, although their role might be to overcome repulsive forces rather than to act as adsorbents or adhesives.

The hydrophobicity of the cell surface is important in adhesion because hydrophobic interactions tend to increase with an increasing non-polar nature of the microbial cell surface and the substratum surface or both. The majority of bacteria are negatively charged but still contain hydrophobic surface components like non-flagellar appendages i.e., fimbriae (called pili). Most of the fimbriae contained a high proportion of hydrophobic amino acid residues. Fimbriae possessed by several bacteria have been found to be involved in bacterial attachment (Hancock *et al*., 2011). Bendinger *et al*. (1993) showed that mycolic acid-containing organisms (*Corynebacterium, Nocardia,* and *Mycobacterium*) were more hydrophobic than nonmycolic acid-containing bacteria. Increase in mycolic acid chain length resulted in increase in hydrophobicity. Thus bacterial cell surface hydrophobicity helps in attachment to the substratum possibly by overcoming the initial electrostatic repulsion barrier that exists between the cell and substratum.

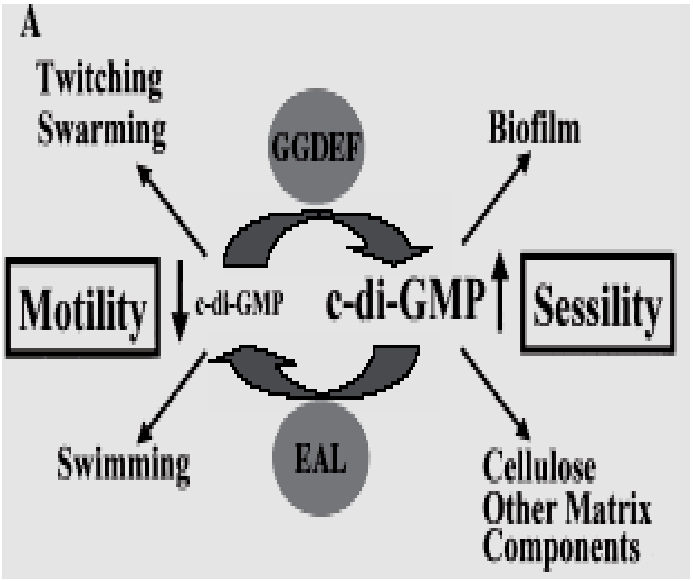
The treatment of adsorbed cells with proteolytic enzymes caused a marked release of attached bacteria from a biofilm suggesting the role of cell surface protein in attachment (Bashan and Levanony, 1988). Marshall *et al*. (1971) based on

scanning electron micrograph (SEM) data, confirmed that attached bacteria were associated with the surface via fine extracellular polymeric fibrils. When attached freshwater bacteria were treated with cations, contraction of the initial adhesives through decrease in the cell distance from the substratum occurred, supporting the idea that the above material was an anionic polymer. In fact, cations have been shown to cross-link the anionic groups of polymers (such as polysaccharides) resulting in their contraction (Fletcher *et al*., 1991).

1.3 Role of quorum sensing in biofilm formation and dispersal

Recently, quorum sensing (QS) has been proposed to play a major role in production of EPS, biofilm formation and differentiation. The QS process in gram negative bacteria involves the production, release and detection of chemical signalling molecules such as N-acyl homoserine lactones (AHLs) which helps cells to regulate gene expression in a cell-density-dependent manner. In Gram-positive bacteria, communication is carried out with modified oligopeptides generating the signals and membrane-bound sensor histidine kinases acting as receptors (Waters and Bassler, 2005; Ruiz *et al*., 2008). Xie *et al*. (2000) revealed that certain dental plaque bacteria can modulate the expression of genes encoding fimbrial expression (*fimA*) in *Porphyromonas gingivalis* by quorum sensing. *Porphyromonas gingivalis* could not attach to *Streptococcus cristatis* biofilms grown on glass slides as *Streptococcus cristatus* was able to modulate *Porphyromonas gingivalis fimA* expression and prevent its attachment to the biofilm.

Figure 1.2 Model of c-di-GMP pathways



(Adapted from: Simm *et al.*, 2004)

In several bacteria GGDEF-EAL domains (gly-gly-asp-glu-phe----glu-ala-leu motif) are reported on bacterial surface. They inversely regulate cyclic–di-GMP (c-di-GMP) levels and in turn regulate the transition process from sessility to motility as shown in **figure 1.2** (Simm *et al*., 2004). In *Yersinia pestis* the expression of the haemin storage system (*hms*) and biofilm formation is dependent on the GGDEF containing HmsT protein (Hare and McDonough 1999; Jones *et al*., 1999). In *Vibrio parahaemolyticus* biofilm formation and polysaccharide production are regulated by a signalling pathway involving the GGDEF-EAL motif-containing ScrC sensor (Guvener and McCarter, 2003) and in *Pseudomonas aeruginosa* the GGDEF proteins WspR and FimX are respectively involved in autoaggregation of cell, and in regulation of twitching motility in response to environmental cues (D'Argenio *et al*., 2002; Huang *et al*., 2003).

1.4 Bacterial surface polysaccharides and extracellular polysaccharide (EPS)

Exopolysaccharides are important component of biofilms acting as cementing substance between cells and substratum. They help biofilm forming bacteria in initial attachment of bacterial cells to surface, formation and maintenance of micro-colony, stabilization of biofilm structure, cell-cell communication and providing resistance towards heavy metal toxicity. The proportion of extracellular polysaccharide (EPS) in biofilm was suggested to be approximately 50 to 90 % of the total organic matter (Hall-Stoodley *et al*., 2004; Branda *et al*., 2005). EPS may be secreted into the medium (slime) or may be attached to the cell (capsular). The composition and quantity of the EPS generally vary depending upon the type of the microorganism, age of the biofilms and environmental conditions such as levels of oxygen and nitrogen, extent of desiccation, temperature, pH, and availability of nutrients. EPS exhibit considerable heterogeneity; from the simple α, 1-4 linked, un-branched glucose polymers called dextrans, to the highly complex, branched, and substituted heteropolysaccharides made up of oligosaccharide repeating subunits such as xanthan and colanic acid.

Most EPS produced by marine bacteria are heteropolysaccharides containing three or four different monosaccharides arranged in groups of 10 or less to form repeating units (Decho, 1990). Marine EPS contain monosaccharide such as pentoses (as D-arabinose, D-ribose and D-xylose), hexoses (D-glucose, D-galactose, D-mannose, D-allose, L-rhamnose and L-fucose), amino sugars (D-glucosamine and D-galactosamine) or uronic acids (D-glucuronic acids and D-galacturonic acids). Organic or inorganic substituents such as sulphate, phosphate, acetic acid, succinic acid and pyruvic acid may also be present. Their existence in such a range of environments suggests that the microorganisms are able to respond to their environments and change their EPS and adhesion abilities, depending on the properties of the surface onto which they attach (Mayer *et al*., 1999). The tremendous diversity existing in structure and composition of the EPS, together with their anionic nature and presence of side chain substituent(s) leads to formation of strong bond with divalent cations such as Ca^{+2}, Mg^{+2} and Fe^{+2} and helps ultimately in stabilization of the developed biofilm.

1.5 Adhesives based on bacterial exopolysaccharides

Currently large quantity of petro-chemical based adhesives is used globally. In 2007, the total world demand for adhesives and sealants was 12 billion Kg (Petrie, 2010). However, usage of toxic chemicals during production, use of volatile organic compounds (toluene, methyl-ethyl ketone and tri-chloro ethane) in their formulation and poor biodegradability are serious problems associated with the synthetic adhesives. In contrast, features such as high performance in nature, unique chemical compositions, economics of production from renewable resources and high biodegradability make bacterial exopolysaccharides attractive candidates for development as adhesive materials. Hence in recent past there is a motivation for exploring biopolymers for use in adhesive applications (Weimer *et al*., 2003; Haag *et al*., 2004; Combie *et al*., 2004a; Combie *et al*., 2004b; Haag 2006; Cha *et al*., 2008).

In biofilms, since EPS are involved in the adhesion of cells to surfaces as cementing substance, they also exhibit better adhesion ability when used as adhesives. The polar and hydrogen 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. The hydroxyl and carboxylate groups of polysaccharides also offer potential sites for synthetic derivatization and crosslinking which can be utilized to modify the adhesive properties. Tertiary structures, such as helices, are formed by some bacterial polysaccharides and account for their notable mechanical properties. Pullulan, an EPS produced by the fungus *Aureobasidium pullulans*, as well as derivatives of natural pullulan, have been described as a paper adhesive and as wood adhesive respectively (Haag *et al*., 2004). The marine bacterium *Alteromonas colwelliana* LST produces an exopolysaccharide which it uses to adhere strongly to surfaces under severe conditions in its natural environment. It also synthesizes tyrosinase, dihydroxyphenylalanine (DOPA), and related quinones which participate in water-resistant adhesive production in higher organisms (Yamada *et al*., 2000**)**. The fermentation residue of ruminal cellulolytic bacteria

contained EPS adhesive, incompletely fermented biomass, adherent bacterial cells has been used in preparation of plywoods (Weimer *et al*., 2003).

Bacterial isolates from the culture collection of Montana Biotech SE, Inc. (Rock Hill, SC, USA) were screened for production of water based adhesive. Testing of adhesive strength was performed on aluminum and coated aluminum substrates. Molecular weight of **Montana Biotech (MB) adhesive** was 10^6 Da and it was composed of >95 % carbohydrate (dry weight basis). Other structural information was not disclosed. Partially methylated or acetylated derivatives of dried MB adhesive were also prepared by reaction with methyl iodide or acetic anhydride, respectively to decrease water solubility. Water solubility of the adhesive decreased as degree of substitution increased (Haag, 2006).

Another bacterial exoplymer-based adhesive was studied by Speciality Biopolymers Corporation (Bozeman, MT, USA) and has shown improved properties over the previously described MB adhesive for bonding wood substrates. The molecular weight of **Speciality Biopolymers (SB) adhe**sive was 500 KDa and was mainly composed of a polysaccharide and formulated as 33 % solids in water (Haag, 2006**)**.

1.6 Yield improvement of a fermentation product

Always improvement of product yield is an essential approach for commercialization of product as it reduces the production cost and thereby product becomes more competitive in the market. Improvement of product yield is achieved by (i) optimizing fermentation conditions (chemical and physical environment), (ii) developing high yielding strains, and (iii) optimizing downstream processing. In above mentioned approaches, optimization of chemical environment i.e. “medium optimization” for growth and product formation gives considerable success in yield improvement as genetic potential for biosynthesis of products is fully expressed phenotypically under particular standardized/optimized medium compositions.

1.6.1 Medium optimization strategies

An optimization strategy that has been conventionally used is the testing of one variable at a time method in which concentration of one independent variable is optimized by changing its concentration while the concentration of the other variables is held constant. The optima for the remaining variables are determined using the same approach, provided the variables do not interact with one another. 'One factor at a time' method for optimization has limitations such as (i) the variables may interact, and (ii) is time consuming and laborious when a large number of variables are being examined. An alternative and statistically more valid optimization strategy, which has been now accepted by scientific community largely, is the use of statistically designed experiments that allows the investigator to evaluate more than one independent variable at a time. Because relatively large numbers of experiments are required to optimize a medium, these experiments are usually performed at shake flask level.

The use of statistically designed experiments involves the following steps:

(i) Identifying the factors affecting the desired response i.e. screening of variables

(ii) Optimization through response surface methodology

In an earlier report, the nutritional requirements for production of xanthan by *Xanthomonas campestris* was investigated by the use of factorial design experiments. The influence of nitrogen, phosphorous, sulphur on xanthan production and nitrogen, phosphorus and magnesium on biomass were found to be significant (Garcia-Ochoa *et al*., 1992).

1.6.2 Response surface methodology

Response surface methodology (RSM) is a collection of mathematical and statistical techniques that are useful for the modelling and analysis of problems in which a response of interest is influenced by several variables and the objective is to optimize this response. The first step in RSM is to find a suitable approximation for the true functional relationship between response and the variables, which yield that

response (Montgomery, 2001). If the response is well modelled by a linear function of the independent variables, then the approximating function is the first order model:

$$Y= \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \ldots\ldots \beta\ k x_k + \varepsilon$$

If there is a curvature in the system, than a polynomial of higher order must be used such as the second-order model:

$$Y= \beta_0 + \sum_{i=1}^{K} \beta i x i + \sum_{i=1}^{k} \beta_{ii} x i^2 + \sum_{i<j} \sum \beta_{ij} x i x j + \varepsilon$$

In literature, RSM has been used for optimization of EPS production by *Bacillus polymyxa* (Lee *et al*., 1997), *Propionibacterium acidi-propionici* (Gorret *et al*., 2001) and *Rhizobium spp.* (Duta *et al*., 2004).

1.7 Biofilms: serious health and industrial problem

Biofilms are serious problem in health, environment and industry. It is responsible for more than 60 % of microbial infections, of which the most prominent being periodontitis, chronic lung infections (as encountered in cystic fibrosis patients), native valve endocarditis and a range of infections linked to biofilm-invaded medical devices (Donlan and Costerton, 2002; Hall-Stoodley *et al.*, 2004). According to the National Institute of Health (NIH), more than half of nosocomial infections reported are predicted to be due to biofilms and the treatment of these biofilm-associated infections costs billions of dollars annually (Mah and O' Toole, 2001).

In addition to their medical consequences, biofilms predispose industrial water distribution systems, pipelines and cooling towers to corrosion (Ludensky, 2003; Chmielewski and Frank, 2003). Biofilms pose a serious problem in paper industry

also. In paper machines, biofilms cause paper defects such as stains and holes, and lead to bad odours both in the mill and on the paper. Biofilms are also responsible for the corrosion of surfaces in the paper mill system and for producing explosive gases such as methane (Blanco *et al*., 1996). Food processing environments are also susceptible to biofilm formation and biofilms can result in losses due to corrosion of equipment, reduction in heat transfer, obstruction of pipelines and spoilage of food products (Trachoo, 2003). Moreover, pathogenic microorganisms present in biofilms are difficult to control due to the increased resistance of biofilm bacteria to disinfectants and can therefore represent a potential health risk (Chmielewski and Frank, 2003).

1.8 Control of biofilms

As a consequence of the economic losses associated with the deleterious effects of biofilms on medical and industrial practices, different methods involving physical, chemical and biological approaches have been used for minimizing the accumulation of biofilms on surfaces. These have included treatment of water to reduce the number of bacteria entering the system, modification of surfaces to prevent their colonization by biofilms, chemical and mechanical treatments that result in removal of biofilms from surfaces, supply of growth factor to microorganisms, so surface attachment has no advantage (Meyer, 2003).

1.8.1 Physical and chemical approaches

For control of biofilms, physical methods such as use of ultrasound, abrasion and chemical methods such as use of ethylene diamine tetraacetic acid (EDTA) have been reported. Oulahal-Lagsir *et al.* (2003) investigated the removal of biofilms formed by two meat spoilage microorganisms (*E. coli* and *Staphylococcus aureus*) by making use of combined ultrasonic and enzymatic (proteolytic or glycolytic enzymes) and/or chelating agent treatment. Compared to ultrasound alone, use of ultrasound in combination with EDTA and/or enzymes resulted in biofilm removal of approximately 75 % and 100 % for *E. coli* and *S. aureus* biofilms, respectively from a stainless steel surface. All above approaches are effective against the biofilm removal from the surfaces but they have significant limitations also. In case of

food/beverages industry they are more prone to develop biofilm due to high nutrient level. The chemical used to remove such biofilms are potential hazard to health if they mixed with food products during sanitation.

1.8.2 Biological methods for biofilm removal

Among biological methods of biofilm removal, the use of bacteriophages, enzymes and quorum sensing inhibitors either individually or in combination have been suggested as an alternative to physical and chemical methods.

1.8.2.1 Use of bacteriophages for control of biofilms

Bacteriophages are ubiquitous in nature and a well known bacteria controlling agent. So they may provide a natural, highly specific, non-toxic, feasible approach for controlling several microorganisms involved in biofilm formation. Host-specific bacteriophages can infect biofilm cells by first degrading the EPS and then ultimately lysing the bacterial cells. However, since bacteriophages and their bacterial hosts exist together in nature, they are not considered a viable tool for biofilm removal (Tait *et al.*, 2002). Another problem to take into consideration is the ability of bacterial strains to become resistant against bacteriophage attack. One way of circumventing these problems is the use of bacteriophages in combination with sanitizers. In this regard, Tait *et al.* (2002) reported that bacteriophage-treated biofilms displayed a reduction in the amount of EPS, making the biofilms more sensitive to subsequent treatment with sanitizers.

1.8.2.2 Inhibition of quorum sensing for control of biofilms

Quorum sensing (QS) is proposed to affect biofilm formation (Parsek and Greenberg, 2005; Harraghy *et al*., 2007). Hence, QS may therefore be a potentially important system to target in order to prevent biofilm formation. It has been reported that an RNAIII-inhibiting peptide, inhibited quorum sensing and subsequently the attachment of *Staphylococcus epidermidis* and *S. aureus* to surfaces (Balaban *et al*., 2003**;** Yarwood and Schlievert, 2003). Enzymatic degradation of quorum sensing molecules has also been investigated as means to prevent biofilm formation (Oulahal-Lagsir *et al.*, 2003; Orgaz *et al.*, 2006). Acyl-homoserine lactones can be degraded by *N*-Acyl homoserine lactonases from

several *Bacillus* species such as *Bacillus cereus*, *B. thuringiensis* and *B. mycoides* (Dong *et al*., 2002), *Arthrobacter* spp. IBN110 and *K. pneumonia* (Park *et al*., 2003), as well as by acylases from *Pseudomonas aeruginosa* PAO1 (Sio *et al*., 2006) and *Ralstonia spp*. (Lin *et al*., 2003).

1.8.2.3 Use of polysaccharases for control of biofilms

As highlighted in the previous sections, most biocides and antibiotics are ineffective in biofilm eradication and control. Moreover, some of these disinfectants produce toxic by-products or have irritant properties (Augustin *et al*., 2004). Thus, there exists a need to identify agents to which microorganisms cannot acquire resistance as well as do not produce harmful by-products. As EPS play an important role in the structural integrity and attachment properties of biofilms, it could be a significant target for detaching and eradicating biofilms (Hentzer *et al.*, 2001; Stoodley *et al.*, 2002). Since polysaccharide hydrolases and polysaccharide lyases both are capable of degrading microbial exopolysaccharides, they have attracted considerable attention as a means to remove bacterial biofilms (Johansen *et al*., 1997; Orgaz *et al.*, 2006). Due to the substrate specificity of enzymes and the heterogeneity of the EPS, the removal of biofilms has been shown to be considerably more effective with enzymes. Moreover, it has been suggested that the use of different types of enzymes, especially combinations of proteases and polysaccharases, may be effective in removing bacterial biofilms (Meyer, 2003).

In mixed biofilm populations, where a range of different exopolysaccharides is present, EPS degradation could be more challenging (Wingender *et al.*, 1999; Tait *et al.*, 2002). In such situations, a mixture of polysaccharases is typically used where degradation products of one enzyme might serve as a substrate for a different enzyme and thus ultimately lead to the degradation of EPS and the removal of biofilms (Wingender *et al.*, 1999). EPS-degrading enzymes can be either endo- or exo-acting (Sutherland, 1999; Wingender *et al.*, 1999). Endo-acting enzymes reduce the polysaccharide chain length rapidly by cleaving internal bonds of the polysaccharide, whereas the exo-acting enzymes cleave bonds at the ends of the polysaccharide and therefore its breakdown is slower. The degradation of homopolysaccharides can be facilitated by a range of endo- and exo-acting enzymes

(Gilbert and Hazlewood, 1993), while heteropolysaccharides are usually degraded by a single, very specific endo-acting polysaccharases.

Polysaccharases can be produced by the same organism (endogenous), produced by other eukaryote and prokaryotic microorganisms and by bacteriophages (exogenous). Only some polysaccharide-producing microorganisms synthesize polysaccharases that are capable of degrading their own polysaccharides towards its use as a source of carbon and energy (Sutherland, 1999; Wingender *et al*., 1999). However, Conti *et al.* (1994) reported that the endogenously-produced enzymes usually do not allow the microorganism to utilize its own EPS as a carbon source, but they can be released into the extracellular environment during cell division and cell lysis (due to autolysis, lytic phages and protozoa). Endogenously-produced polysaccharases such as glycanases or lyases may often form part of the same gene cassettes that are responsible for exopolysaccharide synthesis, polymerization and excretion (Matthysse *et al.*, 1995; Sutherland and Kennedy, 1996). However, it is not clear whether these enzymes are continuously expressed during their growth cycle or if they are expressed under the same physiological conditions as that for genes responsible for EPS synthesis (Sutherland, 1999; Wingender *et al.*, 1999).

On the other hand, exogenously-produced polysaccharases enable bacteria to degrade polysaccharides and utilize the monomers as energy and carbon source. Moreover it could be produced by a single microbial species or mixed bacterial cultures. The above enzymes are secreted out extracellularly and can therefore come into direct contact with the exopolysaccharides produced by biofilm cells of other microorganisms. For example a fungal cellulase caused decrease in the biofilm biomass and colony forming units of *Pseudomonas aeruginosa* (Loiselle and Anderson, 2003).

Actinobacillus actinomycetemcomitans produces an enzyme, known as Dispersin B, which targets polymers that contain *N*-acetylglucosamine and is capable of detaching biofilms of *Actinobacillus pleuropneumoniae* and *Staphylococcus epidermidis.* Dispersin B likewise hydrolyzes the glycosidic linkages in the capsular polysaccharide ß-1,6-*N*-acetyl-D-glucosamine (poly-ß-1,6-GlcNAc) required for

biofilm formation by *E. coli* and *S. epidermidis* (Kaplan *et al*., 2004a and b; Itoh *et al.,* 2005). The synthesis of the above polysaccharide hydrolyzing enzyme is dependent on the presence of *pgaABCD* and *icaABCD* loci. Dispersin B was capable of detaching biofilms from *E. coli*, *Staphylococcus epidermidis*, *Yersinia pestis* and *Pseudomonas fluorescens*, all of which posses either a *pgaABCD* or *icaABCD* locus.

The use of enzymes in biofilm control is still limited due to low prices of the chemicals used today compared to the costs of enzymes. Moreover, the low commercial accessibility of different enzyme activities limits their usage (Johansen *et al.,* 1997). Merritt *et al.* (2000) tested several cleaning agents such as tap water, commercial detergents with and without enzymes, enzymatic and non-enzymatic contact lens cleaning solutions, commercial mouthwash, peroxide-based bleach, bleach containing 5.25 % sodium hypochlorite, Triton X-100 against biofilms of *Staphylococcus epidermidis* and *Candida albicans*, as well as biofilms formed by oral microorganisms grown in 96-well polystyrene plates. The above study showed that the biofilms formed by respective microorganisms were most successfully removed by 'detergents containing enzymes' and sodium hypochlorite (NaOCl).

1.9 Exopolysaccharides of *Bacillus spp.*

Several members of genus *Bacillus* have been reported to produce extracelluar polysaccharides of capsular and slime category. A strain of *Bacillus megaterium* produced exopolymer in a medium containing glucose, ammonium nitrate and various salts in growth associated manner (Gandhi *et al*., 1997). A strain of *Bacillus megaterium* and *Bacillus thuringiensis* of a biofilm community produced EPS having fast attaching ability to glass surfaces. The EPS of *B. megaterium* contained glucose, mannose, galactose and glucuronic acid where the EPS of *B. thuringiensis* was made up of glucose, xylose and ribose. The molecular weight of the EPS from strain *B. megaterium* and *B. thuringiensis* was 2.1 X 10^4 and 5.87 X 10^4 Da, respectively (Kwon *et al*., 2002). *Bacillus cereus* form thick biofilm on steel and polystyrene surfaces (Wijman *et al*., 2007). EPS of haloalkalophilic *Bacillus spp.* exhibiting flocculation activity has been reported The above EPS was acidic in nature containing galactose, fructose, glucose, and raffinose and

uronic acids and possessed molecular weight 2.2 X 10^6 Da (Kumar *et al*., 2004). Lee *et al*. (2007) demonstrated that *Bacillus anthracis* readily formed biofilms which gave resistant to commonly prescribed antibiotics. Also a strain of *Bacillus licheniformis* produced an EPS at a concentration of 0.165 g/l when grew on kerosene as sole carbon source and was resisitant to Cd^{+2}, Zn^{+2}, As^{+2} and Hg^{+2} (Maugeri *et al*., 2002).

1.10 Present study

In majority of biofilms, the extracellular matrix has been reported to be exopolysaccharide in nature. In contrast to two identical amino-acids which can only form one dipeptide, two identical sugars can bond to form 11 different disaccharides. **Compared to 25 different sugars found in plants and animals, more than 200 different sugars have been found in microbial EPS**. **EPS can also be substituted,** normally ester or *N*-linked, with pyruvate, acetate, formate, sulfate, phosphate and other side groups adding to their chemical heterogeneity and thereby to their functional diversity. Functions for this heterogeneity have been ascribed to pathogens such as O-antigen serotypes of Enterobacteria, but not for environmental strains.

Currently, large quantity of **adhesives** is used globally. In 2001, the United States used 2.5 billion Kg of adhesive. In 2007, the total world demand for adhesives and sealants was 12 billion Kg, of which natural adhesives (non-microbial origin) contributed to 0.6 billion Kg. However, there are significant environmental issues like **toxicity and biodegradability**. Many are derived from **non-renewable petrochemicals** and 16 percent of adhesives include **toxic solvents** such as toluene, methyl-ethyl ketone and tri-chloro ethane in their composition/formulation.

Bioadhesives produced by barnacles and mussels have been found with excellent adhesive property, particularly for difficult job of underwater adhesion. However, scale-up of the complex, multi-part system has proven to be cumbersome. A number of biopolymers from bacteria are commercially available for use as a viscosifying agents, emulsifiers, thickeners, stabilizers and gelling agents. However, none of these bacterial polymers are in commercial use as adhesive.

Looking to the possibility of extraordinary diversity just described in the case of exopolysaccharides coupled with the fact that only a few of bioadhesive molecules of environmental bacteria associated with the biofilms of this nature have been characterized, **there is an immense scope for discovery of new and unique bioadhesive molecules (EPS) with different properties and applications.**

Since microbial systems are far less complex than the higher life forms and methods for producing microorganisms in large volume use standard and well established technology, extracellular polymeric substances (EPS) from microorganisms of "biofilm origin" could be a potential source of eco-friendly/biocompatible adhesive. Such EPS or chemically modified molecules can be exploited to develop bioadhesive molecules/materials as a product in a cost effective manner for specific applications such as surgical glue, orthopaedic applications, wood adhesive, underwater surface coatings, marine cements etc.

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.

Previously one/two factors affecting cell attachment and detachment have been studied in different organisms. However variety of physicochemical/physiological/biochemical parameters in relation with biofilm formation and growth and their correlation has not been studied.

Hence, the **objectives** of present study were as follows:

1. Screening for the isolation of bacterial cultures capable of producing bioadhesive exopolysaccharides (EPS).
2. To carryout characterization of polymer's adhesive property keeping its application into consideration.
3. Physical and chemical characterization of EPS to understand its nature and composition.
4. Optimization of physical and chemical environment for the maximum production of a biopolymer.
5. To carry out the characterization and identification of the selected organism.
6. To study the kinetics of growth and product formation at shake flask level.
7. To understand the role of factors involved in and affecting biofilm formation, stability and detachment.

1.11 References

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2.0 MATERIALS AND METHODS

2.1 Screening of bioadhesive producing bacteria

2.1.1 Primary screening based on mucoidal nature of the colony

For this variety of samples from different ecological niches were used (as enlisted in Table 3A). Bacterial isolates producing exopolysaccharide (EPS) were selected on the basis of mucoid phenotype of colonies, grown on a high C:N ratio medium containing (g/l): Sucrose, 40; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0.1, and NaCl, 0.1 with or without yeast extract (0.1 g/l) supplementation. As nitrogen source either KNO_3 (1 g/l) or monosodium glutamate (0.2 g/l) was added. pH of the medium was adjusted to 7.2 ±0.2 and sterilized by autoclaving at 10 psi for 20 minutes. The culture plates were incubated at 30 ±1 °C for 48 h and bacterial isolates producing copious amount of EPS were selected and preserved in the form of agar slopes at 4 °C.

2.1.2 Secondary screening based on adhesive property

Initially using whole cell biomass from culture plate, "paper peel test" was carried out to assess the adhesive nature of the isolates and isolates showing comparatively better adhesive nature were cultivated in above mentioned liquid culture medium, EPS was recovered from culture filtrate by acetone precipitation, dried in oven (50 °C) and aqueous 10 % (w/v) EPS was used for a test. Using printing paper (60 gsm), pieces having dimension of 6 x 5 cm were cut and EPS solution was applied evenly using brush over 5 cm^2 area of paper leaving un-gummed flap of one cm size. Such pieces with applied adhesive were pasted firmly on a large piece of same paper and kept for 1 h at a temperature of 30 ±1 ^{0}C and relative humidity of 50 ±5 %. Using un-gummed flap, adhered paper pieces were pulled apart to assess the adhesive nature of different isolates (Bureau of Indian Standard, 1989).

2.2 Study on EPS-BM adhesive

2.2.1 Cultivation of the organism and production of EPS-BM adhesive at shake flask level

The isolate was grown by inoculating 10 % (v/v) of 0.4 $O.D_{600nm}$ into 50 ml medium (pH 7.2 ±0.2) containing (g/l): Sucrose, 40; KNO_3, 1; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0.1; NaCl, 0.1; and yeast extract, 0.1 in 250 ml Erlenmeyer flask, on a rotary shaker (180 rpm) at 30 ±1 °C for 72 h. Biomass from the culture broth was separated by centrifugation (at 10,000 rpm for 30 minutes) at 4 ºC, the EPS was precipitated from the supernatant using 3 volumes of chilled acetone, re-dissolved in distilled water, precipitation step was repeated, precipitates were dried at 50 °C to a constant weight, ground to powder form and used for adhesion studies.

2.2.2 Characterization of EPS-BM adhesive

2.2.2.1 Gross chemical analysis of EPS-BM adhesive

Thrice precipitated EPS solution was dialysed against distilled water for 18 h freeze-dried and **1 % (w/v) aqueous solution** was used for chemical analysis.

2.2.2.1.1 Estimation of total sugars

This was estimated by following phenol-sulphuric acid method of Dubois *et al*. (1956). To 1 ml of sample, 1 ml of 5 % phenol was added and mixed. Using a fast flowing pipette, 5 ml of concentrated H_2SO_4 was added directing the stream of acid onto the surface of the liquid and shaking the tube simultaneously. The tubes were allowed to stand for 10 minutes, cooled to 30 ±1 °C and the absorbance was measured at 488 nm. Glucose was used as the reference sugar in the range of 20 to 100 µg**.**

2.2.2.1.2 Estimation of uronic acid

This was carried out using the method as described by Bitter and Muir (1962). To 1 ml of sample, 5 ml of borate reagent (0.025 M sodium tetraborate in concentrated sulfuric acid) was added, mixed, heated in a boiling water bath for 10

minutes and cooled to room temperature. 0.2 ml of carbazole reagent [0.125 % (w/v) in ethanol] was then added and the mixture was again heated for 15 minutes in a boiling water bath, cooled and the absorbance was measured at 530 nm. Glucuronic acid (5 to 100 µg) was used as the standard.

2.2.2.1.3 Estimation of pyruvate

Pyruvyl content in EPS-BM adhesive was estimated by the method as described by Slonekar and Orentas (1962). Various aliquotes of sample (2 ml each) were hydrolysed with 1 N HCl for 3 h, and incubated with 1 ml of 2,4-dinitrophenyl hydrazine reagent (0.5 % w/v in 2 N HCl) for 5 minutes. The reaction mixture was extracted with 5 ml of ethyl acetate and the aqueous layer was discarded. The ethyl acetate fraction was further extracted with 5 ml of 10 % (w/v) Na_2CO_3 and the concentration of pyruvic acid was determined by measuring the absorbance at 375 nm. Pyruvic acid was used as a standard in concentration range of 0.01 to 0.05 µg/ml.

2.2.2.1.4 Estimation of acetyl content

The estimation of O-acetyl groups in the EPS-BM adhesive was carried out as described by Hestrin (1949), using acetylcholine (0.1 to 1 mg) as a standard. To 1 ml aliquot of hydrolyzed polysaccharide sample, 2 ml of freshly prepared alkaline hydroxylamine reagent [hydroxylamine-HCl, 2 M and NaOH 3.5 N, 1:1] was added. The pH of the reaction mixture was adjusted to 1.2 ±0.2 using 6 N HCl, 1 ml of $FeCl_3$ solution was added and the absorbance was read at 540 nm.

2.2.2.1.5 Estimation of protein content

The presence of protein in EPS-BM adhesive was analysed by using the method of Bradford *et al*. (1976). To 0.1 ml of sample, 1 ml of Coomassie Brilliant Blue G-250 reagent (10 mg/100 ml, made in a mixture of water, absolute ethanol and O-phosphoric acid in the ratio of 8:1:1) was added. After incubation for 2 minutes, the absorbance was noted at 595 nm. Bovine serum albumin was used as standard in concentration range of 5 to 100 µg.

2.2.2.1.6 Estimation of phosphate

Phosphate, associated/complexed with EPS-BM adhesive was estimated by the method as described by Fiske-SubbaRow (1925). One ml aliquot of polysaccharide samples were treated with 1 ml of 5 N H_2SO_4 followed by the treatment with 1 ml of 2.5 % Ammonium molybdate solution. After mixing thoroughly, 0.1 ml of reducing ANSA (0.2 g 1-amino-2-naphthol-4-sulfonic acid, 1.2 g Na_2HSO_3 and 1.2 g of Na_2SO_3 in 100 ml distilled water) reagent was added and the total volume of the tube was made up to 10 ml with distilled water. The reaction mixture was further incubated for 10 minutes at 30 ±1 °C and the absorbance was measured at 660nm. Phosphate (KH_2PO_4) was used as a standard in concentration range of 0.1 to 1 µM.

2.2.2.1.7 Determination of monomeric composition of EPS-BM adhesive

For this purpose EPS hydrolysate was used. EPS-BM adhesive was hydrolysed with 2 M tri-fluoro acetic acid (TFA) at 100 °C for 2 h and TFA was evaroted in vacuo, freeze-dried and used for analysis.

2.2.2.1.7.1 Paper chromatographic analysis

The monosaccharide composition of the hydrolysed polymer was initially determined by paper chromatography using Whatman filter paper-one and butanol/pyridine/water (6:4:3 by volume) as mobile phase. The developed chromatograms were dipped into silver nitrate reagent for one minute, dried, and were sprayed with ethanolic 0.5 N NaOH solution to visualize the spots. Silver nitrate reagent was prepared as described by Trevelyan *et al*. (1950).

2.2.2.1.7.2 High performance liquid chromatography (HPLC) analysis

The monosaccharide composition of the hydrolyzed polymer was also analyzed by using Waters HPLC model 2410 with Sugar-Pak^{TM} I column and Millennium 32 software. Water containing EDTA (40 mg/l) and $CaCl_2$ (15 mg/l) was used as a mobile phase at a flow rate of 0.4 ml/min, internal oven temperature 40 ^{0}C and the peaks were detected using refractive index detector. 30 µl of either standards (20 mg/ml) or hydrolysate (20 mg/ml) was injected. Authentic monosaccharide standards were also run to determine the identity of the peaks.

2.2.2.2 Fourier transform-infrared (FT-IR) analysis

The pellets for analysis were obtained by grinding a mixture of 1.2 mg of purified EPS-BM adhesive with 150 mg of dry KBr powder followed by pressing the mixture into a mold (Yanping *et al*., 2010). The FT-IR specrum was recorded in the region of 4000–250 cm^{-1}, at a resolution of 1 cm^{-1} by using Perkin-Elmer FT-IR spectrometer (Germany).

2.2.2.3 Determination of ionic nature

The ionic nature of the EPS-BM adhesive was determined by measuring its efficiency of binding to anion (Dowex 1) and cation (Dowex 50) exchange resins as described by Ashtaputre and Shah (1995). Resins (5 g each) were activated by treating with either NaOH (1 N) or HCl (1 N), washed with water and then equilibrated with buffers of 10X strength (100 mM) i.e. Tris-HCl (pH 8.9) for Dowex 50 and acetate buffer (pH 5.0) for Dowex 1. Columns (1x6 cm) were packed with the resins and aqueous solution of EPS-BM adhesive (0.5 mg/ml) was loaded on to each of the columns. EPS-BM adhesive was then eluted with the respective buffers (10 mM), and the fractions (0.5 ml each) collected were subjected to total sugar estimation using phenol-sulphuric acid method. The bound polymer was quantitated using a calibration curve.

2.2.2.4 Molecular weight determination

The molecular weight of the EPS-BM adhesive was determined by gel permeation chromatography performed on sepharose-4B column. Samples (250 μg) were loaded onto the column and eluted with 10 mM phosphate buffer (pH 7.2 ±0.05) at a flow rate of 0.5 ml/min. Fractions (2 ml) were collected and subjected to total sugar estimation by the phenol-sulphuric acid method. Dextrans of different molecular weight ranging from 9 x 10^3 to 2x10^6 Da were used as standards.

2.2.3 Evaluation of the adhesive property

Specimens such as wood, metals and plastics were used individually and in combinations for evaluation of adhesive property. Specimen dimensions were 30×2.5×0.3 cm for wood (*Shorea robusta*) and 30×2.5× 0.1 (metals) /0.2 cm (plastics). Surfaces of adherends were roughed using sand paper, wiped with ethanol and an aqueous adhesive (10 % w/v, having pH 7) was applied (13.33

mg/cm^2) 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 2.5 kg weights for further 18 h to allow the adhesive to set up. Finally bonded specimens were cured for 7 days at 30 ^{0}C ±1 and 50 ±5 % relative humidity. Lap shear strength was determined using Universal Testing Machine (UTM) model Alfred J. Amsler and Co. Schaffhouse (Switzerland) 223/445 and reported in terms of MPa (1MPa = 10.197 Kg/cm^2). Above studies were an adaptation of 'American Standard for testing Material' ASTM D905-03 (2003) (for wood as well as other similar materials) and D1002-10 (2010) (metal to metal). Fevicol (48 to 50 % w/v solids), a commercial wood cum multipurpose adhesive, was used as a positive control for wood specimen. Each individual determination was carried out in triplicate and each complete experiment was carried out twice.

2.2.3.1 Effect of environmental factors (temperature, pH and salinity) on adhesive strength

Curing at 4 °C, 30 °C (Control- relative humidity 50 ±5 %) and 50 °C was carried out for seven days to study the effect of temperature on shear strength of bonded specimen.

An Adhesive solution was prepared in distilled water priorly adjusted to pH 4, 7 (control) and 8 and was used to see the effect of pH on shear strength.

Adhesive used contained none (control), 5 % or 20 % (w/v) NaCl to follow the effect of salinity on shear strength.

2.2.4 Yield improvement of EPS-BM adhesive

2.2.4.1 Box-Behnken design

For media optimization, "Fractional Factorial Design" approach (Box-Behnken design) was used and an attempt was made to standardize the concentrations of main medium components i.e. carbon, nitrogen and phosphate. Factorial design involving **three factors at three levels** was created using Statgraphics plus 3.1software. Each factor was assigned a low (-), middle and a high (+) value. The values of the variables were as given in the **table 2.1**. Accordingly fifteen medium

flasks were prepared along with the other nutrients of the growth medium and were processed as described previously in the section (2.2.1). Two responses i.e. EPS yield (g/l) and biomass (g/l) were determined after 72 h. The order of experiments was fully randomized to provide protection against the effects of lurking variables.

Table 2.1 Levels of independent process variables used for Box-Behnken design

Test	Sucrose (g/l)	KNO_3 (g/l)	K_2HPO_4 (g/l)
1	50	1	0.25
2	50	1	0.75
3	30	0.5	0.5
4	40	1	0.5
5	50	1.5	0.5
6	40	1.5	0.25
7	40	1	0.5
8	30	1	0.75
9	40	0.5	0.25
10	40	1	0.5
11	30	1.5	0.5
12	40	1.5	0.75
13	30	1	0.25
14	40	0.5	0.75
15	50	0.5	0.5

2.2.4.1.1 Growth measurement

Biomass from the culture broth was separated by centrifugation at 10,000 rpm for 30 minutes; cell pellet was washed twice, transferred to pre-weighed aluminium foil and dried at 50 °C to a constant weight. When required, dry weight was also determined by estimating the whole cell protein of cell aliquote and was converted to dry weight using standard relationship established between whole cell protein and dry weight.

2.2.4.1.2 Estimation of EPS

The EPS was recovered from cell free supernatant by precipitation using 3 volumes of chilled acetone, re-dissolved in distilled water, precipitation step was repeated, and precipitates were dried in oven (50 °C) for 2 h to a constant weight.

2.2.4.2 Effect of maintenance of pH and supplementation of phosphate on EPS production and growth

The culture was cultivated as described previously in the section (2.2.1) in the several 250 ml flasks, each containing 50 ml buffered (0.02 to 0.1 M, pH 7.2 ±0.2 phosphate/tris buffer) and also non-buffered but phosphate supplemented (0.02 to 0.1 M K_2HPO_4, pH 7.2 ±0.2) medium for 72 h. The control medium was non-buffered and contained 0.003 M K_2HPO_4 only. Culture flasks were harvested after incubation period; biomass and EPS yield were estimated gravimetrically as described above (section-2.2.4.1.1 and 2.2.4.1.2).

2.2.4.3 Effect of aeration on EPS production

This was observed by making variation in volume of broth/flask volume. The culture was cultivated as described previously in the section 2.2.1 in the several 250 ml Erlenmeyer flasks containing 20, 40, 50 (control), 60 and 100 ml growth medium for 72 h. Culture flasks were harvested after incubation period of 72 h, biomass and EPS production were estimated gravimetrically as described above (section-2.2.4.1.1 and 2.2.4.1.2).

2.3 Characterization of strain ADE-0-1

2.3.1 Morphological, cultural and biochemical characterization

The strain was primarily characterized by morphological and cultural features and biochemical tests and identified following Bergey's Manual of Systematic Bacteriology (Sneath, 1986).

2.3.2 Phylogenetic characterization

"Bacterial Identification Service" based on partial *16S rDNA* gene analysis, provided by Bangalore Genei, Bangalore, India; was used for confirmation of identification of the strain.

For this, i) isolation of genomic DNA, ii) amplification of ~1.5 Kb *16S rDNA* fragment using the "universal" forward primer 27f (5'-AGA GTT TGA TCC TGG CTC AG-3') and reverse primer, 1541r (5'-AAG GAG GTG ATC CAG CCG CA-3') and high-fidelity PCR polymerase (Macrae *et al*., 2000), iii) bi-directional sequencing of PCR product using forward and reverse primers, iv) alignment and analysis of sequence data for finding the closest homologues for strain were carried out.

2.4 Study on biofilm

The culture was cultivated, using the medium and conditions as described previously (in section-2.2.1), in the several (fourteen) 250 ml flask each containing 50 ml medium with two glass slides (7.5X2.5X0.2 cm). Two flasks were harvested at regular interval of time and slides/samples were used for measurement of biomass, biofilm density, EPS production, cell surface polysaccharide and pH change. Samples for cell surface hydrophobicity and cell surface charge were obtained from similar type of separately cultivated culture flask.

2.4.1 Measurement of biofilm density

Biofilm developed on glass slides were quantified using "crystal violet staining procedure" as described by O'Toole *et al*. (1999). At regular interval of time, slides were removed from culture broth, loosely attached cells from the slides were removed by washing thrice with 2 M phosphate buffer saline (PBS-pH 7.4), slides were stained with crystal violet for 2 minutes, washed twice with 0.2 M

PBS (pH 7.4). Slides were exposed to absolute ethanol for 15 minutes and absorbance of eluted dye was measured spectrophotometrically at 595 nm.

2.4.2 Measurement of cell surface hydrophobicity

Relative bacterial cell's surface hydrophobicity measurement procedure, developed by Rosenberg (1980), was followed for this purpose. Cells were separated by centrifugation, washed and resuspended in sterile distilled water to 0.4 $O.D_{600nm}$. Such 3 ml suspension was added to 3 ml hexadecane, mixed on a vortex mixer for 1 minute, incubated at 30º C for 10 minutes and then vigorously mixed again on a vortex mixer for 2 minutes. After settling, the absorbance of the aqueous phase was measured at 600nm after standing at ambient temperature for 20 minutes. The percentage of hydrophobicity (% h) was determined from the absorbance of the bacterial suspension (A_i) and the absorbance of the aqueous phase after mixing with hexadecane (A_f) using the formula:

$$\% h = (A_i - A_f)/ A_i \times 100$$

2.4.3 Measurement of electrochemical potential on cell surface

This was determined as described by Denyer *et al*. (1993). The culture was cultivated in the several 250 ml flask each containing 50 ml liquid growth medium and flasks were harvested at regular interval of time. The cell pellet was washed and resuspended in 1 ml of phosphate buffer (0.1 M, pH 7); the samples were adjusted to 0.7 $O.D_{600nm}$. The electrochemical potential of the cells harvested at different time intervals, was measured with an electrometer (keithley instruments, Model 6514 system electrometer). The mean value of six measurements were calculated and used for correlation with attachment.

2.4.4 Measurement of cell surface polysaccharides (CPS)

This was carried out as described by Zhang *et al*. (1999). Samples (culture broth) were removed at regular interval of time, centrifuged and cell pellet was resuspended in 1:1 (vol/vol) mixture of 0.01 M KCl and 2 % EDTA. The EDTA-cell suspension was incubated at 4 ºC for 30 minutes, followed by centrifugation at 10,000 rpm for 30 minutes at 4 ºC. Released carbohydrate content of the supernatants was determined by phenol-sulphuric acid method.

2.4.5 Microscopic examination of biofilm

2.4.5.1 Light microscopy

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 thrice with 2M Phosphate Buffer Saline (PBS-pH 7.4) to remove planktonic cells/loosely adhered cells, stained by 1 % crystal violet for 2 minutes and washed twice with 0.2 M (PBS-pH 7.4) and observed under light microscope (Accumax UB200i) at 1000 X magnification.

2.4.5.2 Scanning electron microscopy

The culture was cultivated with glass slides as described above (section- 2.4.5.1). Glass slides were removed, rinsed thrice with sterile 2 M Phosphate Buffer Saline (pH 7.4), biofilms were first fixed using glutaraldehyde (2.5 % in PBS buffer) for 1 h, rinsed with PBS for 15 minutes and then using osmium tetroxide (1 % in PBS buffer) for 1 h, dehydrated by treating with ethanol, coated with gold and viewed at 2000X magnification under scanning electron microscope (JSM-5610LU, Hitachi, Japan).

2.4.5.3 Confocal Laser Scanning Microscopy (CLSM)

Culture was cultivated with glass slides as described above in the section (2.4.5.1). Glass slides were removed, rinsed thrice with sterile 2 M PBS (pH 7.4), stained with nucleic acid stain (syto 9) (Invitrogen) according to manufacturer's instructions and observed at 400 X magnification under Zeiss (LSM 510 Meta) confocal laser scanning microscope (CSLM). Three independent slides and three to seven fields from each slide were randomly chosen to acquire images. Raw images were processed by Zeiss software ZEN2010.

2.4.6 Kinetics of biofilm forming ability in buffered and non-buffered medium

The culture was cultivated in the phosphate buffered (0.02 M, pH 7) and non-buffered (Control- only K_2HPO_4 at 0.003 M concentration) media as described

previously in the section (2.4.5.1). Glass slides were removed and biofilm density was estimated by "crystal violet staining procedure" as described previously (Section-2.4.1). The pH was measured through pH meter.

2.4.7 Kinetics of production of extracellular EPS-depolymerase

The culture was cultivated as described previously in the section (2.2.1) in the several 250 ml flask, each containing 50 ml medium, for 96 h. 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 sugars were estimated by following di-nitro salicylic acid (DNS) method as described by Miller (1959).

2.4.7.1 Kinetics of removal of preformed biofilm cells on treatment with EPS-depolymerase

Proteins present in the culture filtrate, obtained after 72 h of growth period, were precipitated 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 (0-5 h) at 30 ±1 °C. Biofilm density on enzyme treated slides and control slides was estimated by "crystal violet staining procedure". From separate set of experiment, such slides were also observed by light microscopy at 1000 X magnification.

2.4.7.2 Assay of EPS-depolymerase activity

EPS-BM adhesive produced as described previously in the section (2.2.1) was used as a substrate for depolymerase. The reaction mixture consisted of 0.5 ml of 1 % EPS, 0.5 ml of acetate buffer (0.2 M, pH 5.6), an aliquote of enzyme preparation and distilled water to make up the total volume 2 ml. After 30 minutes of incubation at 30 °C, liberated reducing sugars were estimated, by following dinitrosalicylic acid (DNS) method as described by Miller (1959).

2.4.7.2.1 Estimation of reducing sugars

Reducing sugars were estimated by the method of Miller (1959). To 3 ml of sample in test tubes, 1 ml of DNS reagent was added and the test tubes were then placed in boiling water bath for 5 minutes, cooled to room temperature and the absorbance at 540 nm was measured. Glucose (up to 250 µg) was used as standard. DNS reagent was prepared by mixing solutions, (A) 300 g sodium potassium tartarate in 500 ml distilled water, and (B) 10 g of 3, 5-dinitro salicylic acid in 200 ml of 2 M sodium hydroxide. The final volume of the solution was made up to 1 litre with distilled water.

2.5 Chemicals

All chemicals used were of analytical grade and were obtained locally. Dextran molecular weights of different range and authentic sugars were obtained from Hi-Media, Mumbai, India. Dowex-1 and Dowex-50 ion exchange resins were obtained from Sigma chemicals Co., USA.

2.6 Reproducibility

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

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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 marine 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**".

Table A: Morphological and cultural characterization of selected isolates

Sample source	Adhesive ability	Gram-reactions	Colony characteristics
Rhizosperic soil (sample 1)	+	-Ve, Short rods,	raised, mucoid, opaque, off-white
Rhizosperic soil (sample 2)	+	+Ve, Short rods	raised, mucoid, translucent, smooth margin
Rhizospheric sea-shore sample (ADE-0-1)	**+++**	**+Ve, Large rods**	**raised, round, smooth, colourless, opaque with the entire edge and mucoid in nature**
sea-shore sample (1-3)	++	+Ve, Cocci	raised mucoid, irregular margin, light brown pigment, opaque
sea-shore sample (3-5)	++	+Ve, Large rods	raised, mucoid, off-white, opaque, serrated margin
sea-shore sample (5-7)	++	-Ve, Cocci	irregular margin, mucoid, raised, opaque, off-white
Garden soil	+	+Ve, Cocci	highly irregular margin, flat, dry, opaque, off-white
Air-condition duct	+	-Ve, Large rods	flat, irregular margin, opaque, off-white

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 Bradford *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^{+2}, Mg^{+2} and Si^{+2} 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_2SO_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).

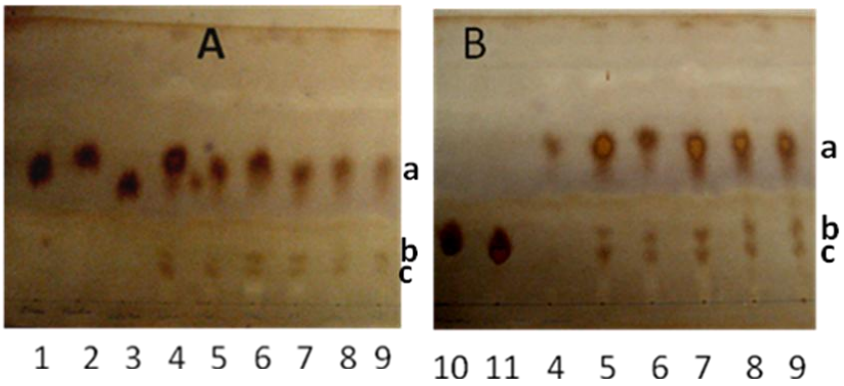
Initially, to determine the time required for complete hydrolysis of EPS adhesive, 2 M trifluoroactetic acid (TFA) was used at 100 °C for different time intervals (0, 0.5, 1.0 1.5. 2.0, 2.5 and 3 h). In an earlier report, for determination of monomeric composition, an EPS of *Pseudomonas spp.* strain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

3.1.2.1.2.1.1 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**).

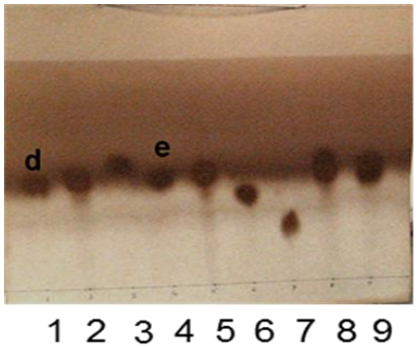
In paper chromatograms in case of EPS hydrolysates, the Spots corresponding to **R_f 0.44, 0.18 and 0.2** indicated the presence of **glucose, glucuronic acid and galacturonic acid, respectively** (**Figure 3.1 A and B**). Similarly in a different paper chromatogram**, mannose and arabinose** (**R_f 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_f for glucose = 0.44, glucuronic acid= 0.18, galcturonic acid= 0.2, respectively.

C



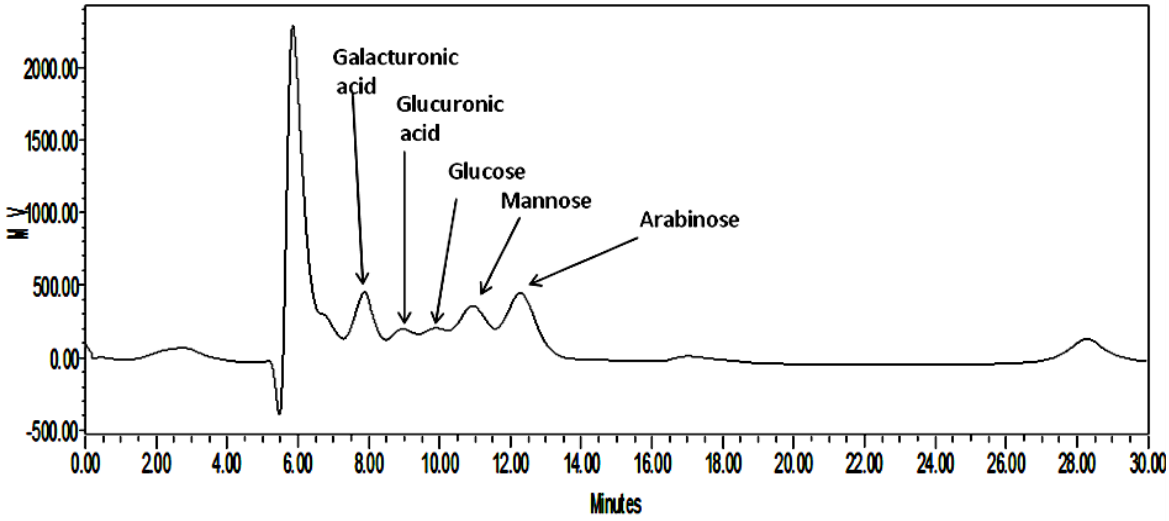
EPS was hydrolysed using 2 M TFA at 100 °C for 2 h. Chromatogram (C) Spot numbers 1- Mannose, 2- EPS hydrolysate, 3- Xylose, 4-Arabinose, 5- EPS hydrolysate, 6-Galactose, 7- Glucosamine, 8- EPS hydrolysate + Xylose and 9- glucose + EPS hydrolysate, In both the chromatograms (C) spots d and e corresponds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

3.1.2.1.2.1.2 High performance liquid chromatography

The monosaccharide composition of BM adhesive was analyzed using HPLC. A comparison of retention times (R_t) 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_t = 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.



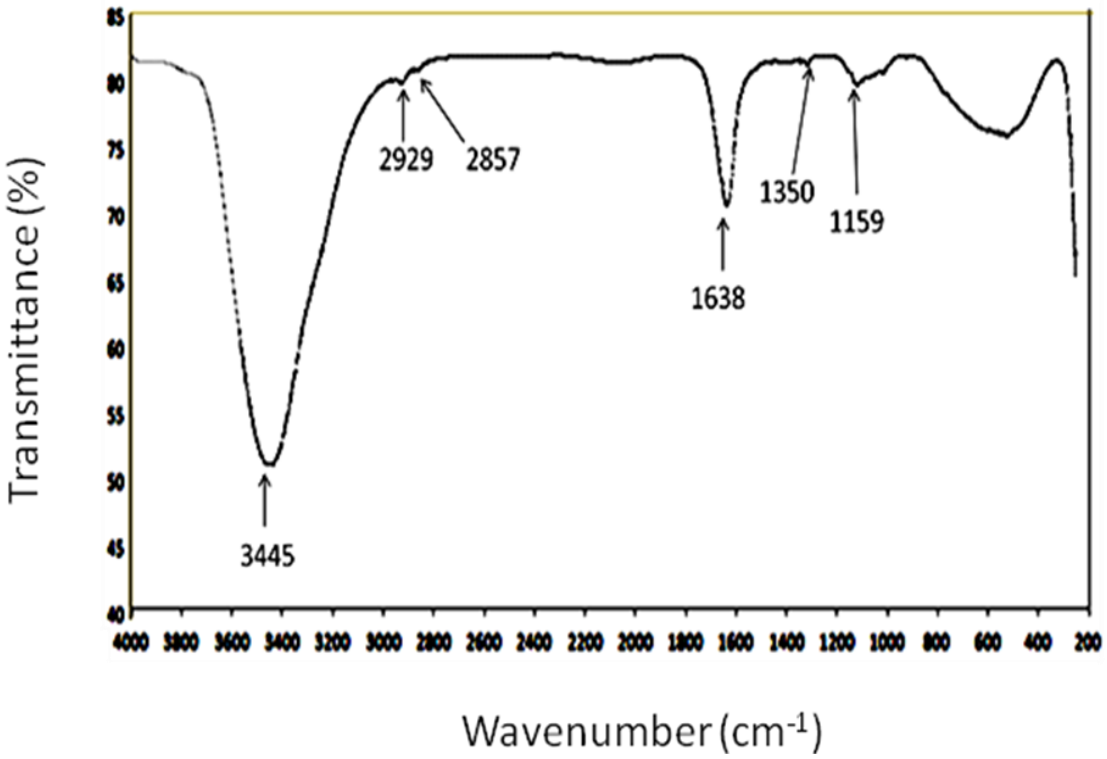
HPLC analysis of the EPS hydrolysate (2 M TFA, 100 °C, 2 h) was performed using a Waters 2410 HPLC system. Water containing EDTA (40 mg/l) and $CaCl_2$ (15 mg/l) was used as a mobile phase at a flow rate of 0.4 ml/min and the peaks were detected using RI detector. 30 µl of either authentic standards (20 mg/ml) or hydrolysate (20 mg/ml) was injected. Peak at R_t =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



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, anioinc 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

Samples	Wood	Smc	Acrylic	Iron	Steel	Al	Tin
Wood	5.46±.04	1.99*	0.79±.05	0.26±.01	0.79±.02	0.53±.01	0.66±.02
Smc	1.99*	0.93±.04	0.39±.05	1.06±.04	0.66±.02	0.79±.05	0.39±.02
Acrylic	0.79±.05	0.39±.05	0.93±.07	0.39±.05	1.06±.06	0.26±.04	0.26±.01
Iron	0.26±.01	1.06±.04	0.39±.05	1.59±.01	0.53±.03	2.79±.08	1.33±.01
Steel	0.79±.02	0.66±.02	1.06±.06	0.53±.03	2.26±.12	0.53±.03	0.39±.02
Al	0.53±.01	0.79±.02	0.26±.02	2.79±.08	0.53±.03	1.59±.02	1.06±.04
Tin	0.66±.02	0.39±.02	0.26±.01	1.33±.01	0.39±.02	1.06±.04	0.79±.02

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/cm^2) 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 2.5 kg weights for further 18 h to allow the adhesive to set up. Finally bonded specimens were cured for 7 days at 30 ^{0}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

Samples	pH 4[a]	pH 8[a]	Salinity[b] (5 %)	Salinity[b] (20 %)	Control[c]	4 °C[d]	50 °C[d]
Wood-Wood	3.3±.4	5.79±.03	4.92±.10	5.85±.02	5.46±.04	NA	**6.12±.03**
Iron-Al	2.13±.15	2.92±.03	2.26±.10	2.92±.23	2.79±.08	NA	3.06±.04
Smc-Wood	1.46±.10	1.99*	1.73 ±.10	1.99*	1.99*	NA	1.99*
Steel-Steel	1.86±.07	2.53±.02	2.26±.10	2.53±.03	2.26±.12	NA	2.66±.04
Iron-Iron	1.33±.07	1.73±.01	1.46±.10	1.86±.05	1.59±.01	NA	1.99±.06
Al-Al	1.2±.11	1.73±.01	1.46±.10	1.86±.08	1.59±.02	NA	1.99±.05

[a]Adhesive used had pH 4 or 7 (control) or 8. [b]Adhesive used contained 5 % or 20 % (w/v) sodium chloride. [c]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. [d]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, hemicelluolose 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 *Altromonas coluuelliana* LST for seven different substrates using lap shear specimens and 6.25 cm^2 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 14.6 MPa shear strength (bond area 19.4 cm^2) 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 cm^2 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 sunmika 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/cm^2 bond area of **both the adherends** compared to 13.33 mg/cm^2 bond area of **only one adherend** as in the case of BM adhesive. Also with wood specimen total bond area was 19.4 cm^2 (MB/SB adhesive) against 7.5 cm^2 in the case of BM adhesive. Taking above mentioned differences into consideration, MB/SB

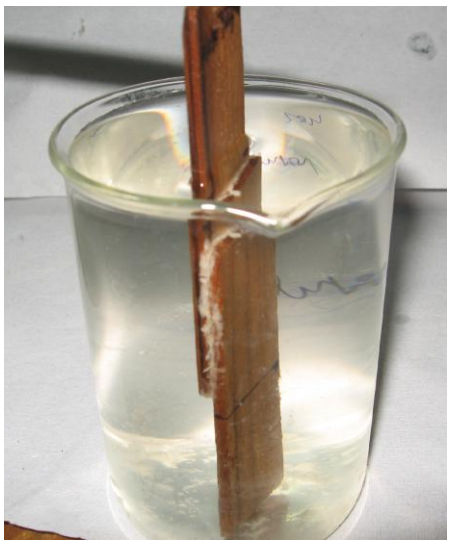
adhesives were tested at **5 to 8 times** higher concentration than that of BM adhesive and hence BM adhesive appeared superior than MB and SB adhesive.

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

Tests	Sucrose (g/l)	KNO_3 (g/l)	K_2HPO_4 (g/l)	EPS Yield (g/l)	Biomass (g/l)
1	50	1.0	0.25	0.40	0.34
2	50	1.0	0.75	0.80	0.25
3	30	0.5	0.50	0.65	0.34
4	**40**	**1.0**	**0.50**	**1.15**	**0.85**
5	50	1.5	0.50	0.51	0.31
6	40	1.5	0.25	1.02	0.37
7	**40**	**1.0**	**0.50**	**1.05**	**0.73**
8	30	1.0	0.75	0.20	0.28
9	40	0.5	0.25	0.40	0.22
10	**40**	**1.0**	**0.50**	**1.16**	**0.88**
11	30	1.5	0.50	0.57	0.31
12	40	1.5	0.75	1.0	0.54
13	30	1.0	0.25	1.03	0.22
14	40	0.5	0.75	0.48	0.40
15	50	0.5	0.50	1.34	0.25

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 (OD_{600nm} = 0.4; 10 % v/v) level and flasks were incubated on orbital shaker at 180 rpm at 30 ±1ºC 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; KNO_3, 1; K_2HPO_4, 0.5; $MgSO_4$, 0.2; NaCl, 0.1;$CaCl_2$, 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 bufferred 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

Figure 3.5 (A) EPS and Biomass production in phosphate buffered media

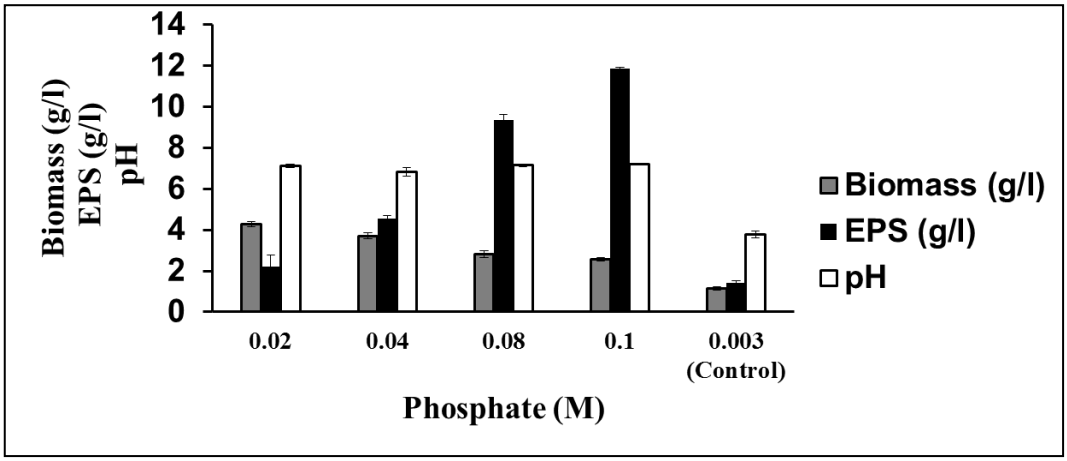
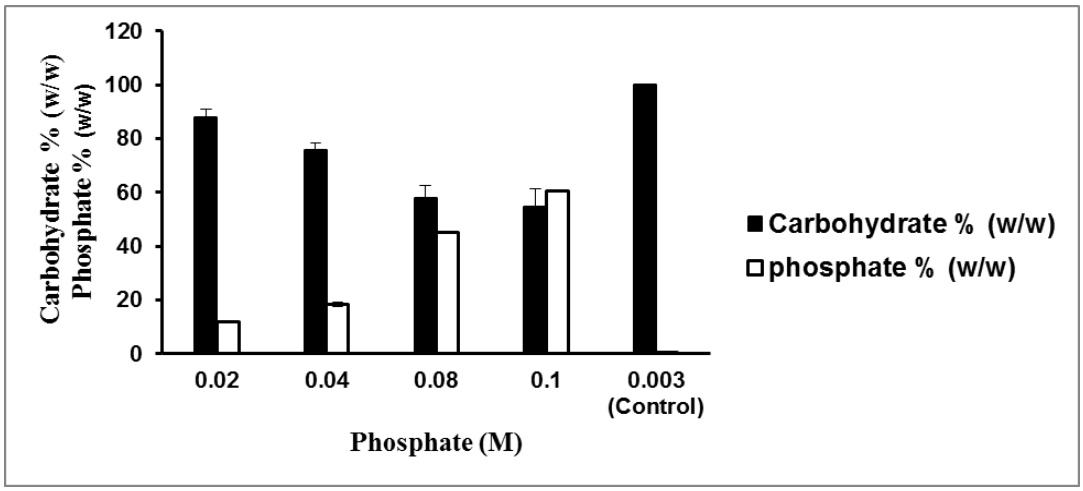


Figure 3.5 (B) Quality of EPS in phosphate buffered media



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_2HPO_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**).

Figure 3.6 (A) EPS and Biomass production in non-buffered media

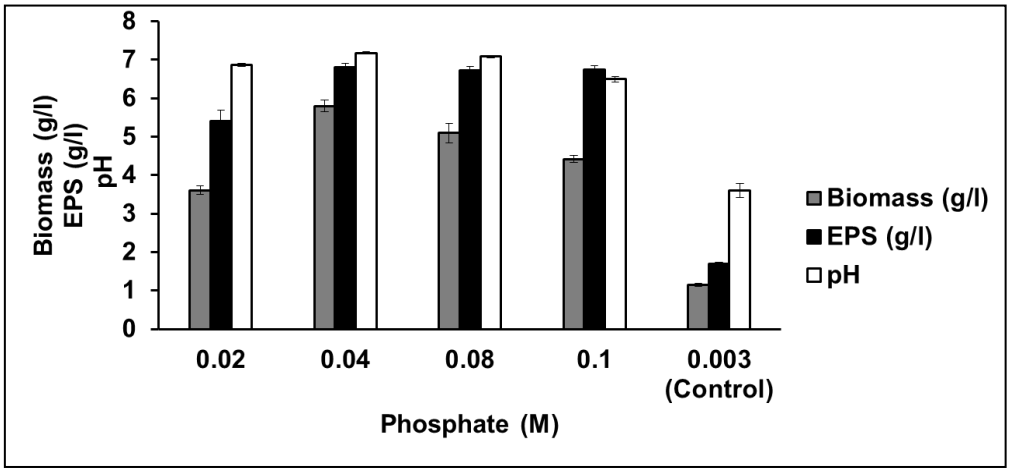
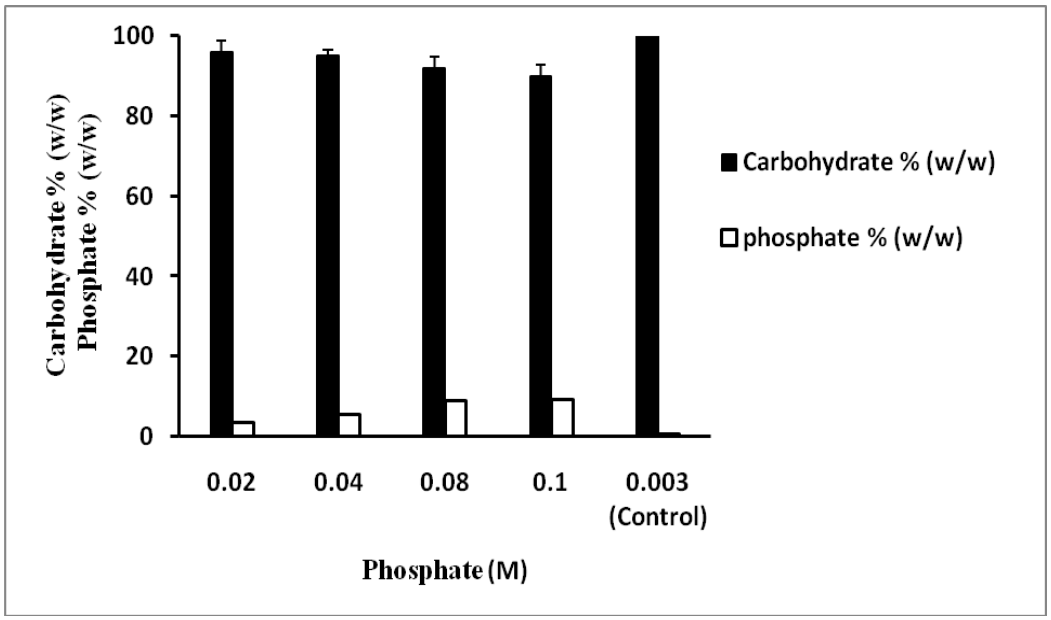


Figure 3.6 (B) Quality of EPS in non-buffered media



Shake flask cultivation at (30 ±1 °C, 180 rpm, for 72 h) was carried out in liquid non-buffered media (where K_2HPO_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 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 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_2HPO_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.

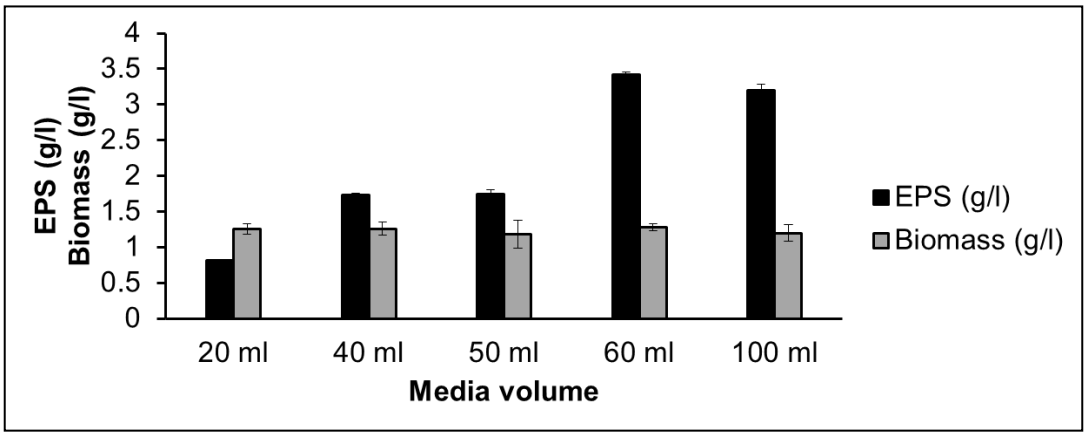
3.1.4.3 Effect of aeration on polymer production

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



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

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

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

Characteristics	**Result**
Inulin fermentation	+
Sodium gluconate fermentation	-
Glycerol fermentation	+
Salicin utilization	+
Glucosamine fermentation	-
Dulcitol fermentation	+
Inositol fermentation	+
Sorbitol fermentation	+
Mannitol fermentation	+
Adonitol fermentation	-
α-methyl-D- glucosamine fermentation	+
Rhamanose fermentation	+
Cellobiose fermentation	+
Melezitose fermentation	+
α- methyl-D-Mannoside fermentation	-
Xylitol fermentation	+
D-arabinose fermentation	+
Citrate utilization	+
Malonate utilization	-
Sorbose fermentation	+

The biochemical tests were performed according to the methods described in information provided in HiCarbohydrate^{TM} Kit (Himedia, India).

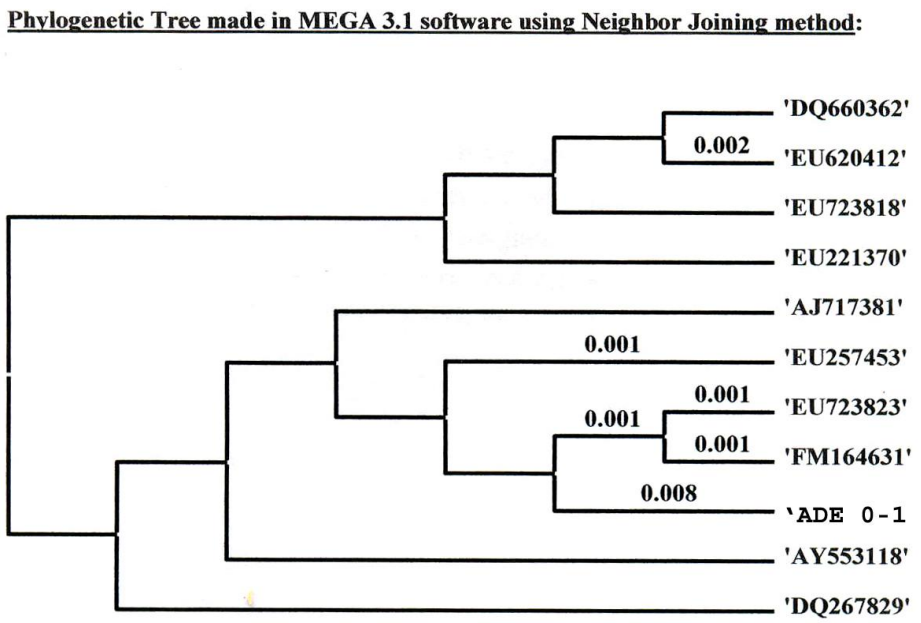
3.1.5.2 Phylogenetic characterization

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



The phylogenetic relationship of *Bacillus megaterium* species and the isolate ADE-0-1 based on *16S rDNA* sequences. The phylogenetic tree was created using maximum-likelyhood 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

Alignment View	ID	Alignment results	Sequence description
	ADE 0-1	0.92	Studied sample
	EU723818	0.97	*Bacillus megaterium* strain HDDMG02
	DQ660362	0.97	*Bacillus megaterium* strain MPF-906 16S
	AJ717381	0.97	*Bacillus megaterium* isolate AC46b1
	DQ267829	0.97	*Bacillus megaterium* strain 2-37-4-1
	EU221370	0.97	*Bacillus megaterium* strain B2P2
	EU257453	0.97	*Bacillus subtilis* isolate C6-1
	AY553118	0.97	*Bacillus megaterium* strain MO31
	EU723823	0.97	*Bacillus subtilis* strain HDDMM01
	FM164631	0.98	*Bacillus megaterium* isolate TS IW 36
	EU620412	0.96	*Bacillus subtilis* strain S8-04

Distance Matrix based on Nucleotide Sequence Homology (Using Kimura-2 Parameter):

Distance Matrix												
		1	2	3	4	5	6	7	8	9	10	11
AJ717381	1	---	0.999	1	1	1	0.999	1	0.999	0.998	1	0.992
EU257453	2	0.001	---	0.999	0.999	0.999	0.998	0.999	0.998	0.997	0.999	0.991
DQ660362	3	0.000	0.001	---	1	1	0.999	1	0.999	0.998	1	0.992
AY553118	4	0.000	0.001	0.000	---	1	0.999	1	0.999	0.998	1	0.992
DQ267829	5	0.000	0.001	0.000	0.000	---	0.999	1	0.999	0.998	1	0.992
EU723823	6	0.001	0.002	0.001	0.001	0.001	---	0.999	0.999	0.997	0.999	0.991
EU723818	7	0.000	0.001	0.000	0.000	0.000	0.001	---	0.999	0.998	1	0.992
FM164631	8	0.001	0.002	0.001	0.001	0.001	0.001	0.001	---	0.997	0.999	0.991
EU620412	9	0.002	0.003	0.002	0.002	0.002	0.003	0.002	0.003	---	0.998	0.990
EU221370	10	0.000	0.001	0.000	0.000	0.000	0.001	0.000	0.001	0.002	---	0.992
ADE-0-1	11	0.008	0.009	0.008	0.008	0.008	0.009	0.008	0.009	0.010	0.008	---

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

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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 system 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 Loosedrecht *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**).

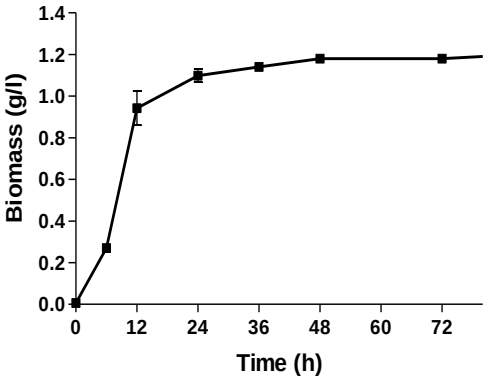
Similar kind of observations have been reported by Walker *et al*. (2005) in the case of *E. coli* D21g and van Loosdrecht *et al*. (1987a and b) in the case of several bacteria, where decrease in acidity/surface titrated charges (with reference to acidic hydrophilic outer membrane proteins) and corresponding 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-cojugated dextrans, 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).

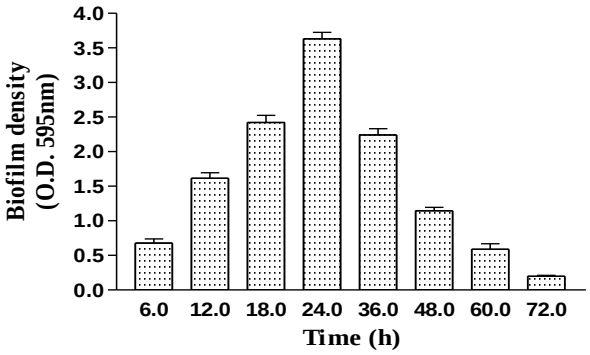
Figure 3.9 Kinetics of change in various parameters in relation with biofilm forming ability and growth of the organism

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.

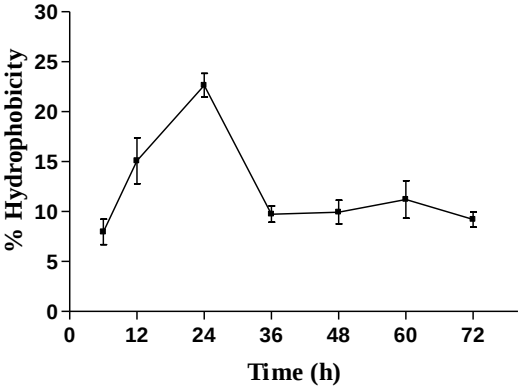
a) Growth



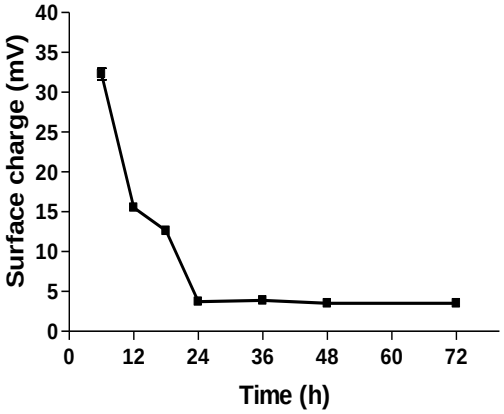
b) Biofilm density



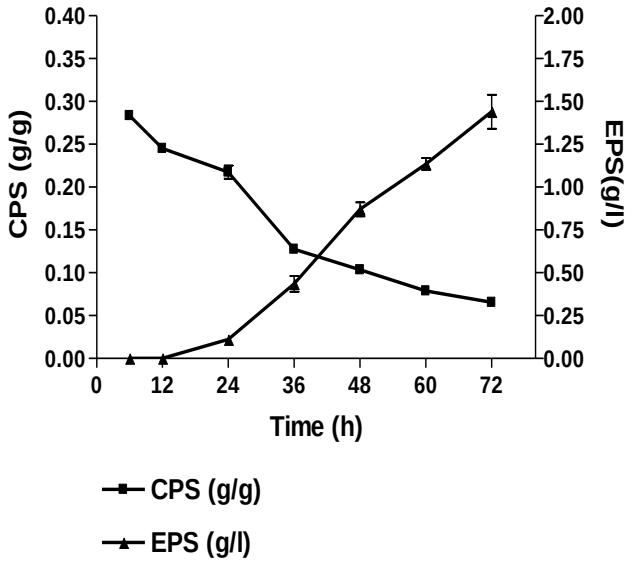
c) Cell surface Hydrophobicity



d) Cell surface charge



e) Cell surface polysaccharide and EPS production



f) pH change

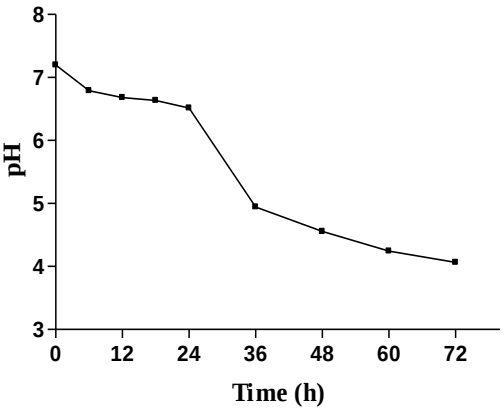
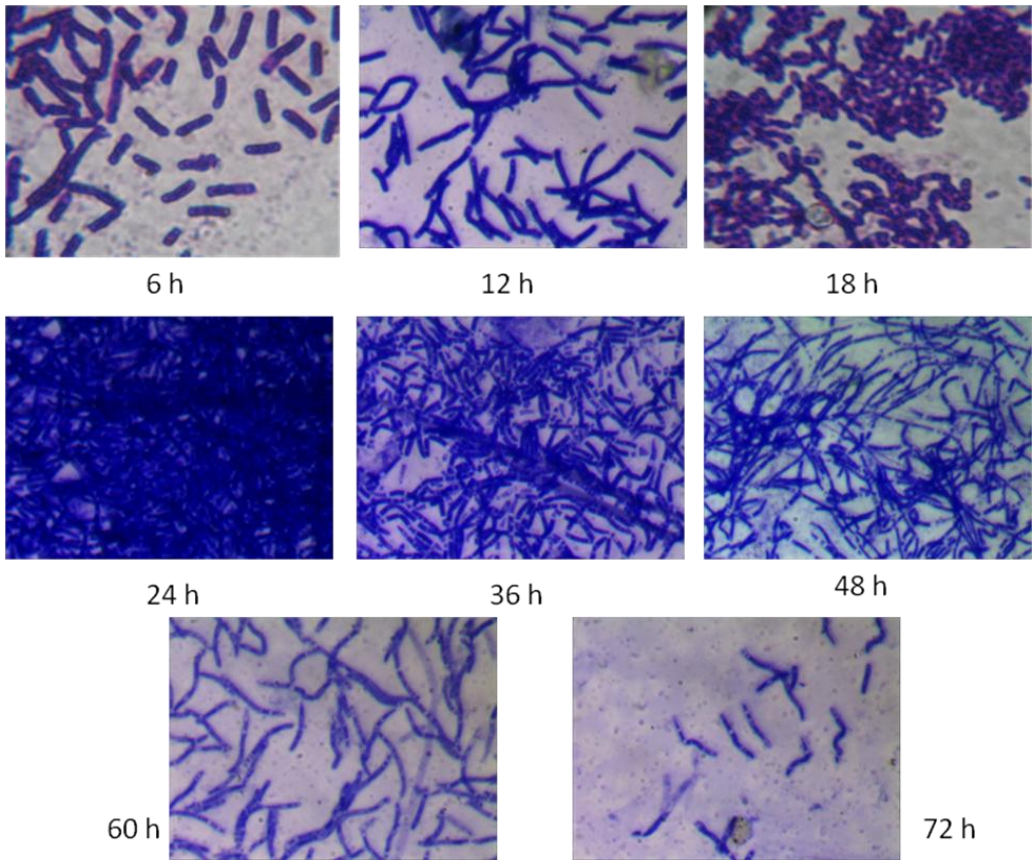
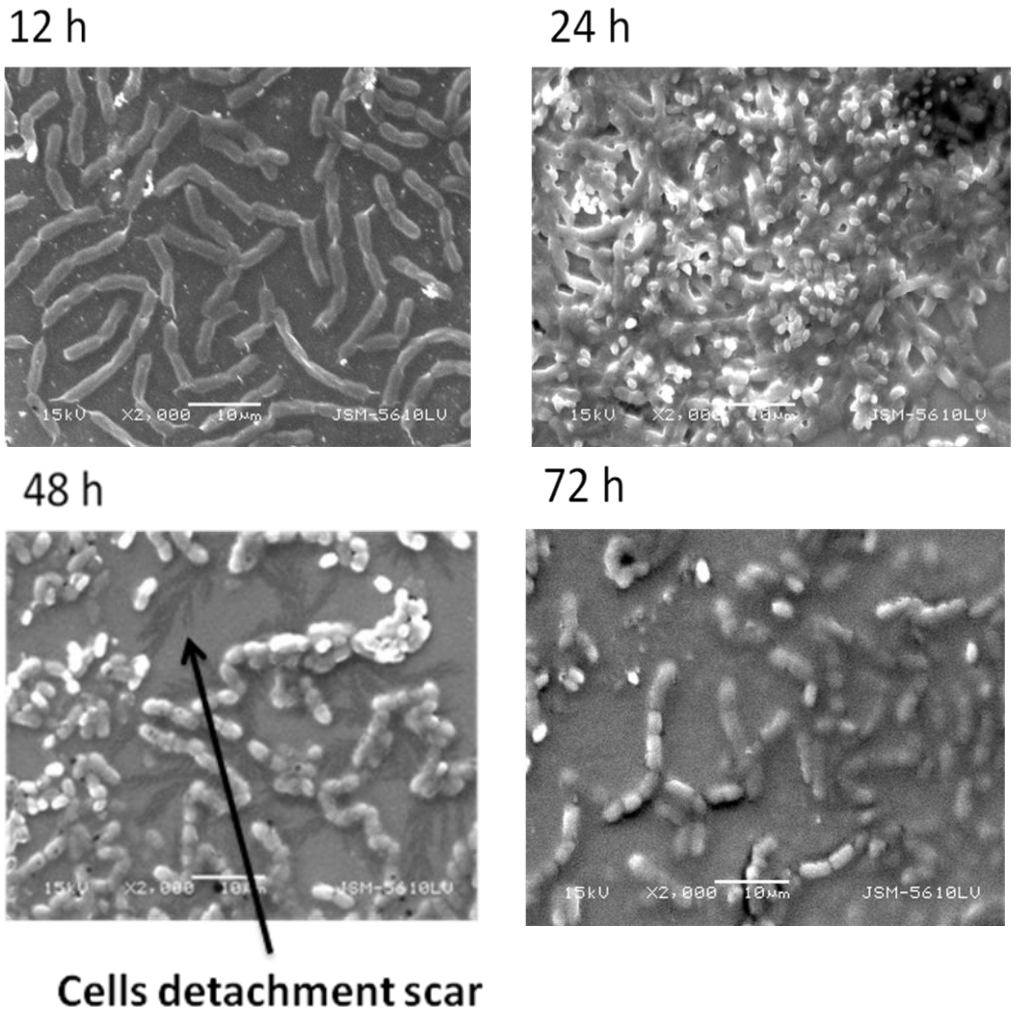


Figure 3.10 Light microscopic observations of biofilm developed on glass slides during growth of *Bacillus megaterium* ADE-0-1



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), bioflms were stained by crystal violet and observed under light microscope at 1000 X magnification.

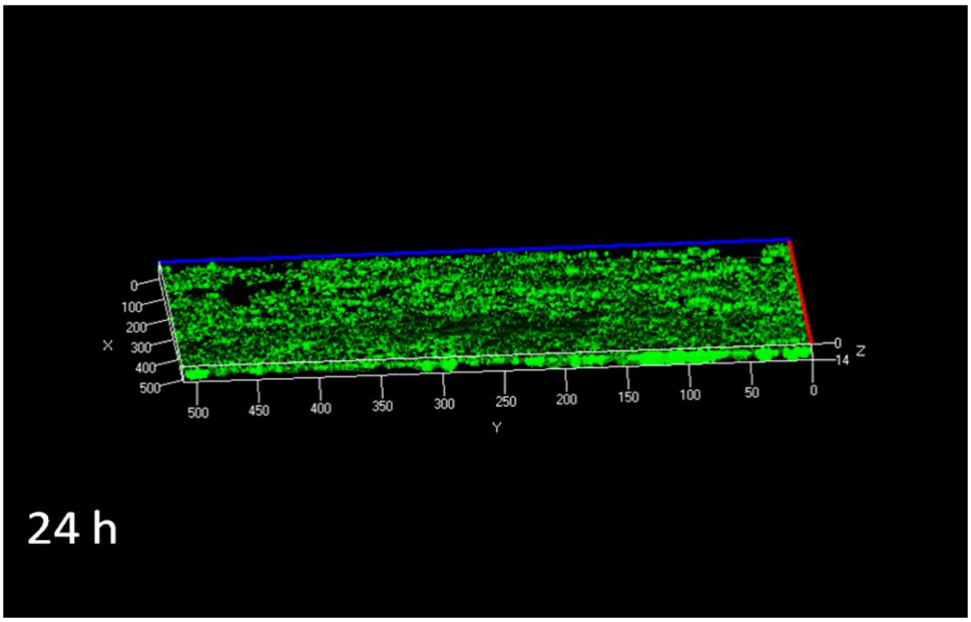
Figure 3.11 Scanning electron microscopic observations of biofilm at different stage of growth of *Bacillus megaterium* ADE-0-1



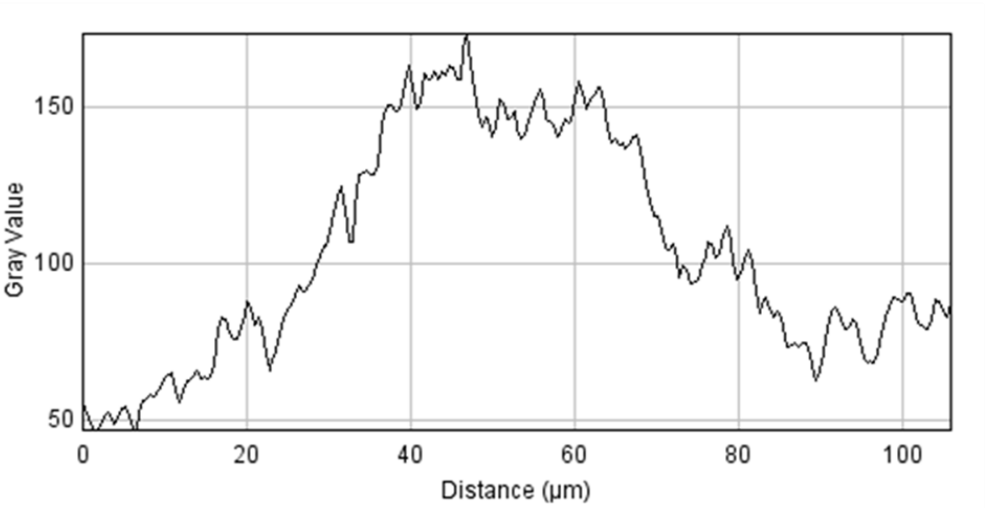
The culture was cultivated with glass slides as described previously in the case of light microscopic observations. Glass slides were removed, biofilms were fixed using glutaraldehyde and osmium tetroxide, dehydrated, gold plated and viewed at 2000X magnification under scanning electron microscope.

Figure 3.12 (A) Confocal Laser Scanning microscopic (CLSM) observations of biofilm at 24 h

a) 24 h biofilm



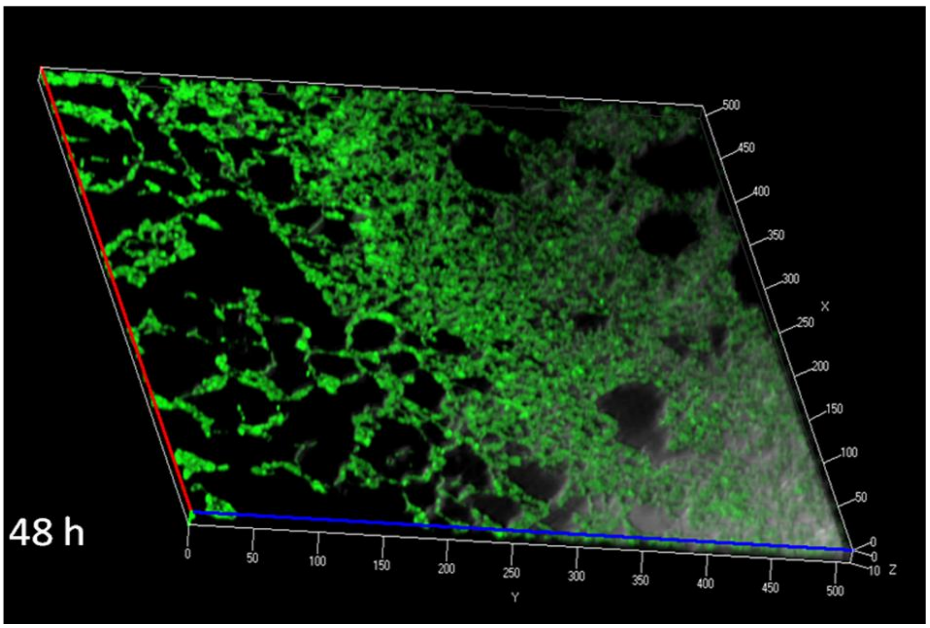
b) 24h Intensity Profile



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)

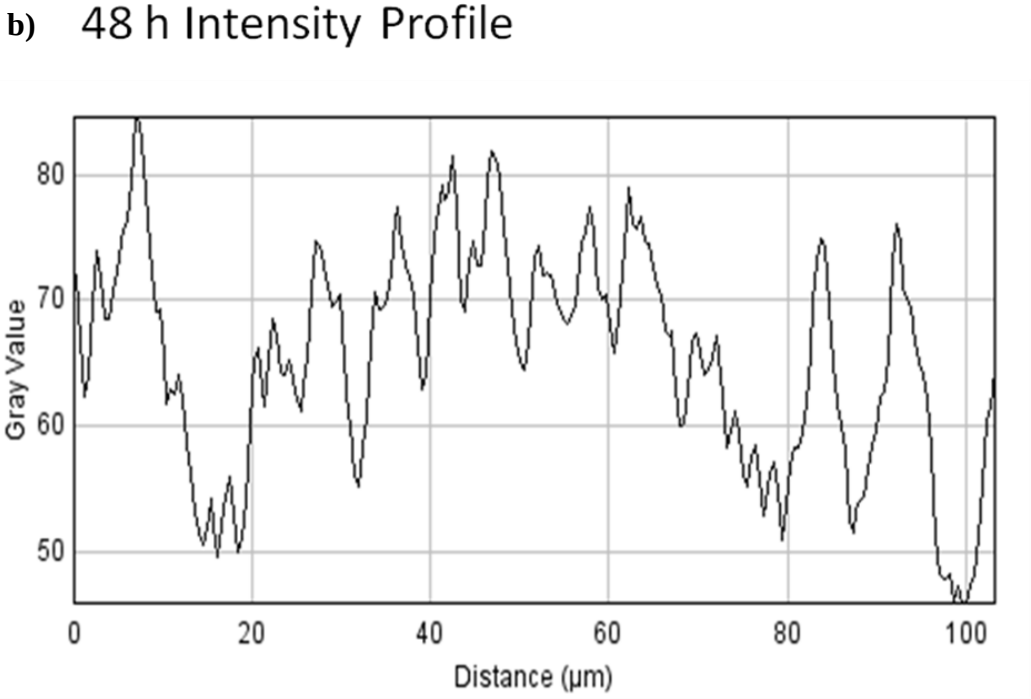
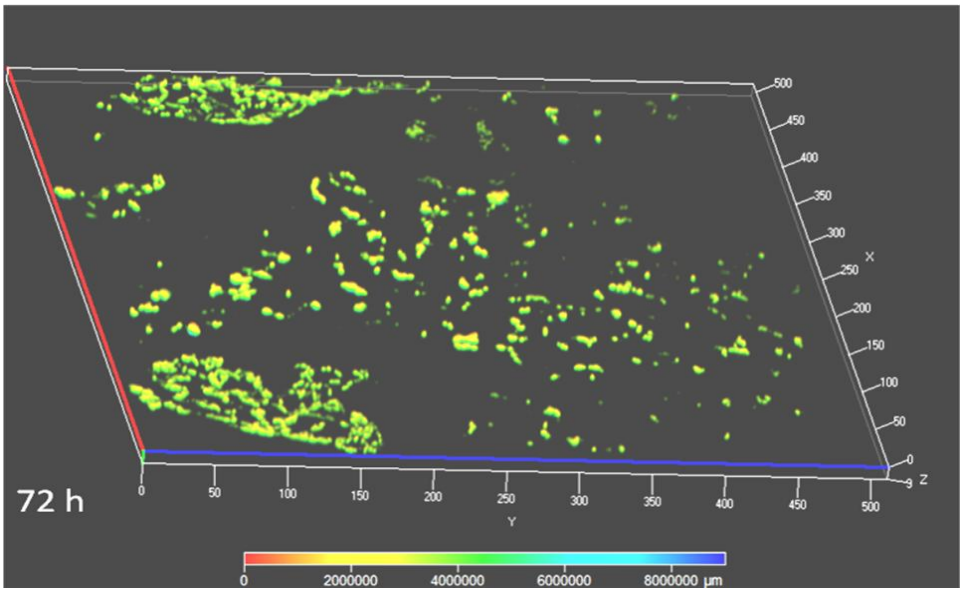
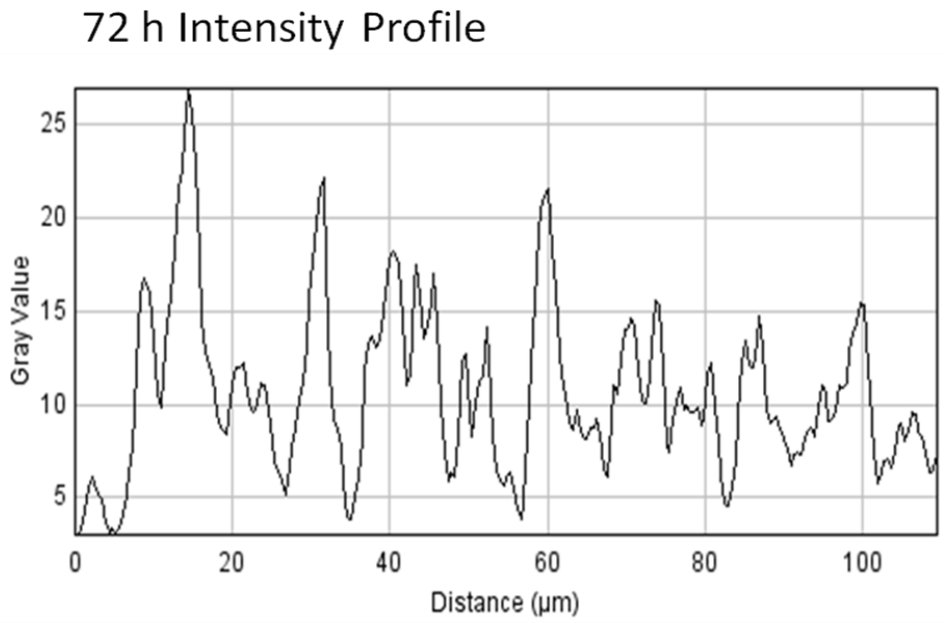


Figure 3.12 (C) Confocal Laser Scanning microscopic (CLSM) observations of biofilm at 72 h

a) 72 h biofilm

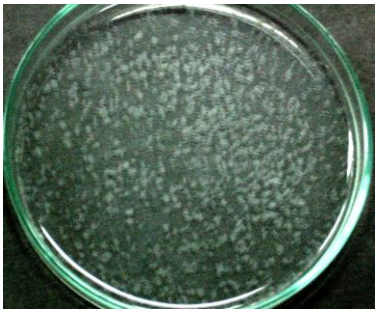


b)



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*, planktonik 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 enzymic 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

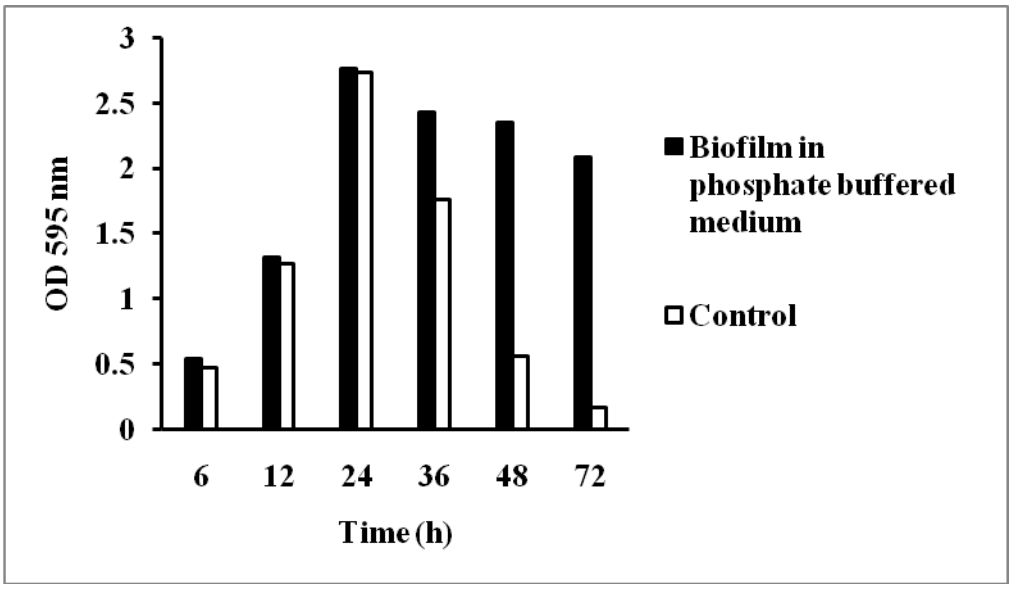
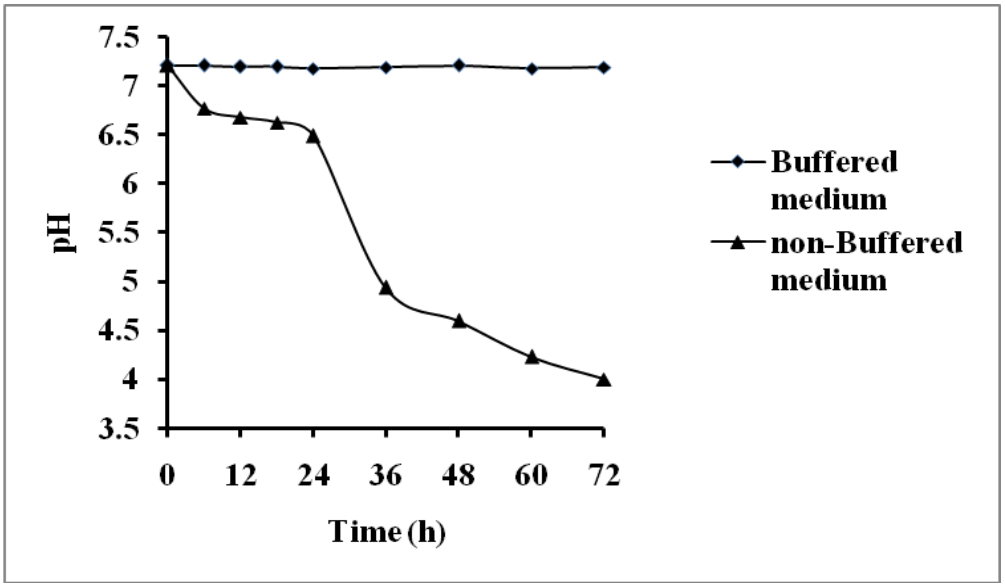


Figure 3.14 b) Kinetics of pH change



The culture was cultivated in the phosphate buffered (0.02 M, pH 7) and non buffered (Control- only K_2HPO_4 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

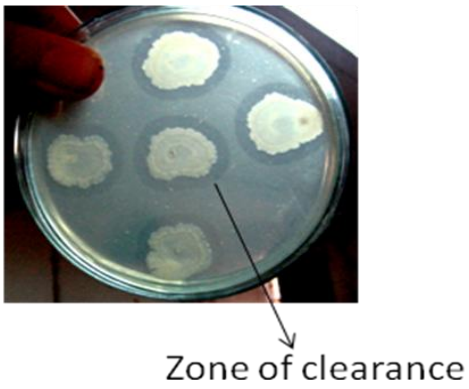
Extrinsic factors such as shear, sloughing, erosion (Rupp *et al*., 2005), change in nutrient (Sauer *et al*., 2004) and oxygen availability (Thormann *et al*., 2005) have been shown to influence the cellular detachment from biofilm. In *Pseudomonas aeruginosa*, production of rhamnoipids has been shown 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-hexosamidine/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 various 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 filterate 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

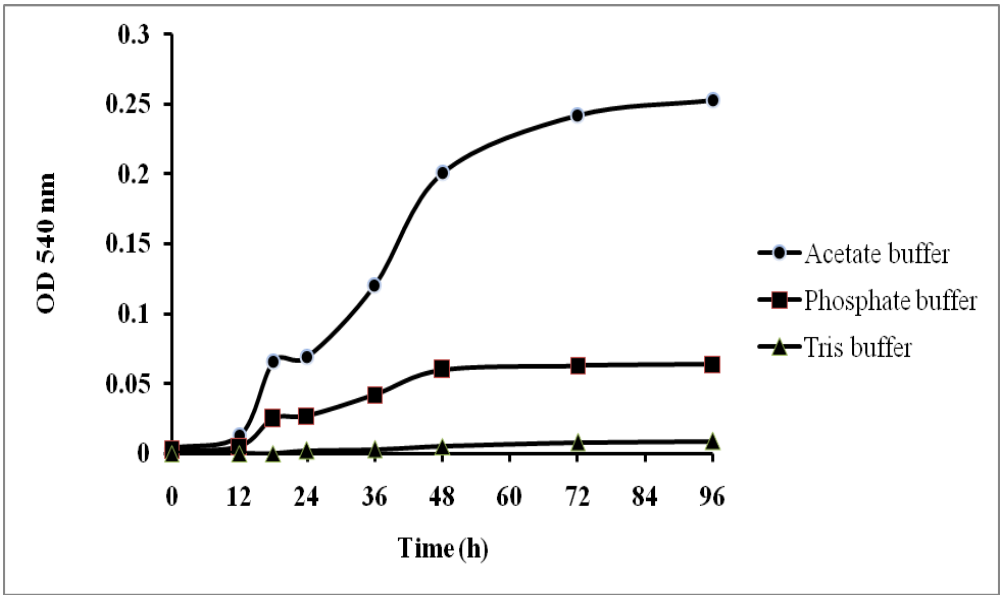


The organism was spot inoculated on a solid synthetic medium containing EPS (1 % w/v) as a sole source of carbon and energy. Plates were incubated at 30 ±1 °C for 72 h. Medium without EPS served as a control.

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.

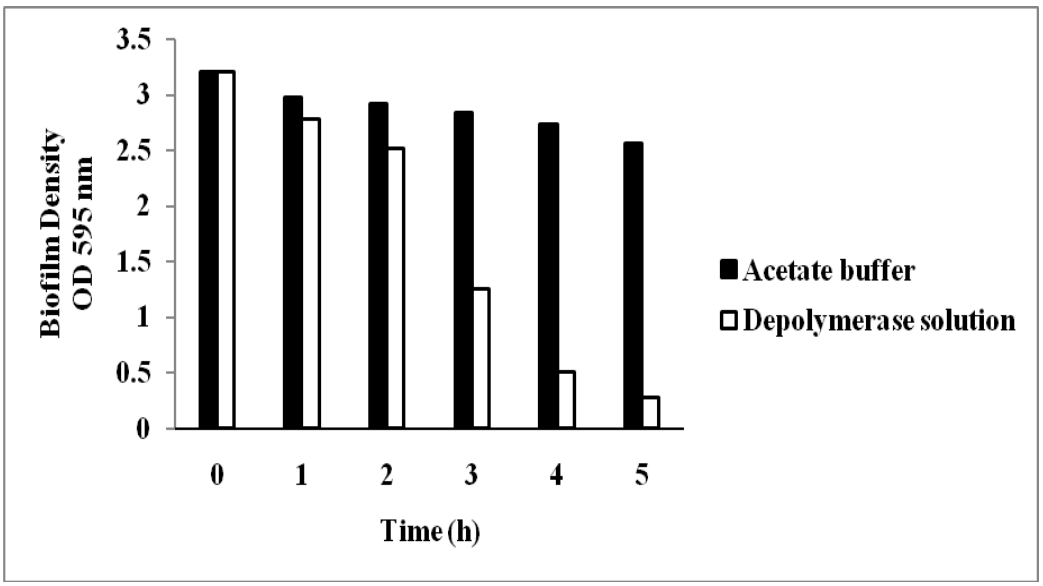
In *in-vitro* experiment time-course treatment of preformed biofilm of 24 h growth stage with concentrated (ammonium sulphate precipitated) EPS-depolymerase preparation showed decrease in biofilm due to detachment of cells and at the end of 5 h only little quantity of attached cells (8 %) left (**Figure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

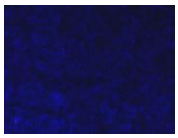
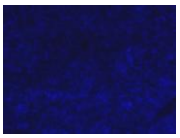
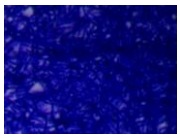
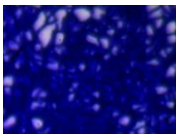
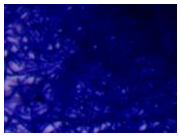
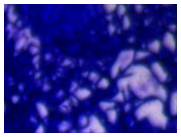
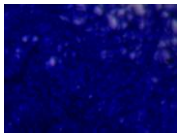
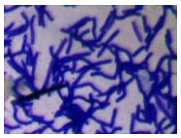
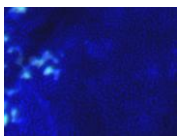
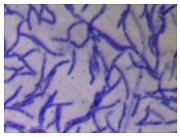
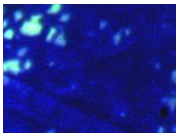
Figure 3.17 Kinetics of removal of preformed biofilm cells on treatment with EPS-depolymerase

(a) Biofilm density



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

Time of enzyme treatment (h)	Control	Experimental
0		
1		
2		
3		
4		
5		

Polysaccharide lyases have been found from same bacteria which produce polysaccharide substrate. *Pseudomonas aeuruginosa*, 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 carboxylayte 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 *Rhizobial* 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 actomycetemcomitans* and not in the above mentioned organisms. Hence detachment of biofilm cells by endogenously produced enzyme is only reported in *Actinobacillus actomycetemcomitans*.

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 (Bruinsma *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

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4.0 SUMMARY

4.1 By following (i) primary screening based on mucoidal nature of the colonies and (ii) secondary screening based on "Paper Peel Test" (BIS), eight bacterial isolates were selected out of sixty isolates and one of them designated here as ADE-0-1 from marine source showed the strongest adhesiveness and has been used in this studies.

4.2 An exopolysaccharide (EPS), recovered from culture filtrate by acetone precipitation, exhibited an adhesive nature and could glue variety of surfaces such as wood, glass, aluminium, iron, steel, tin, sunmika and acrylic plastic individually and in combination also.

4.3 Out of 28 combinations of specimens analysed, maximum lap shear strength observed was 5.46 ± 0.04 MPa in the case of wood-wood specimen at pH 7 and 30 °C (curing temperature). Among the metals, lap shear strength was found in the 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).

4.4 With the exception of steel, it was always noticed that when specimen was prepared using 2 metal surfaces, higher adhesive ability was observed than that prepared using one metal surface and one non-metal surface.

4.5 An adhesive (10 % w/v, solids) exhibited maximum lap shear strength of 6.12 ±0.03 MPa at pH 7 and 50 °C (curing temperature) for wood-wood specimen as compared to 6.54 MPa with fevicol (48-50 % w/v, solids), a commercial wood adhesive and was also found better than some of the reported bacterial bioadhesives.

4.6 Compared to temperature and salinity, increase in pH from 4 to 8 of the adhesive, improved shear strength significantly in the range of 30 to 72 % to that at pH 4 which indicated the role of carboxylate groups of uronic acid in adhesion process.

4.7 Cell biomass itself, grown for 48 h, when used as an adhesive, revealed maximum water resistant adhesive property under moist/water (immersion) condition.

4.8 Based on colorimetric analysis, EPS contained 75 % total carbohydrates, 17 % uronic acid and 0.00125 % pyruvate on w/w basis. Amino sugars, proteins and acetyl content were not detected.

4.9 Paper chromatographic and HPLC analysis of hydrolysate of EPS indicated presence of arabinose, glucose, mannose, galacturonic acid and glucuronic acid.

4.10 During FTIR analysis of EPS, an intense broad stretching peak at 3445 cm^{-1} and strong peak at 1638 cm^{-1} indicated presence of hydroxyl groups (characteristic of polysaccharide) and carboxyl groups (characteristic of uronic acid) respectively.

4.11 Based on the efficiency of binding to ion exchange resins, EPS was found anionic in nature.

4.12 Molecular weight of EPS, by gel permeation chromatography, was found 0.5 X 10^6 Da.

4.13 For improving the yield of the adhesive EPS, statistical method like "fractional factorial design" (Box-Behnken Design) was used for optimizing the medium composition.

4.14 Comparison of yield from un-optimized medium with those from "factorial design experiment" revealed no improvement in the product formation in the concentration range of nutrients tested. EPS and biomass average yield of 1.12 g/l and 0.82 g/l respectively were obtained.

4.15 Use of phosphate buffered medium (0.1 M) increased EPS yield to 11.85 g/l as compared to 1.12 g/l of control medium, due to the maintenance of pH and additional phosphate supplementation.

4.16 However phosphate content (phosphate contamination) in the harvested polymer was found in the range of 10 to 60 % and affected the adhesive property of the polymer.

4.17 Supplementation of only K_2HPO_4 also maintained the pH and increased the EPS yield in the range of 5.5 to 6.5 g/l with lesser degree of phosphate contamination than those of phosphate buffered media.

4.18 In conclusion, supplementation of K_2HPO_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.

4.19 Compared to more aerated condition under less aerated condition, more polymer production was obtained, while the biomass production didn't change significantly.

4.20 Based on morphological, cultural and biochemical characteristics studied and *16S rDNA* genes analysis, the isolate was identified as *Bacillus megaterium* ADE-0-1 (Gene Bank accession number-KF280264).

4.21 Biofilm forming ability, as judged by "crystal violet staining procedure", light microscopic, Scanning Electron Microscopic and Confocal Laser Scanning Microscopic observations of the organism, was found growth associated and declined during stationary phase.

4.22 "Cell-autoaggregation" ability and observations on "cell surface hydrophobicity" and "cell surface polysaccharide" (capsular polysaccharide) suggested their involvement in adhesion process and thereby "biofilm forming ability". While observations on "cell surface charge" showed reciprocal relationship to "biofilm forming ability".

4.23 Considerable decrease in pH of the broth as well as biofilm forming ability was observed after 24 h. However decrease in biofilm forming abilty could be prevented by maintenance of pH.

4.24 Compared to reports on (i) EPS and (ii) adhesive EPS producing organisms from biofilm forming bacteria, a novel finding regarding production of an enzyme "EPS-depolymerase" has been reported.

4.25 Extracellular EPS-depolymerase activity enabled *Bacillus megaterium* ADE-0-1 to utilize its own EPS/EPS(p) as a sole source of carbon for energy and phosphorous for its growth.

4.26 Majority of the EPS-depolymerase activity was found after 24 h growth period and maximum activity was observed at around pH 5.6.

4.27 The observations on 'EPS-depolymerase' in terms of (i) stage/time of production and its association with decrease in pH to 5.5 (favourable for enzyme activity) after growth phase and (ii) its ability to detach cells from preformed biofilms suggested clearly its physiological role in the detachment of cells from biofilm and indeed decrease in biofilm forming ability was observed after growth phase.

4.28 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. Such an enzyme could be exploited as a potential candidate for removal of biofilms.

SUMMARY OF THE THESIS ON

Characterization, production and application oriented studies on bioadhesin and biofilm of *Bacillus megaterium* ADE-0-1

SUBMITTED TO

THE MAHARAJA SAYAJIRAO UNIVERSITY OF BARODA

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IN

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By

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1.0 Introduction

Biofilms (sessile community) are more common than planktonic form (motile) in nature and virtually any surface—biotic or abiotic is suitable for bacterial colonization and biofilm formation. After initial physicochemical interactions between the organisms and the surface, attachment of adhering microorganisms is strengthened through microbial extracellular polymeric substances (EPS) and polymer bridging by the EPS leads to firm adhesion on the surface ultimately leading to scaffold structures of biofilm.

In majority of biofilms, the extracellular matrix has been reported to be exopolysaccharide (EPS) in nature. In contrast to two identical amino-acids which can only form one dipeptide, two identical sugars can bond to form 11 different disaccharides. **Compared to 25 different sugars found in plants and animals, more than 200 different sugars have been found in microbial EPS**. EPS can also be substituted, normally ester or N-linked, with pyruvate, acetate, formate, sulfate, phosphate and other side groups adding to their chemical heterogeneity and thereby to their functional diversity. Functions for this heterogeneity have been ascribed to pathogens such as O-antigen serotypes of enterobacteria, but not for environmental strains.

So far the biofilms and the bioadhesive molecules largely of medical and sanitary importance have been characterized to a considerable extent. However, many bioadhesin molecules (EPS) involved/associated with the biofilms of different/diverse environments, surfaces and ecological niches are still not well studied and characterized and particularly application oriented studies are scarcely reported.

2.0 Present study

Currently, large quantity of **adhesives** is used globally. In 2001, the United States used 2.5 billion Kg of adhesive. In 2007, the total world demand for adhesives and sealants was 12 billion Kg, of which natural adhesives (non-microbial origin) were 0.6 billion Kg. However, there are significant environmental issues like **toxicity and biodegradability**. Many are derived from **non-renewable petrochemicals** and 16

percent of adhesives include **toxic solvents** such as toluene, methyl - ethyl ketone and tri- chloro ethane in their compositions/formulations.

Bioadhesives produced by barnacles and mussels have been found with excellent adhesive property, particularly for difficult job of underwater adhesion. However, scale-up of the complex, multi-part system has proven to be cumbersome. A number of biopolymers from bacteria are commercially available for use as a viscosifying agents, emulsifiers, thickeners, stabilizers and gelling agents. However, none of these bacterial polymers are in commercial use as adhesive.

Looking to the possibility of extraordinary diversity just described in the case of exopolysaccharides coupled with the fact that only a few of bioadhesive molecules of environmental bacteria associated with the biofilms of this nature have been characterized, **there is an immense scope for discovery of new and unique bioadhesive molecules (EPS) with different properties and applications.**

Since microbial systems are far less complex than the higher life forms, and methods for producing microorganisms in large volume use standard technology, extracellular polymeric substances (EPS) from microorganisms of "biofilm origin" could be a potential source of echofriendly/biocompatible adhesive. Such EPS or chemically modified molecules can be exploited to develop bioadhesin molecules/materials as a product in a cost effective manner for specific applications such as surgical glue, orthopaedic applications, wood adhesive, underwater surface coatings, marine cements etc.

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.

Previously one/two factors affecting cell attachment and detachment have been studied in different organisms. However variety of physicochemical/physiological/biochemical parameters in relation with biofilm formation and growth and their correlation has not been studied.

3.0 SUMMARY OF THE WORK DONE

3.1 Study on bioadhesive

- By following (i) primary screening based on mucoidal nature of the colonies and (ii) secondary screening based on "Paper Peel Test" (BIS), eight bacterial isolates were selected out of sixty isolates and one of them designated here as ADE-0-1 from marine source showed the strongest adhesiveness and has been used in this study.

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