Flagellar motility plays important role in Biofilm formation of *Bacillus cereus* and *Yersinia enterocolitica*

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Abstract: Bacteria live either independently as planktonic cells or in organized surface associated colonies called as biofilms. Biofilms play an important role in increased pathogenesis of bacteria and it is assumed that motility is one of the contributing factors towards biofilm initiation. This study was planned to identify the role of flagella in biofilm formation by constructing flagellated (wild type) and physically disrupted variants (non-motile). Total 10 clinical bacterial strains were isolated and characterized. Morphological and biochemical study identified these strains as *Enterobacter* spp., *Pseudomonas* spp., *Yersinia* spp., *Escherichia* spp., *Salmonella* spp., *Proteus* spp., *Staphylococcus* spp., *Streptococcus* spp., *Lactobacillus* spp. and *Bacillus* spp. Among all strains, two strains including *Yersinia* spp and *Bacillus* spp. showed higher antibiotic resistance, hence studied at molecular and physiological level. Biofilm formation capacity of strains was analyzed using three methods including Congo red assay, Test tube assay and Liquid-interface coverslip assay. Afterwards, flagellar disintegration was induced by blending and centrifugation for 5, 10 and 15 minutes. 16S rRNA sequencing showed two strains as *Bacillus cereus* and *Yersinia enterocolitica.* Both strains produced significant biofilm by all three above mentioned methods. A motility test of these blended variants showed partial/diminished motility with increased blending time. The significant loss in biofilm formation after 15 minutes blending confirmed the important flagellar contribution to the initiation of biofilm formation. This biofilm defect observed in flagella paralysed/minus variants presumably may be due to defects in attachments to surface at early stages. This study indicated that flagellar motility is crucial initially for surface attachment and subsequently for biofilm formation.

Keywords: Biofilm formation, planktonic mode, MIC, flagellar motility, physical disruption.

INTRODUCTION

The world around us is enriched with most wonderful miniatures called bacteria that occupy almost every habitat in biosphere. More than 99% of bacteria present on the earth exhibit biofilm formation. Biofilm is a community of bacteria enclosed in the protective covering of extracellular polymeric substances (EPS) (Karatan and Watnick, 2009). Biofilm mode of life defends bacteria against the stresses offered by their immediate environment like desiccation and radiation. The bacterial biofilms consist on 76-96% EPS and 4-24% bacterial mass approximately (Huang *et al.,* 2011). The infrastructure of biofilm is made up of various bacterial colonies, EPS, fluid channels and a very intricate communication system. The bacterial colonies in the biofilm may belong to the same or different species and may also be aerobic, anaerobic or both. Along with bacteria, biofilms may also contain various other microbial populations like protozoans, algae and fungi that collectively form a complex mutualistic environment (Branda *et al*., 2005).

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Biofilms have a broad worldwide impact on global economy. They are beneficial as well as harmful for various industries. Biofilms have ability to degrade many organic molecules; in this way they are helpful for soil and water to get them rid of enormous organic pollutants naturally (Lawrence and Neu, 2003). Bacterial biofilms that stick on various surfaces causes the biofouling that adversely affect the capabilities of heat exchangers. Many industries including food manufacturing and processing, polymer production, dairy processing, paper industry, power generation plants, oil and gas refineries are suffering from the most evident problem of fouling of heat exchange surfaces (Coetser and Cloete, 2005).

Biofilms has become the cause of massive economic losses to various above mentioned industries worldwide. Biofilms have important clinical significance because they provide protective barrier against disinfectants, antibiotics and unfavourable conditions. Many physical, chemical and medicinal methods have been developed against these destructive biofilms and most of them have failed completely or partially to overcome this problem. For developing new strategies to overcome these biofilms we need to capture the root of this problem (Belas, 2014; Rasamiravaka *et al*., 2015).

It is said that motility and biofilm formation are exclusive events and transition from motile to sessile biofilm mode is triggered by surface sensing. Bacteria do this surface sensing using flagella. So flagella are essential to provide adhesion between cell and surface by overcoming the repulsive forces. Furthermore flagella induced motility is significantly needed to recruit planktonic cells in biofilm mode and making them resistance against various disinfecting agents/antibiotics. Flagella are therefore suspected to play a very important role not only in biofilm initiation but also spreading (Houry *et al*., 2009). The fact that when bacteria have to switch from motile to sessile form is sensed by flagella made us to investigate the actual role that flagellar motility plays in biofilm formation.

MATERIALS AND METHODS

Pure cultures of ten bacterial strains isolated from soil, air and water environment were obtained from our laboratory. These strains were isolated, purified and biochemically tested up to genus level.

Morphological, biochemical and physiological characterization of isolated strains

Morphological tests like acid fast staining, gram's staining and motility test were performed to determine morphological characters of isolated strains. Biochemical characterization was done by performing various tests including H_2S production test, methyl red test, catalase test, citrate utilization test, voges proskauer test, tryptophan deaminase (TDA) test, indole test, urease test and denitrification tests.

Antibiotic resistance profile (Kirby Baeur method)

Antibiotic resistance profile of isolated 10 strains was performed using disc diffusion assays. Six different disks with different concentrations were used as [Ampicillin (Am-10μml^{-1}), Trimethoprim (TMP-1μml^{-1}), Cephrodoxil (Cd-10μml^{-1}), Tetracycline (Te-30μml^{-1}), Chloramphenicol (Cl-30μml^{-1}) and Erythromycin (Er-25μml^{-1})]. Physiological characterization was done on the basis of growth curve, temperature and pH.

16S rRNA gene sequencing of isolated bacterial strains

16S rRNA gene sequencing was done to identify two highly resistant strains upto species level. PCR was performed to amplify 16S rRNA gene under standard conditions using Techne (PROGENE) thermal cycler. Invitrogen PureLinkTM kit was used to purify amplified PCR product. The amplified DNA was sent to Axil scientific Singapore for sequencing. Dendrograms of identified strains were constructed with the help of ClustalW software.

Biofilm formation

To access the biofilm forming capability of the bacterial isolates, three methods were used. These three methods include Congo red Assay (Mathur *et al.,* 2006), Test tube assay (Liaqat *et al.,* 2009) and Air-Liquid interface coverslip assay (Mathur *et al.,* 2006). In Congo red method, Congo red medium was streaked with fresh culture of bacterial isolates and incubated for 24 hours at 37°C. Appearance of black colonies on the media indicated the positive results. In Test tube assay, biofilm formation was monitored following Liaqat *et al*. (2009) with modification. Briefly, nutrient broth solution was prepared and inoculated with overnight bacterial culture. The test tubes were then incubated in shaking incubator at 37°C for 48, 72, 168 hours. The experiment was run in triplicates. After 48 hours, first set of tubes was taken out; culture medium was decanted and washed with 0.85% saline solution. Attached biofilms was stained with 0.1% crystal violet and dissolved in 33% glacial acetic acid. O.D was measured at 523 nm. In Air-Liquid interface method, nutrient broth solution was inoculated with 300 µl of pre inoculum. Then 15 ml of this mixed culture was poured in petri plates and coverslips were very cautiously placed under sterile conditions for 12, 24 and 48 hours. The petri plates were placed in incubator at 37°C after 18 hours of incubation. Afterwards, cover slips were removed and stained with 0.1% crystal violet. All experiments were run in triplicates.

Construction of physically disrupted bacterial variants

Blending technique was used to induce physical disruption in bacterial isolates. For this purpose, sterilized flasks containing media were inoculated with fresh bacterial cultures and placed in incubator at 37°C for 18 hours. For induction of physical disruption, a blender cleaned with ethanol and exposed to UV light for 45 minutes was used to disrupt strains for 5, 10 and 15 minutes. Blended broth culture was centrifuged at 6000 rpm for 40 minutes. Supernatant was discarded and the pellet was stored at -20°C. A motility test was performed afterwards to check the motility of blended variants comparing with controls.

STATISTICAL ANALYSIS

Means and SDs of whole data were calculated using Microsoft Excel software (Microsoft Corporation). Results obtained in these experiments were analyzed statistically applying Student 't' test to determine the significance of data. The results were considered statistically significant when $P \leq 0.05$.

RESULTS

Bacterial characterization

Total 10 bacterial isolates were obtained from our laboratory and named as $F_{1(1)}$, $F_{2(2)}$, $F_{3(3)}$, $F_{4(4)}$, $F_{5(5)}$, $F_{6(6)}$, F_{15}, F_{16}, F_{17}, F_{18}. Morphological analysis involved the study of colony morphology, color, texture and margins etc. Both morphological similarity and diversity was

Table 1: Congo red method for biofilm formation by *Y. enterocolitica* and *B. cereus*

Strain	Variant types	Colony color	Inference
Bacillus cereus	F_{16} C	Dark Black	Heavy biofilm formation
	F_{16} 1	Black	Biofilm formation
	F_{16} 2	Dull black	Less biofilm formation
	F_{16} 3	Red	Absence of biofilm formation
Yersinia enterocolitica	$F_{5(5)}$ C	Dark Black	Heavy biofilm formation
	$F_{5(5)}$ 1	Black	Biofilm formation
	$F_{5(5)}$ 2	Dull black	Less biofilm formation
	$F_{5(5)}$ 3	Red	Absence of biofilm formation

observed among isolates. Most of bacterial strains exhibited circular colonies with entire margins. Biochemical characterization of 10 isolates showed most strains positive for catalase, urease and dentrification tests (Data not shown). From above results, these strains were inferred as *Enterobacter* spp., *Pseudomonas* spp., *Yersinia* spp., *Escherichia* spp., *Salmonella* spp., *Proteus* spp., *Staphylococcus* spp., *Streptococcus* spp., *Lactobacillus* spp. and *Bacillus* spp. Two strains (*Bacillus* spp. and *Yersinia* spp.) showed high resistance against tetracyclin, chloramiphenicol, trimethoprim and erythromycin, hence were further characterized upto species level.

16S rRNA gene Sequencing and Growth Optimization

16S rRNA gene sequencing results of two aforementioned strains were submitted to the NCBI nucleotide BLAST software and GenBank database. BLAST showed that strains F_{16} and $F_{5(5)}$ revealed 100% sequence homology with *Bacillus cereus* and *Yersinia enterocolitica* and their accession numbers were KT182077 and KT182076 respectively. Growth curve of two strains indicated that *B. cereus* showed 4 hours long log phase followed by stationary phase. Growth curve of *Y. enterocolitica* showed 3 hours long log phase followed by stationary phase (fig. 1). *B. cereus* showed best growth at 37°C temperature except for *Y. enterocolitica* which showed best growth (at 25°C). Optimum pH was observed to be 9 for both strains (figs. 2a, b).

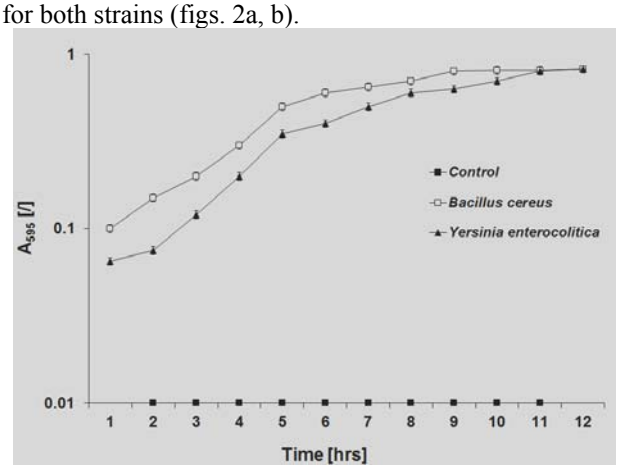


Fig. 1: Growth curve of bacterail strains. Bacteria were grown in nutrient broth for 14 hours. O.D was measured after respective time intervals at 595nm.

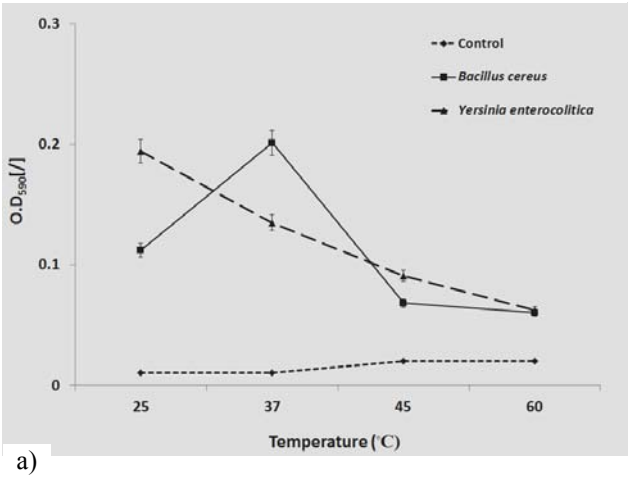
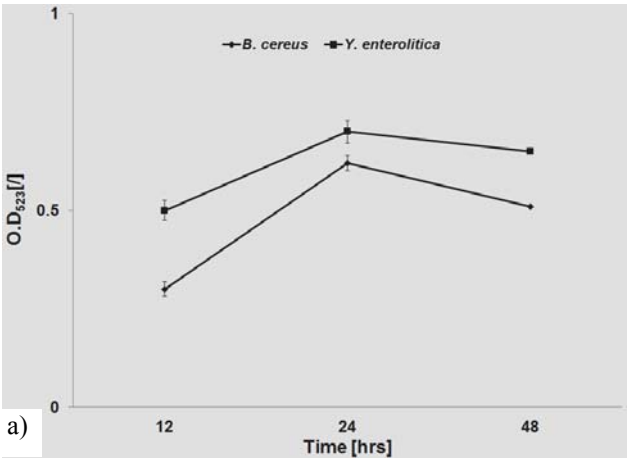


Fig. 2: Effect of temperaure and pH on growth of bacteria. Bacteria were grown in nutrient broth at (a) various temperaures (25, 37, 45 and 60°C) and (b) pH (3, 5, 7, 9 and 11). O.D was measured at 590nm.

Kinetics of biofilm formation

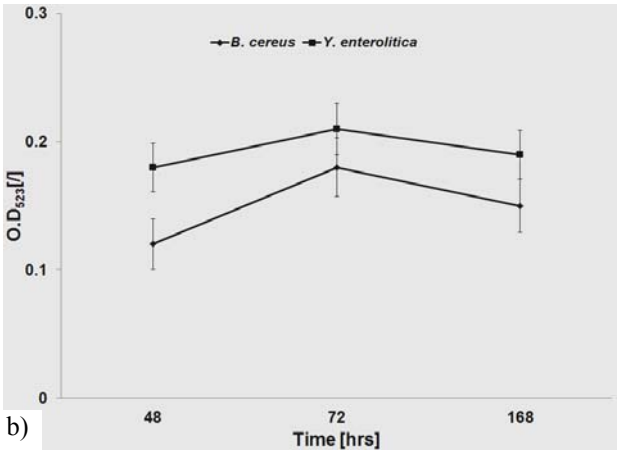
Congo red method indicated both *B. cereus* and *Y. enterocolitica* are strong biofilm formers. Both Test tube and Liquid interface coverslip methods are ideal to quantify the biofilm formation. Both assays showed that *Y. enterocolitica* is strong biofilm former compared to *B. cereus.* However, maximum biofilm formed by Test tube method was observed after 72 hours whereas coverslip assay proved that both strains produced good biofilm after 24 hours (figs. 3a, b).



a)

Motility test of physically disrupted variants

Both *B. cereus* and *Y. enterocolitica* were blended for 5, 10 and 15 minutes to construct variants. Motility test performed afterwards revealed that 5 minute variants of both strains were considerably less motile as compared to the wild types of both respective strains. On the other hand, 10 and 15 minutes variants of both strains showed no growth indicating that blending induced physical disruption in bacterial strains causing reduced motility and hence decreased biofilm formation.



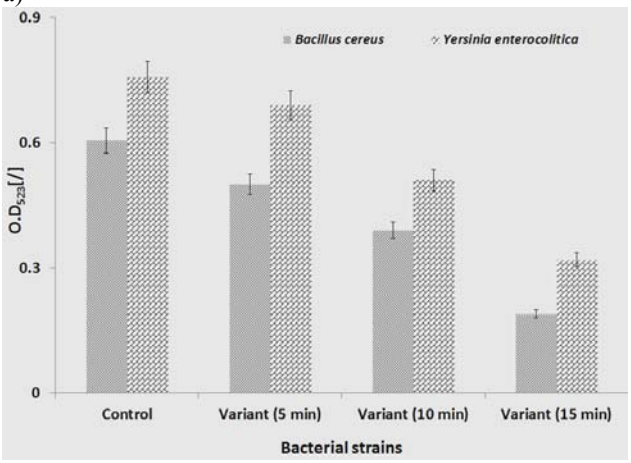
b)

Fig. 3: Time kinetics for biofilm formation. (a) Air-Liquid interface cover slip assay for biofilm formation and (b) Test tube assay for biofilm formation. Both strains were grown on cover slip immersed in nutrient broth in petri plates and test tubes respectively for various time intervals. Biofilm was stained with 0.1% crystal violet. O.D was measured at 523nm.

Biofilm formation by physically disrupted variants

Congo red method showed positive results for both *B. cereus* and *Y. enterocolitica* wild type strains by producing black colored growth on the Congo red media. In contrast, physically disrupted variants of both strains showed dark red and red colonies indicating decreased trend of biofilm formation particularly after 15 minutes (table 1). Cover slip method revealed that the wild types of both strain have excellent ability to form massive biofilms. The 5 minute variants of both strains showed moderate biofilm formation ability. The 10 minutes variants showed considerably less biofilm formation whereas 15 minutes variants displayed no biofilm formation ability (Fig. 4a). Test tube method assay of variants (5, 10and 15 minutes) showed considerable reduction in biofilm formation with increased blending time compared with control. Significantly reduced biofilm formation was observed after 15 minutes blending (fig. 4b).

a)



b)

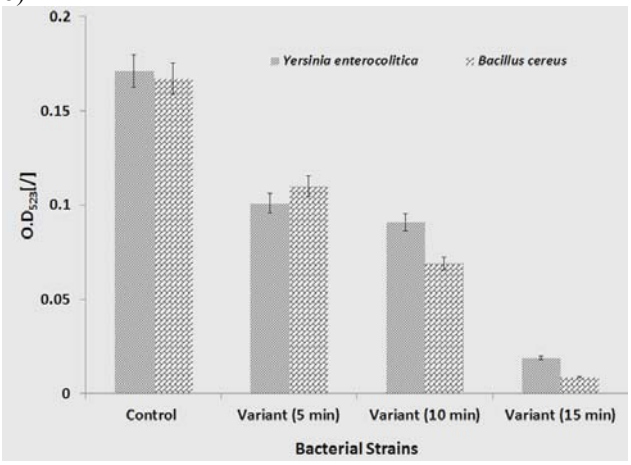


Fig. 4: Biofilm formation assays by wild type and blended variants of *B. cereus* and *Y. enterocolitica*. (a) Air-Liquid interface coverslip assay (b) Test tube method. Both strains and their blended variants (5, 10 and 15 minutes) were grown in nutrient broth for 72 hours. O.D was measured at 523 nm. Significant decrease ($p>0.05$) in biofilm formation was observed after 10 minutes and this decrease was highly significant ($p>0.001$) after 15 minutes. Experiment was run in triplicates.

DISCUSSION

Biofilm is defined as the bacterial community encased in the protective mucoid covering of extracellular polymeric substances EPS (Beech, 2004). Biofilms are responsible

for about 60% of all infections caused by microbes. According to the clinical microbiologists, bacterial biofilms are omnipresent in nature and are responsible for a number of infectious diseases and health issues. In food industry, biofilms are becoming the cause of a wide range of food borne diseases. Standard laboratory criteria was followed to identify and characterize environmental isolates. Bacteria identified upto genus level include *Escherichia* spp.*, Staphylococcus* spp.*, Lactobacillus* spp.*, Streptococcus* spp., *Bacillus* spp. and *Yersinia* spp. Previously, Stoodley *et al.* (2004) also reported similar strains from environmental settings.

Among ten strains, two belonging to genera *Bacillus* and *Yersinia* were found to have high antibiotic resistance profile. Assuming that these should have strong biofilm forming ability, 16S rRNA gene sequencing revealed that two strains were *B. cereus* and *Y. enterocolitica*. Physiological characterization of these two strains was done by plotting growth curve, determining optimum pH and temperature. 16S rRNA sequencing identified two strains as *B. cereus* and *Y. enterocolitica*. Biofilm formation phenomenon is a chief determinant factor for bacterial pathogenicity conferring them protection against host immune responses and antibiotics (Đapa *et al*., 2013). Biofilm formed by bacteria was quantified by three methods i.e. Congo red assay, Liquid interface assay (coverslip method), Test tube method. Both strains formed strong biofilm as observed by Congo red method. The Congo red method is however, is not a very precise and reliable method for the quantification of biofilm formation; similar results were reported by other studies (Mathur *et* al., 2006). The results by Congo red assay method were quantified using other two methods of biofilm formation (Liaqat *et al*., 2009). Liquid interface assay and coverslip assay revealed that both strains produced maximum biofilm after 72 and 24 hours respectively. *Y. enterocolitica* was a strong biofilm former compared to *B. cereus* (Kim *et al*., 2008).

For the detection of biofilms, the accuracy of tube method is near 80% and its sensitivity is reported to be almost 73%. The results of test tube method are more authentic then the Congo red assay method. Ruzicka *et al*. (2004), compared the Test tube method and Congo red assay and claimed Test tube method to be more accurate and sensitive method then Congo red assay on the basis of percentage of biofilm formed i.e. 54% and 44% respectively. Baqai *et al*. (2008), used Test tube method to detect biofilm formation by uropathogens, their results exhibited biofilm formation by 75% isolates while the Congo red assay method detected 11 biofilm producing bacteria and 99 as non-biofilm formers out of 110, with (11%) sensitivity, specificity (92%) and accuracy (41%) (Mathur *et al*., 2006).

Physical disruption was induced in environmental isolates using traditional blending technique. For degenerating bacterial motility we subjected all strains for variable blending time. This technique may decrease biofilm formation assuming that flagella are disrupted or lost. Results of motility test showed that physically disrupted strains showed reduced/no motility. Biofilm formed by the control and physically disrupted variables were again exposed to above mentioned three biofilm assays. Congo red test results revealed that both *Y. enterocolitica* and *B. cereus* wild type strains gave black colored growth on the Congo red media, whereas physically disrupted mutants of both strains produced dark red and red colonies indicating decreased trend of biofilm formation with increased blending time.

Test tube method results of physically disrupted mutants of *Y. enterocolitica* and *B. cereus* revealed a considerable reduction in biofilm formation with increased blending time. The 5 minute variants showed moderate biofilm formation ability. The 10 minute variants of both strains showed considerably less biofilm formation whereas the 15 minute variants displayed complete lack of biofilm formation ability. It has already been reported that minimal motility is sufficient to establish contact with abiotic surfaces, leading to subsequent development of biofilms as observed in 10 minutes disrupted variants. Significantly reduced biofilm formation observed by 15 minutes variants might be due to complete lack of flagella as confirmed by motility test. Previously Houry *et al*. (2009) reported that both flagella and motility are necessary for biofilm formation in *B. cereus*. Kim *et al*. (2008) also investigated the role of flagella in biofilm formation by *Y. enterocolitica*. Their results indicated the important contribution of flagella in biofilm initiation and progression under variable culture conditions.

CONCLUSION

Due to the continuous economic risk posed to industry and health by these biofilms, scientists and researchers have profound concern in developing alternative techniques to hinder this problem. This study was conducted to find out the possible role of flagella in biofilm formation. We constructed physically disrupted bacteria with their flagella disintegrated/removed and observed a considerable decrease in biofilm formation. The results of this study revealed that flagellar motility is important in biofilm initiation and in agreement with our accepted study (Liaqat *et al*., 2019). Though flagella might not be the only factor in biofilm formation. Other factors including fimbriae, pili, curli have also been reported to contribute to enhanced biofilm formation under varying environmental conditions (Reisner *et al*., 2006; Kim *et al*., 2008). The findings of this study indicate that flagellar expression may be a mean for *B. cereus* and *Y. enteroclitica* to persist in environment by forming biofilms. Though in future it will be interesting to

investigate the role of other factors including fimbriae, pili etc in biofilm formation.

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