CHAPTER 1

Studies on Biofilm Formation by *A. junii* BB1A

1.1. Introduction

In any microbial biofilm studies it is the primary requirement to use easy and reliable quantification techniques. Numerous methods have been used routinely in different laboratories for the cultivation and quantification of biofilms such as tissue culture plate (Christensen et al., 1982), tube method, congo red agar (Freeman et al., 1989), bioluminescent assay (Oliveira and Cunha, 2010), piezoelectric sensors (Aparna,2008), and fluorescent microscopic examination (Zufferey,1988). Each of these methods has several advantages or disadvantages, but no singly standardized protocol for assessment of biofilm formation by any bacterial species has been established so far.

1.1.1.Congo Red Agar (CRA) method

CRA method (Freeman et al., 1989) has been widely used for the screening of biofilm forming bacteria from diverse environments. The medium is composed of brain heart infusion agar supplemented with 0.8 g/l of Congo red. Congo red is known to have the ability to bind the extracellular matrix component, including polysaccharide (Romling et al., 1988). As a result biofilm positive strains are recognized as black colonies on the red solid medium; however, the assessment of variability in colony color may be difficult (Arciola et al., 2001; Mathur, 2006).

1.1.2. Tube method

Tube method is another phenotypic method used to study biofilm formation. It involves visual examination of biofilm formation in tubes after staining with crystal violet. Both tube method and CRA method has been extensively used as a screening method for biofilm forming bacteria (Rossi et al., 2007). However, the use of these tests for characterization of biofilm formation has led to conflicting results by different investigators (Knobloch et al., 2002).

1.1.3.Tissue culture plate (TCP) method

TCP method devised by Christensen et al. (1982) is one of the most widely used methods to identify biofilm producing strains and biofilm quantification.TCP also known as microtitre plate assay involves

growing the culture in 96 well microtiter plate and measuring the optical density of the formed biofilm. The biofilm grown in microtitre plate can be analyzed and quantified in different ways such as staining with fluorogenic dye Syto 9: a nucleic acid stain (Honraet et al., 2005; Honraet and Nelis, 2006), tetrazolium salts 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (Berit et al., 2002; Pettit et al., 2005;Honraet et al., 2005), fluorescein diacetate (Honraet et al., 2005; Prieto et al., 2004) and crystal violet (CV) staining (Christensen et al. 1985). CV staining for quantification of biofilm is the most widely used method. CV is a basic dye which binds to the negatively charged molecules, including cell surfaces and EPS (Li et al., 2003). By staining with crystal violet, rinsing and consequently solubilizing the bound dye in ethanol, the biofilm can be semi-quantitatively measured using a spectrophotometer. Merrit et al. (2005) has described a good correlation between crystal violet readings and viable counts.

Table 1.1 Typical conditions for developing and performing microtiter plate biofilm assay/TCP assay.

Organism	**Incubation temperature (°C)**	**Solvent for solubilization of stained biofilms**	**Reference**
Pseudomonas fluorescens	30	95% ethanol	O'Toole and Kolter 1998
Escherichia coli	25	80% ethanol/20% acetone	O'Toole et al., 1999
Pseudomonas aeruginosa	25-37	30% acetic acid	Zegans et al., 2009
Staphylococcus aureus	37	33% glacial acetic acid	Stepanovic et al., 2001

1.1.4. Environmental factors affecting biofilm formation

Bacteria are thought to form biofilm when they sense environmental conditions that activate the conversion of free floating planktonic form to surface attached biofilm growth (Watnick, 1999; Stanley, 1983; Wang, 1996; Pratt 1998; Palmer, 1997; O'Toole, 1998; O'Toole, 2000; Fletcher, 1986). These environmental conditions can vary, depending upon the type of bacteria and include nature of the attachment surface (Verran and Boyd, 2001), nutrient availability (Allan et al., 2002; Ebrey et al., 2004; Huang et al., 1994) etc. The environmental factors may vary among organisms but in many cases it is still not clear exactly how these factors interact, or which factors governs the biofilm formation in particular bacterial genera.

1.1.4.1. *Surface*

It is the natural affinity of microorganisms to adhere to surface as a survival mechanism and bacterial colonization of surfaces has been described as a crucial and natural strategy in diverse ecosystem (Donlan, 2002; Dunne, 2002). The surface could be a dead or living tissue, or any inert surface. The microbial attachment to the surface is a complex process, affected by many variables (Pratt, 1998). The attachment surface may have several characteristics that are significantly affects the attachment process. Microbial colonization appears to increase as the surface roughness increases (Characklis et al., 1990). Other characteristics of substrata such as hydrophobicity and the non polar nature of plastic and teflon was shown to promote bacterial adherence as compared to hydrophilic metals (Fletcher and Loeb, 1979; Pringle and Fletcher, 1983; Bendinger, 1993). Prior to attachment to a surface, conditioning takes place with the development of organic film on the surface. This conditioning may alter the physico-chemical properties of substrata and thus affecting the rate and extent of microbial attachment (Loeb and Neihof, 1975; Kumar and Anand, 1998).

1.1.4.2. *Cell surface hydrophobicity*

Cell surface hydrophobicity is one of the physico-chemical factors that contribute to the process of attachment of microbial cell to solid surfaces in natural environment (Marshall and Cruickshank, 1973; Busscher et al., 1990). It is an important factor which determines the electrostatic interaction between the cell and the substratum (van Loosdrecht et al., 1990). Most bacteria are negatively charged and contain hydrophobic surface components like fimbriae (Rosenberg and Kjelleberg, 1986). These hydrophobic components may play essential role in the interaction with the substrata (Busscher and Weerkamp, 1987). Rosenberg (1986, 1990) revealed that the cell surface hydrophobicity is a major determinant of bacterial cell adherence to the wide variety of surfaces. Many investigators have found that microorganisms attach more rapidly to hydrophobic and non-polar surfaces than hydrophilic surfaces (Donlan, 2002; Flemming and Wingender, 2001). Increase in surface hydrophobicty has been shown to increase the adhesion of vegetative cells (Sinde and Carballo, 2000) and freshwater bacteria (Pringle and Fletcher, 1983).

Cell surface hydrophobicity of the bacteria can be measured by several methods, such as contact angle measurement (Absolom et al., 1983; Busscher et al., 1984), hydrophobic interaction chromatography (Pedersen, 1981; Stenström, 1989), and microbial-adhesion-to-hydrocarbon (MATH) testing (Rosenberg et al., 1980). The selection of appropriate test depends on the purpose of

measurement since the results of these tests are applicable only for particular populations of microbial strains (Dillon et al., 1986; Rosenberg et al., 1980; Van der Mei et al., 1987).

1.1.4.3. *Nutrients*

Nutrient levels are another environmental factor which has profound effect on biofilm formation and known to regulate the depth of biofilm (Stanley and Lazazzera, 2004). Moreover the structure and amount of biofilm production has also been related to the nutrient availability (Wimpenny and Colasanti, 1997). Biofilms can be formed under varying nutrient levels, ranging from nutrient rich to nutrient poor (Prakash et al., 2003). In nutrient rich environment, biofilms are more profuse, densely packed and thicker (Allison et al., 2000; Prakash et al., 2003; Roche and Lebeault, 2007). O'Toole et al. (2000) has revealed that the high concentration of nutrient supports the transition of bacterial cells from the planktonic to biofilm state. Many investigators have shown that the increase in nutrient level is associated with an increase in the number of attached bacterial cells (Cown et al., 1991; Dunne, 2002; Prakash et al., 2003). However, some of the research shows the formation of biofilms under low nutrient concentration (Hsueh et al., 2006; Rice et al., 2005).

1.1.4.4. *Characteristics of growth medium*

The physico-chemical properties of growth medium may have a profound effect on the process of bacterial cell such as attachment to the surface. These factors include the type of growth medium, presence of different electrolyte species (type and concentration of cations), pH (McEldowney and Fletcher, 1986; Stanley, 1983), temperature (Fletcher, 1977; Harber et al., 1983) and ionic strength of the medium. Studies on the biofilm formation by some *Escherichia coli* and *Klebsiella pneumoniae* isolates have shown that the growth medium has a significant effect on the biofilm formation (Hancock et al., 2011). In a study, Fletcher (1988) found that an increase in the concentration of several cations (sodium, calcium, lanthanum, ferric iron) affected the adhesion of *Pseudomonas fluorescens* to glass surfaces, most probably by neutralizing the negative charge on cell surface and thus reducing the repulsive forces between the bacterial cells and the glass surfaces. The effect of pH levels of the suspending medium has received comparatively little attention. Although the details of the environmental signals triggering biofilm formation may vary from organism to organism, it is now clear that the environmental parameters have a profound impact on the transition between planktonic and biofilm growth.

1.2. Materials and Methods

1.2.1. Reagents and Chemicals

All the chemicals and reagents used in the experiment were of analytical grade (AR) and purchased from E. Merck, India. The growth media or its components used were purchased from HiMedia, India.

1.2.2. Maintenance of bacterial strain

For short-term preservation and routine use, stock bacterial culture of *Acinetobacter junii* BB1A was maintained on Luria-Bertani(LB) agar slant containing peptone,10 g/l; yeast extract, 5 g/l; NaCl, 10 g/l; and agar 15g/l, at pH 7.0. LB agar medium was sterilized by autoclaving at 121 °C for 20 minutes and 4 ml was transferred into the test tubes to form LB agar slant. A loopful of bacterial cultures from the stock samples was streaked on LB agar slant and incubated at 30 °C for 24 h. The culture was stored refrigerated at 4 °C. For long-term preservation culture in 10-15% glycerol was stored at -20 °C and sub-cultured after every 3 months.

1.2.3. Inoculum preparation

The strain BB1A was sub cultured into LB broth (peptone,10 g/l; yeast extract, 5 g/l; and NaCl, 10 g/l; pH 7.0),or Brain Heart Infusion (BHI) (calf brain infusion from, 200 g/ l; beef heart infusion from, 250 g/ l; proteose peptone, 10 g/ l; glucose, 2 g/ l; NaCl, 5 g/ l; and Na_2HPO_4, 2.5 g/ l; pH 7.4 ± 0.2) or Trypticase soy broth (TSB) (pancreatic digest of casein, 17 g/l; papaic digest of soy, 3 g/l; NaCl, 5 g/l; K_2HPO_4, 2.5 g/l; glucose, 2.5 g/l; pH 7.2 ± 0.2). The culture broth was incubated aerobically at 30 °C for 24 h under static condition. The bacterial growth was measured by taking absorbance at 600 nm and was adjusted so as to give $O.D_{600}$ value of 0.30. This was then used as inoculum for further experiments.

1.2.4. Standardization of protocol for biofilm assay

1.2.4.1.Tissue culture plate method (TCP)

The TCP assay described by Christensen et al. (1982) is the most widely used method and was considered as standard test for detection of biofilm formation. For the experiment 50 ml LB broth in a 250 ml Erlenmeyer flask was inoculated with 2% (v/v) overnight grown culture of BB1A. 0.2 ml aliquot from this was then used to fill individual wells of sterile 96 well flat-bottom polystyrene tissue culture plates (Tarson, Kolkata, India) and only broth served as control to check sterility and non-specific binding of media. The tissue culture plates were incubated for 48 h at 30 °C without shaking. After incubation, content of each well was gently removed by inverting the plates over tissue paper. The wells

were then washed two times with 0.2 ml of phosphate buffer saline (NaCl, 8 g/l; KCl, 0.2 g/l; Na_2HPO_4, 1.44 g/l; KH_2PO_4, 0.24 g/l; pH 7.4±0.2) to remove free-floating 'planktonic' bacteria. Biofilm formed by the adherent 'sessile' organisms in plate were fixed and stained with crystal violet. Excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying. The stain was then solubilized and optical density (OD) of the resulting solution of each well was measured at 540 nm (OD_{540}) using an automated microtitre plate reader (MERCK MIOS junior, USA). These OD values were considered as an index of bacteria adhering to surface and forming biofilms. Experiment was performed in triplicate with different conditions of staining, fixing and de-colorization.

Staining of biofilm was done with 0.2 ml of 0.1% or 0.05% CV for 1 min. For fixation of biofilm in the plates, three protocols were compared. In first trial, cells were heat fixed at 80 °C for 30 min, in 2nd case, wells were flooded with 2% sodium acetate (Mathur, 2006) for 15 min and in the third case methanol (Stepanovic et al., 2001) was employed for 5 min. Stain solubilization was done with 95% ethanol or 33% glacial acetic acid. To compensate for background absorbance, the mean OD value obtained from media control well was deducted from all the test OD values. The experiment was repeated thrice and the optimized protocol was further used for biofilm assay.

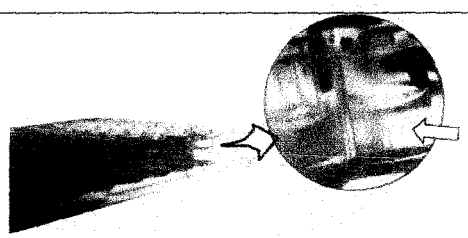
1.2.4.2. *Tube method*

As described by Christensen et al., 1982, this is a qualitative method for biofilm detection. A loopful of test organisms was inoculated in a test tube containing 10 ml of LB broth. The tubes were incubated at 30 °C for 48 h. After incubation, tubes were decanted and washed with phosphate buffer saline (pH 7.3) and dried. Tubes were then stained with crystal violet (0.1%). Excess stain was washed with deionized water. Tubes were dried in inverted position. Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube.

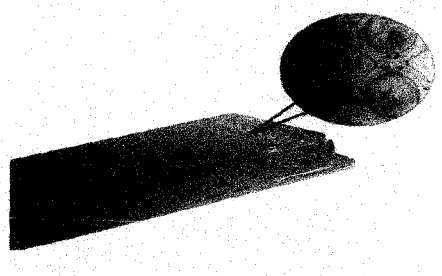
1.2.4.3.*Congo Red Agar method*

Freeman et al. (1989) has described a simple qualitative method to detect biofilm producing strains, using Congo Red Agar (CRA) medium. CRA medium was prepared with brain heart infusion 37 g/l, sucrose 20 g/l, Congo red indicator 0.8 g/l and agar 15 g/l. Congo red stain was prepared as a concentrated aqueous solution and autoclaved (121 °C for 15 min) separately from the other medium constituents. Then it was added to the autoclaved brain heart infusion agar. CRA plates were inoculated with test organisms and incubated at 30 °C for 24 h aerobically. After incubation the colony characteristics was recorded.

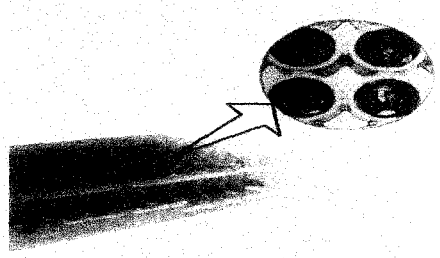
Bacteria cultured in 96 well tissue culture plate (flat bottom). Wells showing clear supernatant due to adherence of cells to the bottom.



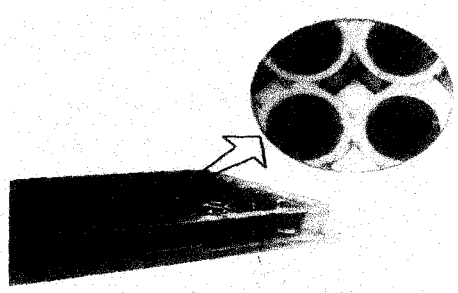
Biofilm sticked to the bottom of well (Biofilm after washing and fixing).



Biofim stained with crystal violet.



Solubilization of stain



Measurement of OD at 540 nm using Elisa reader.

Fig. 1.1: Biofilm assay procedure using TCP method

1.2.5. Determination of cell surface hydrophobicity.

The surface hydrophobicity of the bacterial cells was examined by the Microbial Adhesion to Hydrocarbon (MATH) assay (Geertsema-Doornbusch et al., 1993). Culture broth from different hours (6, 24, 48, 72 and 96 h) of incubation at 30 °C, was harvested by centrifugation (2000 x *g*, 15 min, 4 °C), washed twice in phosphate buffer saline (PBS) and finally suspended in the same buffer. The initial absorbance (A_0) at 600 nm of the suspension was adjusted to 0.40 ±0.02 units. 5 ml of cell suspension in PBS buffer was dispensed in clean and dry round bottom test tubes followed by addition of 5 ml of n-hexadecane. The content was vortexed for 2 min. The tubes were then left undisturbed for 15-20 min at room temperature to allow the phase separation. The lower aqueous phase was carefully removed with a sterile Pasteur pipette and absorbance (A_1) was recorded at 600 nm. Cell surface hydrophobicity in terms of per cent was calculated using the following formula:

$$(\%)\ \text{Hydrophobicity} = (1 - A_1/A_0) \times 100$$

1.2.6.Influence of environmental factors on biofilm formation by *A. junii* BB1A

1.2.6.1. *Influence of growth media*

To compare the effect of nutrients availability on biofilm formation, *A. junii BB1A* was grown in three different media i.e TSB, LB, and BHI. 0.2 ml of each media in 96 well microtiter was inoculated with 2% of the overnight culture grown in the respective media and incubated at 30 °C for different time intervals (24, 48 and 72 h) as it considerably influences the amount of biofilm produced and highest density of cells in biofilm reach at optimum incubation period. The amount of biofilm formed was determined by optimized TCP method.

For testing the effect of nutrient starvation, the media (LB, TSB and BHI) was diluted to 1:1, 1:10 and 1:20 times in distilled water to make a final volume of 10 ml each and sterilized. Each dilution as well as control (without dilution) was inoculated with 2% overnight grown culture. Prior to inoculation, cell pellets were harvested from 10 ml overnight grown culture. The cell pellets were washed twice with PBS buffer (pH 7.4±0.2) and re-suspended in 10 ml of the same buffer; this was then used as inoculum. The experiment was done in 96 well microtitre plate using 0.2 ml inoculated media in each well and in two sets. After incubation at 30 °C for 48 h, one set was used for biofilm quantification using optimized TCP method as described previously and the other set for estimation of growth by measuring optical density at 600 nm (OD_{600}).

1.2.6.2. ***Influence of substrata (abiotic surfaces)***

10 ml of LB broth in borosilicate (15x125 mm), polystyrene (12x75 mm) or polypropylene (12x75 mm) sterile Petri-dish was inoculated with 2% of an overnight grown culture. Each Petri-dish containing media was inoculated in duplicates and were incubated for 48 h at 30 °C. After 48 h, plates were compared for the percentage of cells adhered to the plate. This was measured by the method described by Sarkar and Chakraborty (2008). To determine the % adherence, absorbance of the attached cells and absorbance of the complete content (total biomass) of the plates were determined. For attached cells, the medium from each plate was discarded without disturbing the attached cell and then 10 ml of fresh LB broth added followed by vigorous shaking and repeated aspiration up and down using a micropipette. For total biomass, complete content from one set of plate was withdrawn and homogenized by vigorous shaking. The optical density was then measured at 600 nm (OD_{600}). Percentage adherence was measured using the formula:

% Adherence= (attached cell biomass)/ (total biomass) x 100

1.2.6.3. ***Influence of incubation temperature***

The ability of *A. junii* BB1A to form biofilm at different temperatures (20, 25, 30, 35 and 40 °C) was studied in 96 well microtitre plate containing 0.2 ml of LB media/ well. After incubation for different hours, the biofilm was quantified through the optimized TCP method.

1.2.6.4. ***Influence of carbon source***

Cells were grown in LB, LB+ 0.05M glucose, LB+0.05M sucrose, LB+0.05M lactose, LB +0.05M galactose, LB+0.05M arabinose and LB+0.05M rhamnose. The biofilm formed in polystyrene microtitre plates was quantified as described earlier.

1.2.6.5. ***Influence of pH***

The effect of pH was investigated by allowing biofilm formation in LB broth, adjusted before the autoclaving to pH values of 4-10 with buffers. pH was determined after autoclaving to ensure that the values were maintained. The experiment was done in microtitre plate and biofilm was quantified after 48 h of incubation at 30 °C with optimized TCP method.

1.2.6.6. ***Influence of ionic strength***

In order to determine the effect of ionic strength on the biofilm formation, *A. junii* BB1A was grown in LB broth supplemented with different concentration of NaCl (1 to 4% NaCl). The experiment was done

in 96 well polystyrene microtitre plate. Biofilm formation was quantified after 48 h of incubation at 30 °C.

1.2.6.7. *Influence of static and dynamic condition*

Influence of static and dynamic condition on the biofilm formation was evaluated by incubating the inoculated media in microtitre plates with or without shaking (100 rpm on horizontal shaker). 10 ml LB broth was inoculated with 2% of overnight grown culture. From this 0.2 ml, was dispensed in each well of microtitre plate. One set of microtitre plate was kept at 100 rpm on horizontal shaker at 30 °C and the other set was kept without shaking. After incubation period of 48 h, quantitative analysis of biofilm was done by the optimized protocol.

1.3. Results

1.3.1. Standardization of protocol for studying biofilm

1.3.1.1. *TCP method*

Fixation: Comparative fixation of adhered cell on microtitre plate by sodium actetae, methanol and by application of heat revealed that, fixing with either heat or methanol gives the same result with more absorbance at 540 nm. Fixation with sodium acetate resulted in lower $O.D_{540}$ values.

Staining: It was observed that staining with 0.1% crystal violet yielded better result in comparison to staining with 0.05% crystal violet for 1 min.

De-colorization: De-colorization of the stained biofilm with ethanol or glacial acetic acid revealed that glacial acetic was the better de-colorizing agent since it completely decolorized the stained biofilm while ethanol resulted in incomplete decolorization.

Thus TCP method using methanol fixation, 0.1% CV staining and 33% glacial acetic acid as de-colorizing agent was found to be effective for quantification of biofilm formed by *A. junii* BB1A. The optimized parameters were followed in other experiments.

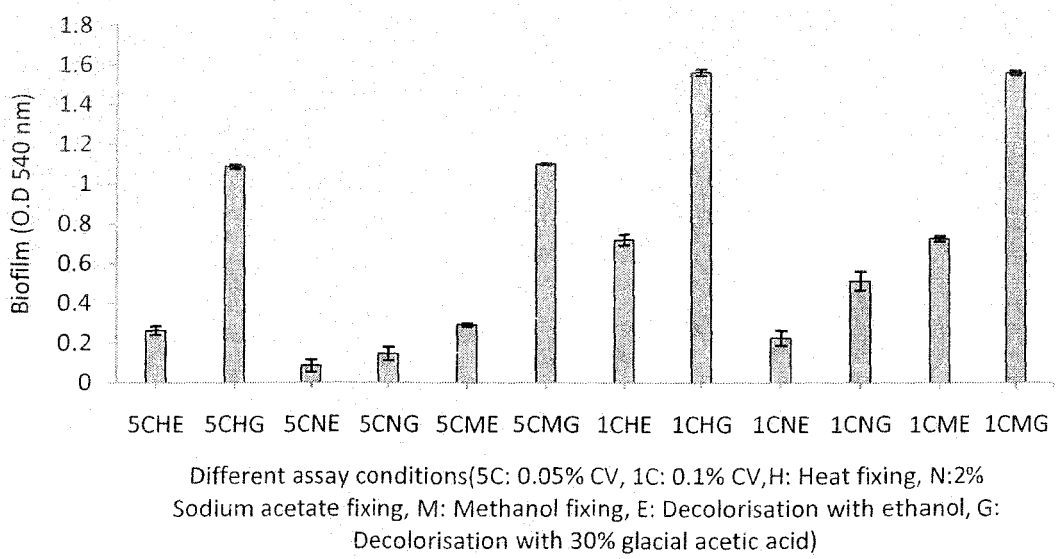


Fig.1.2: Quantification of biofilm by TCP method: comparison of different assay conditions such as fixing, staining and solubilization.

1.3.1.2. *Tube Method*

In tube method, biofilm was formed at the bottom of the glass tube. Large portion of the biofilm get detached during the crystal violet staining and it gets poorly stained (Fig. 1.3).

Fig. 1.3: Biofilm detection by Tube method.

1.3.1.3. *Congo Red Agar method*

In CRA method the colonies of *A. junii* BB1A displayed dark red colored colony without dry crystalline morphology.

1.3.2. *Cell surface hydrophobicity*

The hydrophobicity of the bacterial cell surfaces was determined by measuring the percentage of adhesion to n-hexadecane. It was found that the cell surface hydophobicity of *A. junii* cell increases with the growth phase. They are least hydrophobic (42%) during 12 h growth while the hydrophobicity increases to 93% after 24 h and remains constant upto 96 h of growth (Fig. 1.4).

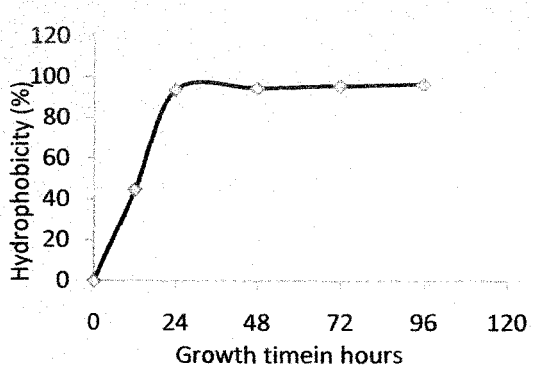


Fig. 1.4: Cell surface hydrophobicity of *A. junii* BB1A measured after different growth periods.

1.3.3. Influence of environmental factors on biofilm formation by *A. junii* BB1A

1.3.3.1. *Influence of growth media*

The biofilm formation by *A. junii* BB1A in three different media namely LB, TSB and BHI was compared. As revealed from the figure 1.5a, the amount of biofim formed in the three growth media increased in the following order: BHI ($O.D_{540}$ of 2.31±0.1)>TSB ($O.D_{540}$ of 1.83±0.05)>LB ($O.D_{540}$ of 1.56±0.01). Growth of the culture was also found to be in the similar order i.e., BHI ($O.D_{600}$ of 0.68±0.03)> TSB ($O.D_{540}$ of 0.58±0.02)> LB ($O.D_{540}$ of 0.44±0.02). Dilution of the growth medium resulted in a decrease in both growth and biofilm formation as shown in the figure. 1.5b.

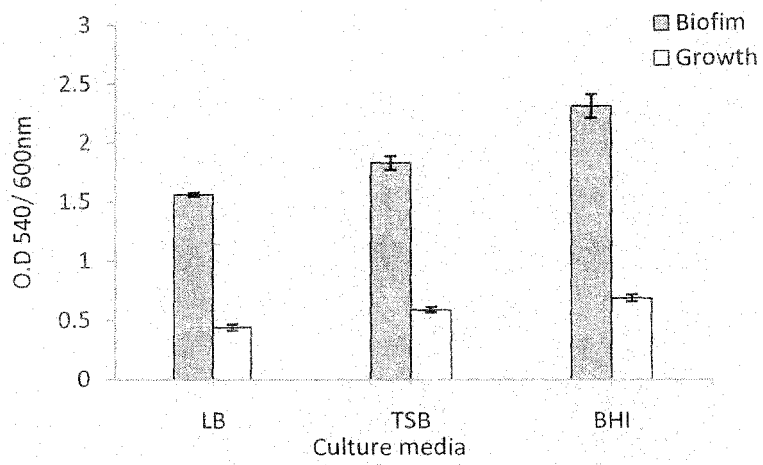


Fig. 1.5: a) Effect of different media on growth and biofilm formation.

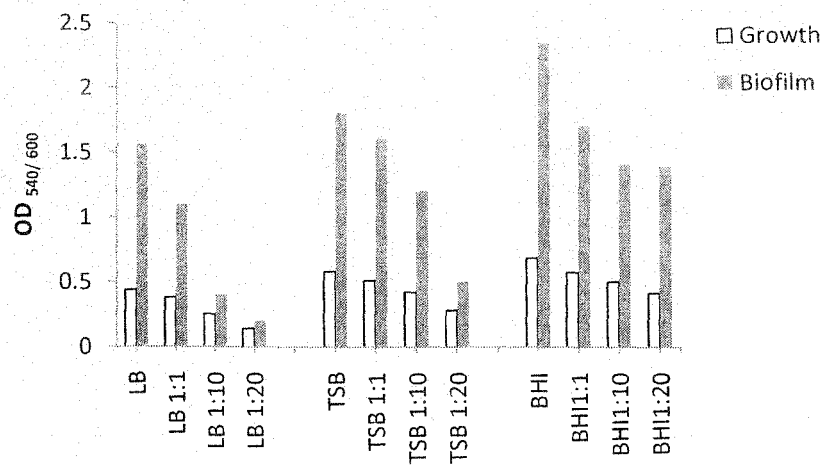


Fig. 1.5: b) Effect of media dilution on growth and biofilm formation.

1.3.3.2. *Influence of substrata (abiotic surfaces)*

Biofilm formation by *A. junii* BB1A in LB medium was studied in three different Petri-dishes made of borosilicate glass or polystyrene or polypropylene. Quantitative analysis showed that the amount of *A. junii* BB1A biofilm formed on polystyrene and polypropylene are quite similar however they differ significantly with the biofilm formed on borosilicate glass (Fig.1.6). The percentage of attached cells were higher on polystyrene (% adherence= 80.9) and polypropylene (% adherence = 81.5) surface while least adherence was observed on glass surface (% adherence =72.2).

1.3.3.3. *Effect of incubation temperature on biofilm formation*

Biofilm formation and growth of *A. junii* BB1A was tested at various growth temperatures (20, 25, 30, 35 and 40 °C). The mean quantity of growth as well as biofilm formed at 30 °C after 48h were higher than those formed at 20, 25 35 and 40 °C (Fig.1.7).

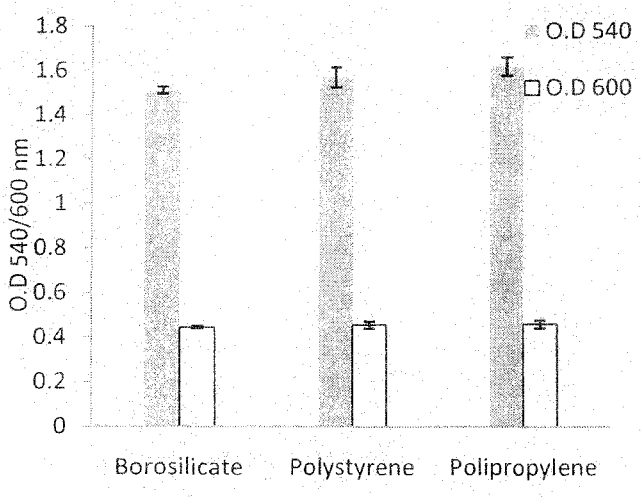


Fig. 1.6: Biofilm formation on different abiotic surfaces.

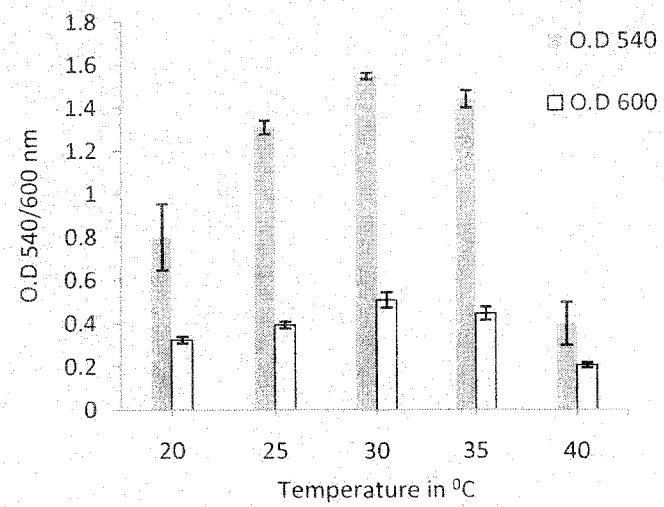


Fig. 1.7: Biofilm formation at different temperatures in LB media.

1.3.3.4. *Effect of pH*

The growth at pH 7 and 8 shows no marked differences, however the amount of biofilm was highest at pH 8 (Fig. 1.8). No growth or biofilm formed at pH below 6 and pH above 9.

1.3.3.5. *Effect of carbon source*

Effect of carbon source on the ability of *A. junii* BB1A to form biofilm on polystyrene surface was investigated at the optimum growth temperature (Fig. 1.9). It was found that quantitatively more biofilm formation occurred when cells were grown in LB media supplemented with sucrose ($O.D_{540}$= 2.3±0.15)

and reached a maximum ($O.D_{540}$= 2.53±0.15) when supplemented with glucose. Thus, the amount of biofilm formation in *A. junii* BB1A varied with the nature of carbon source.

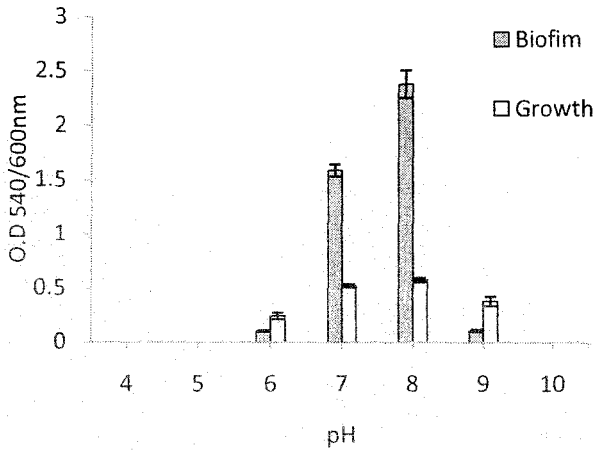


Fig. 1.8: Biofilm formation at different pH of the medium.

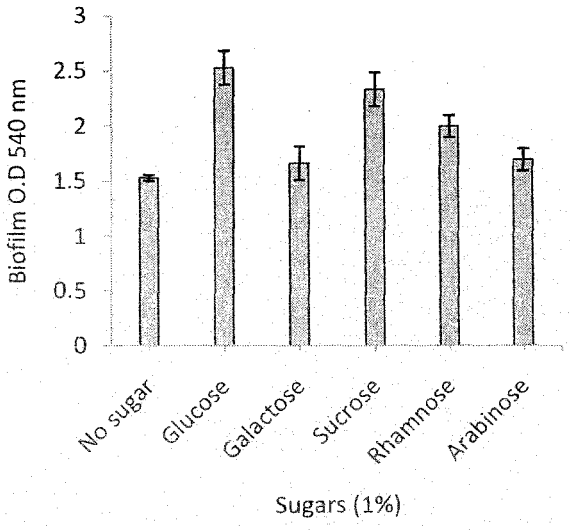


Fig. 1.9: Biofilm formation in LB broth supplemented with different sugars.

1.3.3.6. *Effect of ionic strength*

The growth and biofilm formation of *A. junii* BB1A was determined in LB media supplemented with different concentrations of NaCl (1-4% w/v) during 48 h of incubation at 30 °C. It was observed that the amount of biofilm was increased in osmotic stress condition (Fig.1.10).The effect of increasing ionic strength on the biofilm formation was strongest between 3 and 3.5% NaCl on polystyrene surfaces. The growth of *A. junii* BB1A only increased up to 1.5% NaCl and further decreased with increasing salt concentration.

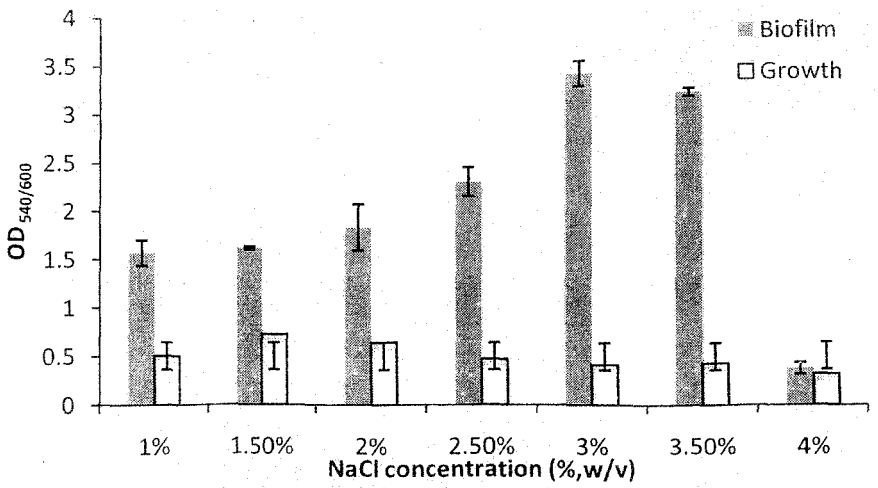


Fig. 1.10: Biofilm formation in LB broth supplemented with different concentration of NaCl.

1.3.3.7. *Effect of dynamic condition*

The biofilm production under static and agitation was comparable (Fig.1.11). The dynamic condition significantly inhibited the biofilm formation whereas; static condition favored the biofilm formation reaching maximum at 120 h of incubation at 30 °C.

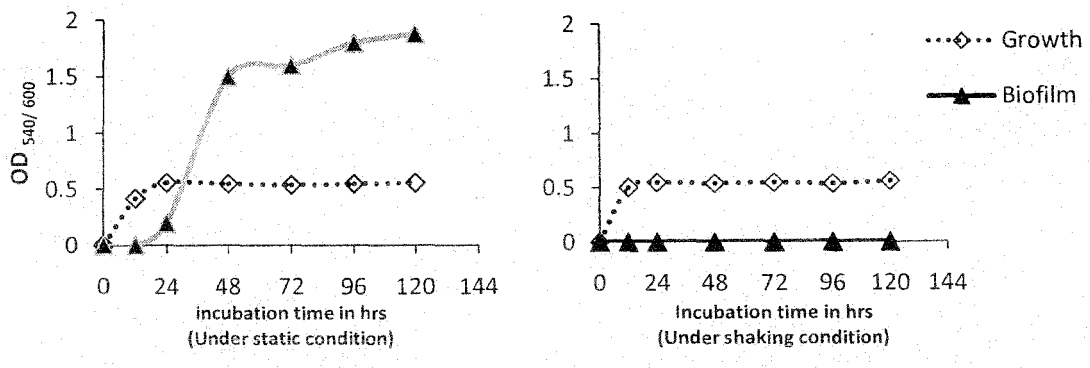


Fig. 1.11: Growth and biofilm formation under static or shaking condition.

1.4. Discussion

A variety of methods have been developed and used for the evaluation of bacterial biofilm formation. The most commonly used method is the microtiter plate system with colorimetric assessment. Microtiter plate-based systems are simple and inexpensive yet permit evaluation of the effects of multiple factors on biofilm formation. Hence, in the present study microtiter plate-based method most commonly known as TCP or crystal violet assay (CV) was standardized to assess the biofilm formation abilities of *A. junni* BB1A.Crystal violet is most widely used for the staining of biofilm cells and its different concentrations (0.1%, 0.5% and 1%) were used by various workers (Pour et al., 2011; Marti et al., 2011; de Breij et al., 2010). In this study two different concentrations of crystal violet stain (0.1% and 0.05%) were compared for staining of biofilm. Staining with 0.1% revealed better results in comparison to staining with 0.05% crystal violet for 1 min. Prior to staining, fixation of biofilm is sometimes required, this is often helpful in case of weakly adhered biofilms. In the present study fixation of biofilm was compared by treating biofilm with heating or treatment with sodium acetate or with methanol. Genevaux et al. (1996) used heating temperature of 80 °C for fixation of adhered cells. The fixation of attached biofilm by exposing them at 80 °C for 30 min revealed similar result as that with the fixation by methanol however methanol fixation takes lesser time. The fixation by 2% sodium acetate was proved to inadequate as it resulted in the loss of biofilm during subsequent washing. Solubilization of the dye was compared by treating the crystal violet stained biofilm with 95% ethanol or 33% glacial acetic acid. The solublization by 33% glacial acetic was found to be comparatively better than by 95% ethanol. It was found that the glacial acetic acid can completely solubilize the dye.

The process of biofilm formation by microorganisms is influenced by various environmental factors such as nutrients level, pH, temperature, ionic strength, solid surface etc. Interactions between bacterial cells and solid surfaces are different for adhesion onto hydrophobic or hydrophilic surfaces (Sommer et al., 1999). The adherence of *Acinetobacter* to various abiotic surfaces has been studied by various investigators (Tomaras et al., 2003; Brossard and Campagnari, 2011; Costa et al., 2006). These studies are mostly limited to the medically important *Acinetobacter baumanii* strain. Only few studies have carried out on other species particularly the environmental isolates such as *A. junii*. In this study, plastic surface such as polystyrene and polypropylene was found to be more efficient for bacterial attachment. The suitability of plastic surfaces for bacterial attachment is due to its hydrophobic non-polar nature, while other materials like glass, stainless steel, mica are known to be hydrophilic (Sinde and Carballo, 2000; Djordjevic et al., 2002). It is obvious that the bacteria with hydrophobic cell surface colonies prefer plastic materials more than the hydrophilic bacteria. Previous studies have already shown

the correlation between affinity of bacteria to polystyrene and surface hydrophobicity (Rosenberg, 1981). The hydrophobic nature of *A. junii* BB1A was established by the MATHS test which shows the ability of *A. junii* BB1A cells to adhere to hydrocarbon. Hrenovic et al. (2011) has projected the role of deoxy sugars, amino acids and branched polymers in the hydrophobicity *A. junii*. According to Pompilio et al. (2008) hydrophobicity is one of the determinant of adhesion and biofilm formation on polystyrene surfaces.

Availability of nutrient is one of the most common factors that can influence biofilm formation (Ghannoum and O'Toole, 2004). Among the three growth media (LB, TSB, and BHI) selected for the comparison, TSB and BHI supported maximum growth as well as biofilm formation. Diluted medium was found to be least effective in promoting biofilm formation. Similar effect where higher nutrient concentration favored the biofilm formation has been reported in some bacteria such as *Listeria monocytogenes* (Stepanovic et al., 2004) *Elizabethkingia meningoseptica* (Jacobs and Chenia, 2011) and in *Hafnia alvei* (Vivas et al., 2008). TSB and BHI are considered as nutrient rich media and most commonly used for biofilm formation among various bacterial genera including *Acinetobacter* (Can et al., 2009; Hood et al., 2010; Sechi et al., 2004). BHI and TSB contained large quantities of proteins and sugars (BHI contains 460 g/l of protein and 2 g/l of dextrose; TSB contains 23 g/l of protein and 2.5 g/l of dextrose). The nutrient-limiting LB medium contains protein (10 g/l) and yeast extract (5 g/l) and is without any glucose. Cowan et al. (1991) proved in a laboratory study that an increase in nutrient concentration is correlated with an increase in the number of attached bacterial cells. Moreover, biofilm formation is found to be associated with the synthesis of extracellular polymers, which is energetically demanding and carbon-expensive (Chakrabarty, 1996). The present study suggests that *A. junii* BB1A forms biofilm under nutrient rich condition. In this study maximum amount of biofilm formation was observed in nutrient rich medium and after 48 h of incubation at 30 °C, this may be due to the fact that nutrient rich media support maximum growth as in the case of *A. junii* BB1A while growth in LB medium was less compared to TSB and BHI (Fig. 1.4A). The amount of biofilm formation of this strain was also found to be dependent of the type of carbon source. Since BHI and TSB medium already contain glucose, LB medium supplemented with different sugars was used to study the effect of carbon source. *A. junii* BB1A formed highest biofilm in LB medium supplemented with either glucose or sucrose as a carbon source. The result suggests that the carbon source available for nutrition can have an important effect on biofilm maturation. In a multi-drug resistance clinical isolate of *Acintobacter baumanii,* the adhesion to polystyrene was found to be strongly affected by growth conditions, and is favored in glucose-based medium (Nucleo et al., 2009).

The temperature of incubation also considerably influences the amount of biofilm produced as biofilm density increases with the incubation temperature. For comparison the strain was incubated at different temperatures (20, 25, 30, 35, and 40 °C). Maximum biofilm as well as growth was observed at 30 °C after 48h of incubation. The result of this study is in agreement with other studies, where 30 °C of incubation temperature was used for biofilm formation or growth of certain *Acinetobacter sp.* (Tomaras et al., 2003; Pour et al., 2011; Park and Park, 2011). Maximum amount of biofilm production was observed at pH 8 and to a lesser extent at pH 7 while above and below these pH values, biofilm formation was less or negligible, and this corresponds to the growth of *A. junii* BB1A at different pH and temperatures. The strain was also found to tolerate high salt concentration up to 3.5% NaCl and form more biofilm in presence of high concentration of NaCl. The ionic strength of the medium is generally thought to promote biofilm formation by decreasing the electric diffuse double layer, which is a barrier against bacterial movement toward a surface (Gu et al., 2000). The increase in bacteria adhesion with ionic strength has also been observed by many investigators (Camesano and Logan, 1998; Jewett et al., 1995; Gross and Logan, 1995). The result suggests that the strain have the potential to form biofilm under stressful conditions (high salt and pH). *A. junii* BB1A was found to form biofilm only under static condition. The other studied species such as *A. baumanii* forms biofilm under both static and dynamic condition, although the later result in less biofilm (Tomaras et al., 2003).

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