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Research Article

Standardization and Comparative Evaluation of *In-vitro* Methods for Bacterial Biofilm Formation

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ABSTRACT

Biofilms are communities of microorganisms embedded in an extracellular matrix, which enhance their survival, virulence, and resistance to antimicrobial treatments. Due to their importance in clinical and environmental settings, effective *in vitro* biofilm models are crucial for studying biofilm biology and assessing antibiofilm strategies.

This study is focused on standardizing and comparing various *in vitro* methods for biofilm formation using six bacterial strains: *Escherichia coli MTCC 1687, Klebsiella pneumoniae MTCC 432, Staphylococcus aureus MTCC 3160, S. epidermidis MTCC 3615, Pseudomonas aeruginosa MTCC 2453*, and *Bacillus subtilis MTCC 2423*. Biofilm formation was evaluated through qualitative, semi-quantitative, and quantitative methods, including the Congo red agar assay, tube method with optimized inoculum density, biofilm formation on glass slides and coverslips using tube, plate, and 6-well plate methods, and the 96-well microtiter plate assay. The Congo red assay detected exopolysaccharide production in *E. coli, K. pneumoniae, S. aureus*, and *P. aeruginosa*, while *S. epidermidis* and *B. subtilis* did not exhibit the characteristic blackening. The optimal inoculum density for biofilm formation differed among species, highlighting the need for culture-specific standardization. Plate-based methods consistently facilitated stronger and more uniform biofilm formation compared to tube-based systems. Among the methods tested, the 6-well plate coverslip method and the 96-well microtiter plate assay generated dense, reproducible biofilms within 24 to 48 hours, with *P. aeruginosa* showing the highest biofilm-forming capability. Statistical analyses using one-way and two-way ANOVA revealed significant differences between methods and among bacterial strains ($p < 0.0001$). In summary, this study offers standardized and reproducible *in-vitro* biofilm models that can be effectively used for comparative biofilm research and for testing antibiofilm and antimicrobial agents.

Keywords: Extracellular matrix, Antibiofilm, Exopolysaccharide, Congo red assay

INTRODUCTION

Biofilms are defined as group of microorganisms which adhere to biotic or abiotic surfaces with cells embedded in an extracellular matrix (ECM) which consists of biomolecules like exopolysaccharides, extracellular DNA, proteins and lipids[1]. The cells constitute 10% of the dry mass while the ECM accounts for the rest 90%. This ECM provides the three-dimensional structural framework of the biofilm. The cells use ECM to adhere, aggregate, retain water, create a barrier against antimicrobial agents and ensure continuous nutrient availability[2].

The formation of biofilm occurs in the following way: 1) Planktonic cells attach themselves to a suitable surface. They metabolize nutrients, gain energy, and multiply in number to attain a quorum. They secrete components that make up the ECM. 2) This leads to firm attachment of the cells, aggregates the cells and forms a thick matrix. 3) The films evolve into a mature structure consisting of microcolonies and water channels. 4) At full maturity, these films have maximum cell density and operate as three-dimensional communities. 5) Mature biofilms rupture and release cells, which then colonize new surfaces[1,2].

More than 80% infections are biofilm-associated and pose a challenge in treatment. Cells that are part of a biofilm acquire resistance strategies to antimicrobial agents via horizontal gene transfer mechanisms[3]. The biofilm niche favors such genetic transfer, making the cells resist a wide variety of antimicrobials. This niche also enhances the expression of virulence factors and increases the severity of an infection[4].

It is the need of the hour to develop strategies to combat biofilm-based infections. To determine the efficacy of these novel agents, it is important to mimic biofilm formation in a laboratory setup. Given the complex nature of biofilms, it is necessary to optimize and develop a standard strategy for biofilm development in the lab. This study explores different methods to assess the biofilm-forming potential and techniques to develop strong biofilms for in vitro analysis.

MATERIALS AND METHODS

Congo red assay

A qualitative method to detect the potential of biofilm formation of bacterial strains is the Congo red agar method. This involves the use of a medium which consists of brain heart infusion (52 g/L), agar (36g/L), and Congo red (0.8g/L). This medium was prepared with varying concentrations of sucrose (3.5% and 5%)[5,6]. Congo red stock was prepared and autoclaved separately. It is added to the basal medium when it is cooled to 50℃ and then poured into Petri plates. The test organisms *Escherichia coli MTCC 1687*, *Klebsiella pneumonia MTCC 432*, *Staphylococcus aureus MTCC 3160*, *S. epidermidis MTCC 3615*, *Pseudomonas aeruginosa MTCC 2453*, and *Bacillus subtilis MTCC 2423,* having an optical density of 0.1 at 620nm was streaked on these plates and incubated for 24 and 48 hours at 37℃. Black colouration around the colonies was considered to be a positive test, while growth without any blackening was considered to be negative for biofilm formation.

Density optimization for biofilm formation by the tube method

This is a semi-quantitative method of biofilm formation. About 3 mL of Trypticase soy broth having 2% sucrose was inoculated with 20 μL culture suspension of the above-mentioned bacterial strains having different optical density (0.01, 0.05, 0.1, 0.2 and 0.5) at 620 nm. The tubes were incubated for 24 hours and 48 hours at 37℃. Post this, the tubes were decanted and washed with sterile saline and were air-dried. The tubes were then stained with 3mL of 0.1% crystal violet solution for 20 minutes, washed with sterile distilled water and destained using 3 mL of 33% acetic acid. 100 μL of this acetic acid wash was transferred to 96-well plates and the absorbance was measured at 599 nm using a plate reader[7].

Biofilm formation on glass slides

Slide in tube method

Glass slides (76.2 x 25.4 mm width x length, 1.0mm thickness) were placed in 50 mL Falcon tubes and autoclaved. Sterile Trypticase soy broth containing 2% sucrose was added to the tube such that the slide is completely immersed in the broth. 100 μL of the culture suspension having standardized optical density for respective strains at 620 nm was inoculated into the tubes. The tubes were incubated for 24 hours and 48 hours at 37℃. Post incubation, the slides were gently removed from the tube, gently washed with sterile saline, air dried and subjected to gram staining(8). The intensity of the biofilm formed was observed using a 100X oil immersion objective of a compound microscope.

Slide in plate method:

Glass slides (76.2 x 25.4 mm width x length, 1.0 mm thickness) were placed in 90 mm glass Petri dishes and autoclaved. Sterile trypticase soy broth containing 2% sucrose was added to the plates such that the slide is completely immersed in the broth. In 100 μL of the culture suspension having standardized optical density for respective strains at 620 nm was inoculated into the plates. The plates were incubated for 24 hours and 48 hours at 37℃. Post incubation, the slides were gently removed from the plate, gently washed with sterile saline, air dried and subjected to gram staining[8]. The intensity of the biofilm formed was observed using a 100X oil immersion objective of a compound microscope.

Biofilm formation on glass coverslips

Coverslips in tube method

Glass coverslips (20 x 20 x 0.15 mm) were autoclaved and placed in a sterile 50mL falcon tube. Sterile trypticase soy broth containing 2% sucrose was added to the tube such that the coverslip is completely immersed in the broth. 100 μL of the culture suspension having standardized optical density for respective strains at 620 nm was inoculated into the tubes. The tubes were incubated for 24 hours and 48 hours at 37. Post incubation, the coverslips were gently removed from the tube and transferred to a 6 well plate, gently washed with sterile saline, air dried and stained with 2 mL of 0.1% crystal violet for 20 minutes. The stain was decanted; the coverslip was washed with sterile distilled water and destained using 2 mL of 33% acetic acid. The absorbance of this was read at 599nm using a plate reader[9,10].

Coverslips in plate method

Glass coverslips (20 x 20 x 0.15 mm) were autoclaved and placed in sterile 90 mm Petri plates. Sterile trypticase soy broth containing 2% sucrose was added to the plate such that the coverslip is completely immersed in the broth. 100 μL of the culture suspension having standardized optical density for respective strains at 620 nm was inoculated into the plates. The plates were incubated for 24 hours and 48 hours at 37℃. Post incubation, the coverslips were gently removed from the plate and transferred to a 6 well plate, gently washed with sterile saline, air dried and stained with 2 mL of 0.1% crystal violet for 20 minutes. The stain was decanted; the coverslip was washed with sterile distilled water and destained using 2mL of 33% acetic acid. The absorbance of this was read at 599 nm using a plate reader[9,10].

Coverslips in 6-well plate method

Glass coverslips (20 x 20 x 0.15 mm) were autoclaved and placed in sterile 6-well plates. Sterile trypticase soy broth containing 2% sucrose was added to the wells such that the coverslip was completely immersed in the broth. 100 μL of the culture suspension having standardized optical density for respective strains at 620 nm was inoculated into the wells. The plates were incubated for 24 hours and 48 hours at 37℃. Post incubation, the coverslips were gently removed from the wells and transferred to another sterile 6 well plate, gently washed with sterile saline, air dried and stained with 2 mL of 0.1% crystal violet for 20 minutes. The stain was decanted; the coverslip was washed with sterile distilled water and destained using 2 mL of 33% acetic acid. The absorbance of this was read at 599 nm using a plate reader[9,10].

Biofilm formation in 96-well plate

Over 180 μL of sterile trypticase soy broth was added to the wells of a sterile 96 well plate. In 20 μL of the culture suspension, having standardized optical density for respective strains at 620 nm was added to the wells. Uninoculated wells containing only the broth were maintained as a negative control. The plates were incubated for 24 hours and 48 hours at 37℃. Post incubation, the broth was decanted and the wells were washed with sterile saline. The wells were air-dried and stained with 200 μL 0.1% crystal violet for 20 minutes. Excess stain was decanted, washed with sterile distilled water and destained

with 200 μL of 33% acetic acid. The absorbance of these wells, along with the negative control treated in a similar manner, was measured at 599 nm[11].

RESULTS

Congo red assay

The ability to form exopolysaccharides was exhibited by *E. coli MTCC 1687, Klebsiella pneumoniae MTCC 432, S. aureus MTCC 3160, and P. aeruginosa MTCC 2453,* as blackening was observed within 24 hours of incubation around the colonies as shown in Figure 1. While *S. epidermidis MTCC 3615* and *B. subtilis MTCC 2423* did not exhibit blackening around the colonies. The extent of EPS formation was similar in both 3.5 and 5% sucrose within 24 hours, while the blackening intensified comparatively in plates containing 5% sucrose when incubated for 48 hours.

Density optimization for biofilm formation by the tube method

As shown in Figure 2 the culture density required to form dense biofilms was determined to be 0.05 for *Klebsiella pneumoniae MTCC 432* and *P. aeruginosa MTCC 2453,* while for *E. coli MTCC 1687, S. aureus MTCC 3160, and S. epidermidis MTCC 3615* was found to be 0.1 and for *B. subtilis MTCC 2423*was found to be 0.5 at 620 nm. One-way ANOVA was performed and the *p-value* was found to be <0.0001.

Biofilm formation on glass slides

The plate method was found to be effective for all organisms to form biofilms on glass slides as compared to the tube method as seen in Figure 3. In a few organisms, the intensity of biofilms was also found to reduce by 72 hours.

Biofilm formation on coverslips

The well plate method was found to be effective for biofilm formation on coverslips as compared to the tube and petri dish methods for all the bacterial strains used as seen in Figure 4. The statistical evaluation to compare the effect of different methods of biofilm formation on coverslips was performed using two-way ANOVA. It was found that all methods don't have the same impact on the different bacterial strains used in the study. But each method differs significantly from the others in its potential for biofilm formation. Also, biofilm formation differs strongly among the strains used, indicating intrinsic culture-dependent differences. The p- value was found to be <0.0001.

Biofilm formation in 96 well plate

Prominent biofilms were formed by all the bacterial strains within 24 hours of incubation. *P. aeruginosa MTCC 2453* was found to form intense biofilms as compared to the other strains. Biofilms of all the bacterial strains intensified further post 48 hours of incubation as seen in Figure 5. One-way ANOVA was performed and the p- value was found to be <0.0001.

DISCUSSION

The formation of biofilms is a complex and multifactorial process that is affected by parameters such as the type of bacteria, the composition of nutrients, the characteristics of the surface, the duration of incubation, and the initial density of the inoculum[12]. This study systematically assessed and standardized various in vitro techniques to evaluate biofilm formation in six bacterial species that are significant in both clinical and environmental settings. The findings indicate

Fig. 1: Congo red assay (3.5% sucrose) a) *S. aureus* exhibiting blackening, b) *Klebsiella pneumoniae* exhibiting blackening and c) *B. subtilis* exhibiting only growth

Table 1: Potential of strains to produce exopolysaccharides using Congo red assay

Organism	*24 hours*		*48 hours*	
	3.5% Sucrose	5% Sucrose	3.5% Sucrose	5% Sucrose
E. coli MTCC 1687	Growth with blackening	Growth with blackening	Growth with intense blackening	Growth with intense blackening
Klebsiella pneumoniae MTCC 432	Growth with blackening	Growth with blackening	Growth with intense blackening	Growth with intense blackening
S. aureus MTCC 3160	Growth with blackening	Growth with blackening	Growth with intense blackening	Growth with intense blackening
S. epidermidis MTCC 3615	Only growth	Only growth	Only growth	Only growth
P. aeruginosa MTCC 2453	Growth with blackening	Growth with blackening	Growth with blackening	Growth with blackening
B. subtilis MTCC 2423	Only growth	Only growth	Only growth	Only growth

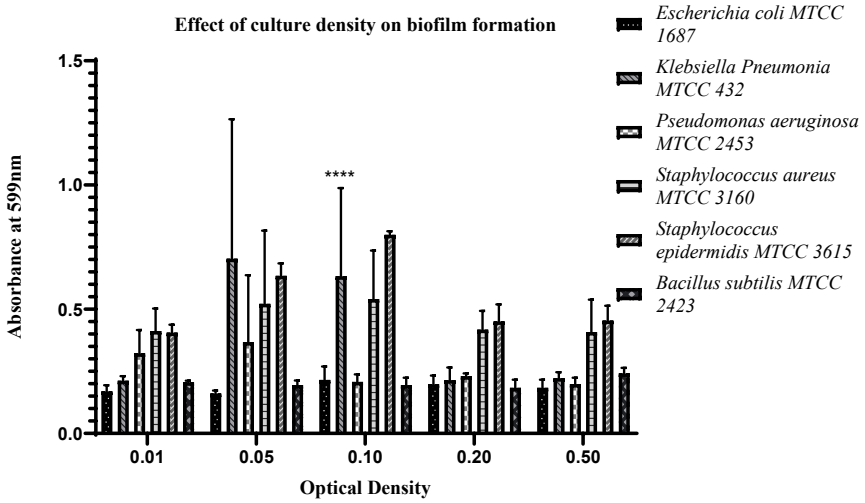


Fig. 2: Optimization of culture density for biofilm formation in tubes

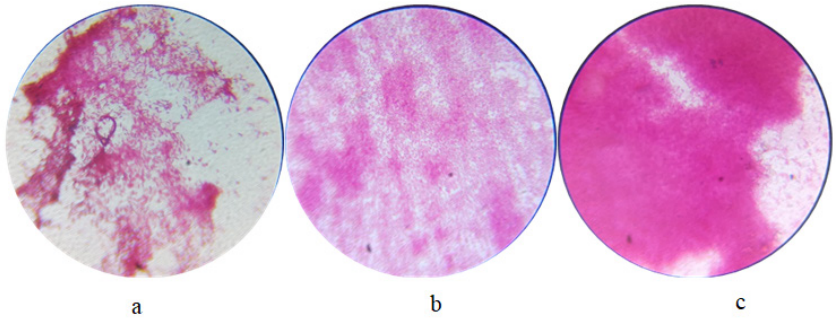


Fig. 3: Biofilm formation of *P. aeruginosa MTCC 2453* on glass slides using the plate method: a- 24 hours, b- 48 hours, c- 72 hours on incubation

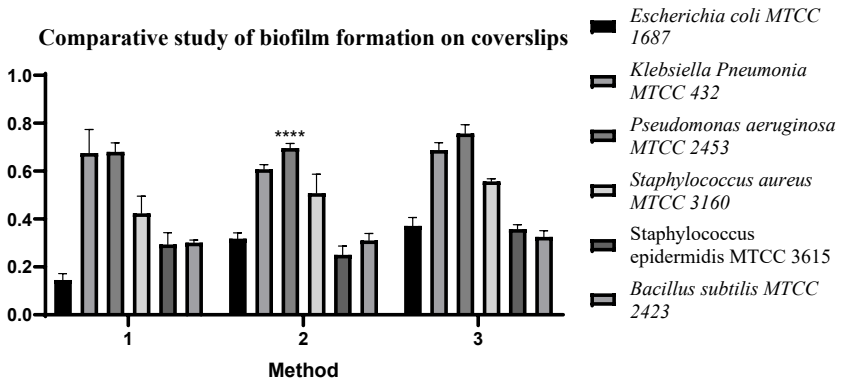
Table 2: Biofilm formation on glass slides using tube and plate method

Organism	*Tube method*			*Plate method*		
	24 hours	*48 hours*	*72 hours*	*24 hours*	*48 hours*	*72 hours*
E. coli MTCC 1687	++	++	++	+	++	++
Klebsiella pneumoniae MTCC 432	+	++	++	++	+++	+++
S. aureus MTCC 3160	++	+++	+++	+++	+++	+++
S. epidermidis MTCC 3615	-	-	+	-	+++	+++
P. aeruginosa MTCC 2453	+	++	++	+	++	+++
B. subtilis MTCC 2423	+	++	+	+	++	+++

Key: '-' indicates only scattered cells, '+' indicates weak films with scattered cells, '++' indicates patchy films, '+++'indicates intense films

that no single method is completely effective and that the potential of biofilm formation depends on the features of the organism used and the experimental conditions.

The Congo red agar assay performed in this study is a qualitative method to evaluate the production of exopolysaccharides (EPS), which are significant components for forming the biofilm matrix. *E. coli, Klebsiella pneumoniae, S. aureus, and P. aeruginosa*exhibited distinct blackening, indicating their capability to produce EPS. While, *S. epidermidis and B. subtilis* did not display black pigmentation, even though they are recognized as biofilm formers under certain conditions[6]. This highlights the limitation of the Congo red assay; while it is effective for initial screening, it may underestimate the biofilm potential in organisms that depend less on Congo red–binding polysaccharides or generate different matrix components, indicating the sensitivity and specificity of the assay[13]. The



Key: 1:Tube method, 2: Petridish method, 3: 6 well plate method

Fig. 4: Comparative analysis of methods to form biofilms on coverslips

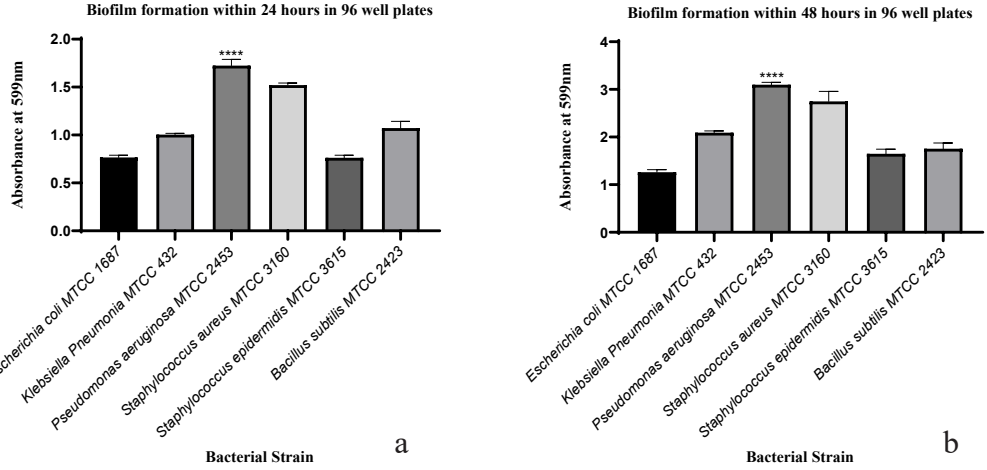


Fig. 5: Biofilm formation in 96 well plate a) 24 hours and b) 48 hours

increased intensity of blackening observed at 48 hours suggests that EPS production is dependent on time, whereas the differences between 3.5% and 5% sucrose concentrations indicate that there is a threshold effect as shown in Table 1 [14].

The tube method used to optimize the inoculum density highlighted the requirements of different strains for effective biofilm formation. For *K. pneumoniae and P. aeruginosa*, lower optical densities were adequate for intense biofilm formation, while *B. subtilis* required a higher inoculum density. These results exhibit the impact of inoculum density on biofilm development as it affects quorum sensing, nutrient availability, or surface attachment[12]. The statistically significant differences observed indicate the necessity of standardizing inoculum density when comparing biofilm formation across different strains or experimental setups.

In a comparative study of biofilm formation on glass slides, the petri dish method was consistently found to be more effective than the tube method as seen in Table 2. This could be due to enhanced aeration, greater surface area, and reduced shear stress in the petridish setup. A decrease in biofilm intensity in some strains after 72 hours suggests biofilm dispersal or nutrient depletion, reflecting the natural biofilm life cycle[2]. In the case of the coverslip-based methods, the 6-well plate method was found to be effective for all the tested strains. This may be due to an optimal surface-to-volume ratio, improved oxygen availability, and minimal disturbance during incubation. The strain-dependent variation observed across all coverslip methods indicates intrinsic differences in adhesion properties, matrix composition, and growth kinetics of the strains[12].

The 96-well microtiter plate assay was found to be an effective and reproducible quantitative method, producing prominent biofilms within 24 hours for all strains. The biofilm-forming ability of *P. aeruginosa* aligns with its well-documented clinical relevance and its capacity to rapidly establish structured biofilms[15]. The further intensification at 48 hours confirms that extended incubation enhances biofilm formation.

This study highlights that biofilm formation is highly dependent on both bacterial species and experimental conditions. The findings highlight the importance of standardizing parameters such as inoculum density, incubation time, and surface configuration to ensure reproducibility and biological relevance. These standardized and optimized *in vitro* models provide a reliable platform for future

investigations aimed at understanding biofilm physiology and evaluating novel antibiofilm strategies.

CONCLUSION

The current research effectively standardized and compared various *in vitro* techniques for bacterial biofilm formation using both gram-positive and gram-negative bacteria. The results clearly indicate that biofilm formation is specific to the organism and dependent on the method used, highlighting that no single assay is universally applicable to all bacterial species.

The Congo red assay was successful in qualitatively identifying strains that produce exopolysaccharides, but it underestimated the biofilm-forming ability of some organisms, pointing out its limitation as a standalone method. Optimizing the inoculum density was found to be crucial for strong biofilm formation, with each bacterial species requiring a unique inoculum concentration for optimal biofilm growth.

Among surface-based methods, plate-based approaches consistently facilitated stronger and more uniform biofilm formation compared to tube-based systems. Biofilm development on glass slides and coverslips was significantly improved in plate and well-plate formats, likely due to better aeration, surface exposure, and reduced physical disturbance. The 6-well plate method for coverslips and the 96-well microtiter plate assay proved to be the most reliable and reproducible quantitative methods, producing dense biofilms within 24 to 48 hours across all tested strains.

Overall, this study establishes a set of optimized, reproducible *in-vitro* biofilm models that can be reliably used for biofilm characterization, comparative studies, and evaluation of antibiofilm agents. The standardized methodologies described here provide a robust experimental framework for future research aimed at understanding biofilm biology and developing effective strategies to combat biofilm-associated infections.

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