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 \(\times\) 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 K₂HPO⁴ 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 K₂HPO⁴ 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”.

104
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 ability 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

FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
MICROBIOLOGY

By
Santosh Kumar

DEPARTMENT OF MICROBIOLOGY AND BIOTECHNOLOGY CENTRE
FACULTY OF SCIENCE
THE M. S. UNIVERSITY OF BARODA
VADODARA- 390 002
October 2013
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 vicosifying 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 ecofriendly/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.

- 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.

- 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).

- 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.

- 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.

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

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.

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

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.

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

Molecular weight of EPS, by gel permeation chromatography, was found 0.5 X 10\(^{6}\) Da.

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

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.

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

Supplementation of only $\text{K}_2\text{HPO}_4$ 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.

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

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

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).

### 3.2 Study on biofilm

- “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”.

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

• 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.

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

• 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.

• 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.