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STANDARDIZING METHODS TO EVALUATE THE EFFICACY OF ANTIBIOFILM AGENTS

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ABSTRACT: Microbial biofilms are a major cause of hospital-acquired infections and play a critical role in the dissemination of antimicrobial resistance. Cells within biofilms exhibit altered physiology and resistance to antimicrobial agents, making biofilm associated infections challenging to treat. Although numerous compounds are being evaluated for antibiofilm activity, lack of standardized and optimized evaluation methods often result in poor reproducibility and inconsistent interpretation of efficacy. This study aimed to optimize *in-vitro* methods for assessing the efficacy of antibiofilm agents against clinically relevant biofilm forming bacteria. Biofilms of *Klebsiella pneumoniae* MTCC 432, *Pseudomonas aeruginosa* MTCC 2453, *Staphylococcus aureus* MTCC 3160 and *Staphylococcus epidermidis* MTCC 3615 were evaluated using model antibiofilm agents like acetic acid and sodium dodecyl sulphate. Assays for minimum biofilm inhibitory concentration (MBIC), minimum biofilm eradication concentration (MBEC), biofilm dispersion, viability of biofilm embedded cells, extracellular DNA (eDNA) release and exopolysaccharide production and emulsification were standardized. Species-specific differences in biofilm eradication and dispersion thresholds were observed signifying organism specific evaluation of the antibiofilm agents. The optimized methods exhibit enhanced reproducibility and revealed species specific differences in biofilm eradication and dispersion. These methods assure reliable evaluation of antibiofilm agents and increase the comparability of the agents.

INTRODUCTION: Microbial biofilms are the major cause of several hospital acquired infections and also are the core niche for the dissemination of antimicrobial resistance genes [1]. These biofilms are communities of microbial cells embedded in a protective blanket of extracellular polymeric substances (EPS). This EPS makes the biofilm a challenging microenvironment to penetrate and disintegrate [2].

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Several antimicrobial agents which work effectively against the planktonic cells fail to act on the biofilms formed by these cells, thereby making biofilm-based infections difficult to treat [3]. Several natural and antibiofilm agents are being evaluated for their efficacy in inhibiting the formation of biofilms or in dispersing a preformed biofilm.

There is a need to optimize and standardize the methods used for such evaluations for the following reasons: Firstly, biofilms are heterogenous and dynamic structures. Their architecture, thickness, metabolic activity and extracellular matrix composition differ with bacterial species, surface type, nutrient conditions, incubation time [4].

Secondly, planktonic susceptibility does not mean effective action against biofilms. Cells in biofilm exhibit altered gene expression, growth rates and increased tolerance to antimicrobial agents [5]. Thirdly, methodological variations lead to poor reproducibility and difficulty in comparing efficacy of the agents [6]. Differences in inoculum density, surface material, incubation conditions, biomass quantification techniques can produce diverse results across laboratories [2]. Additionally, maturity of the biofilm influences the treatment outcomes. Antibiofilm agents may act differently on early, developing or mature biofilms. Finally, regulatory and translational relevance depends on robust models. Optimized *in-vitro* methods provide reliable platforms for screening novel antibiofilm agents, reducing errors and bias in the study.

These reasons backed this research study so as to avoid formation of weak, immature and inconsistent biofilms which could lead to inaccurate assessment of antibiofilm activity. An agent may be labelled as effective just because the biofilm itself is poorly formed. This study involves optimizing methods to determine minimum biofilm inhibitory concentration, minimum biofilm eradication concentration, Dispersion activity, viability of cells in biofilms, extracellular DNA and the amount of EPS produced.

MATERIALS AND METHODS:

Maintenance of Biofilm Forming Cultures: *Klebsiella pneumoniae* MTCC 432, *Pseudomonas aeruginosa* MTCC 2453, *Staphylococcus aureus* MTCC 3160 and *Staphylococcus epidermidis* MTCC 3615 were grown in Tryptic soy broth and maintained on Tryptic Soy agar plates. Culture suspension of 24 hours old strains having an optical density of 0.05 for *Klebsiella pneumoniae* MTCC 432 and *Pseudomonas aeruginosa* MTCC 2453, while optical density of 0.1 for *Staphylococcus aureus* MTCC 3160 and *Staphylococcus epidermidis* MTCC 3615 at 620nm were used for all the experiments.

Determination of Minimum Biofilm Inhibitory Concentration (MBIC): In a 96-well microtiter plate, 180 µL of tryptic soy broth containing 2% acetic acid was added to a well. A two-fold serial dilution was performed using plain tryptic soy broth to achieve concentrations of 1%, 0.5%, 0.25%, and 0.125% in the subsequent wells. To all wells except the negative control, 20 µL of inoculum was added. For the control, 20 µL of inoculum was mixed with 180 µL of broth. The plates were then incubated at 37°C for 24 hours. After incubation, the spent broth was aspirated, and the wells were washed with sterile saline to remove unattached cells. The wells were dried for an hour before adding 200 µL of 0.1% crystal violet to the wells. The plate was incubated at room temperature for 30 minutes. The stain was then removed, and the wells were washed with sterile distilled water. Subsequently, 200 µL of 33% acetic acid was added to the wells, and the plates were placed on a plate rocker for 20 minutes. Absorbance was measured at 599 nm using an ELISA plate reader. This setup was replicated five times for each strain [7-9].

Determination of Minimum Biofilm Eradication Concentration (MBEC): In a 96-well microtiter plate, 180 µL of tryptic soy broth was added to each well, followed by 20 µL of the inoculum. The plates were then incubated at 37°C for 24 hours. After incubation, the spent broth was aspirated, and the wells were washed with sterile saline to remove any unattached cells. Subsequently, 200 µL of sodium dodecyl sulphate at concentrations of 2%, 1%, 0.5%, 0.25%, and 0.125% were sequentially added to the wells, except for the control.

The plates were incubated again at 37°C for 6 hours. Following this incubation, the sodium dodecyl sulphate solution was aspirated, and the wells were washed with sterile distilled water. The wells were then dried for an hour before adding 200 µL of 0.1% crystal violet. The plate was incubated at room temperature for 30 minutes. After removing the stain, the wells were washed with sterile distilled water. Then, 200 µL of 33% acetic acid was added to the wells, and the plates were placed on a plate rocker for 20 minutes. Absorbance was measured at 599 nm using an ELISA plate reader. This setup was replicated five times for each strain [7-9].

Determination of Dispersal of Cells from the Biofilm: In a 6-well microtiter plate, 900 µL of tryptic soy broth was added to each well, followed by 100 µL of the inoculum. The plates were then incubated at 37°C for 24 hours. After incubation,

the spent broth was aspirated, and the wells were washed with sterile saline to remove any unattached cells. Subsequently, 1mL of tryptic soy broth containing sodium dodecyl sulphate at concentrations of 2%, 1%, 0.5%, 0.25%, and 0.125% were sequentially added to the wells, except for the control well. In the control well, 1mL tryptic soy broth is added. The plates were incubated again at 37°C. 100 µL aliquot was removed from the wells at 0, 0.5,1,2,4,6 hours and presence of dispersed cells was checked at these timepoints using TTC assay. For TTC assay, the aliquots were transferred to a new plate. 50µL of 24mM 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) is added to the wells. The plate is incubated at 37°C for 1 hour. 1mL dimethyl sulfoxide is the added to the well to solubilize the formazan product. The absorbance is measured at 510 nm [10, 11].

Determination of Viability of Cells in the Biofilm Post Treatment with Antibiofilm agent: In a 96-well microtiter plate, 180 µL of tryptic soy broth was added to each well, followed by 20 µL of the inoculum. The plates were then incubated at 37°C for 24 hours. After incubation, the spent broth was aspirated, and the wells were washed with sterile saline to remove any unattached cells. Subsequently, 200 µL of 1% sodium dodecyl sulphate was added to the wells, except for the positive control. The plates were incubated again at 37°C for 6 hours. Following this incubation, the acetic acid was aspirated, and the wells were washed with sterile distilled water. 200µL sterile saline was added to the wells and vigorous pipetting was done to remove the adhered cells in the well. Ten-fold dilution of 100µL aliquot withdrawn from the wells was performed and cells were plated on tryptic soy agar by Miles and Mishra technique. The plates were incubated at 37°C for 24 hours and post incubation the colonies were counted [12-14].

Qualitative Detection of Extracellular DNA: In a microcentrifuge tube, 200 µL of the bacterial suspension was inoculated in 1800 µL of tryptic soy broth containing 1% acetic acid. Tubes were incubated 37°C for 24 hours. Post incubation the tubes were centrifuged at 5000rpm for 10 minutes. Supernatants were subjected to qualitative test for presence of DNA by diphenylamine method. 1mL of the supernatant was added 4mL of diphenylamine reagent and the tube was incubated in boiling water bath for 15 minutes. The presence of DNA is indicated by blue color. Absorbance is measured at 595nm. Control in this experiment were cells grown in the absence of antibiofilm agent [15, 16].

Determination of Effect of Antibiofilm agent on Production of Exopolysaccharide: 3mL of Tryptic soy broth having 2% sucrose and 0.2% acetic acid was inoculated with 20µL culture suspension in glass tubes. The tubes were incubated for 24 hours at 37 °C. Post this the tubes were decanted and washed with sterile saline. 1mL of 5% phenol and 5mL of sulfuric acid was added to the tubes. The tubes were sonicated and vortexed to disrupt the film. Tubes were incubated in dark for 1 hour. Absorbance was measured at 490nm. Untreated tube was maintained as control [17].

Determination of Effect of Antibiofilm agent on Emulsification of Exopolysaccharide: 3mL of Tryptic soy broth having 2% sucrose was inoculated with 20µL culture suspension in glass tubes. The tubes were incubated for 24 hours at 37°C. Post this the tubes were decanted and washed with sterile saline. 3mL of 0.5% SDS was added to the tubes. The tubes were incubated for 24 hours at 37°C. Post this the tubes were decanted and washed with sterile saline. 1mL of 5% phenol and 5mL of sulfuric acid containing 1% phenyl hydrazine was added to the tubes. The tubes were sonicated and vortexed to disrupt the film. Tubes were incubated in dark for 1 hour. Absorbance was measured at 490nm. Untreated tube was maintained as control [17].

RESULTS AND DISCUSSION:

Determination of Minimum Biofilm Inhibitory Concentration (MBIC): The minimum biofilm inhibitory concentration of acetic acid against all the bacterial strains was found to be 1%. The data was statistically analyzed using non-linear regression (Inhibitor vs response curve). The R^2 values for the assay against *Klebsiella pneumoniae* MTCC 432, *Pseudomonas aeruginosa* MTCC 2453, *Staphylococcus aureus* MTCC 3160 and *Staphylococcus epidermidis* MTCC 3615 was found to be 0.9858, 0.9845, 0.9943, 0.9847 respectively.

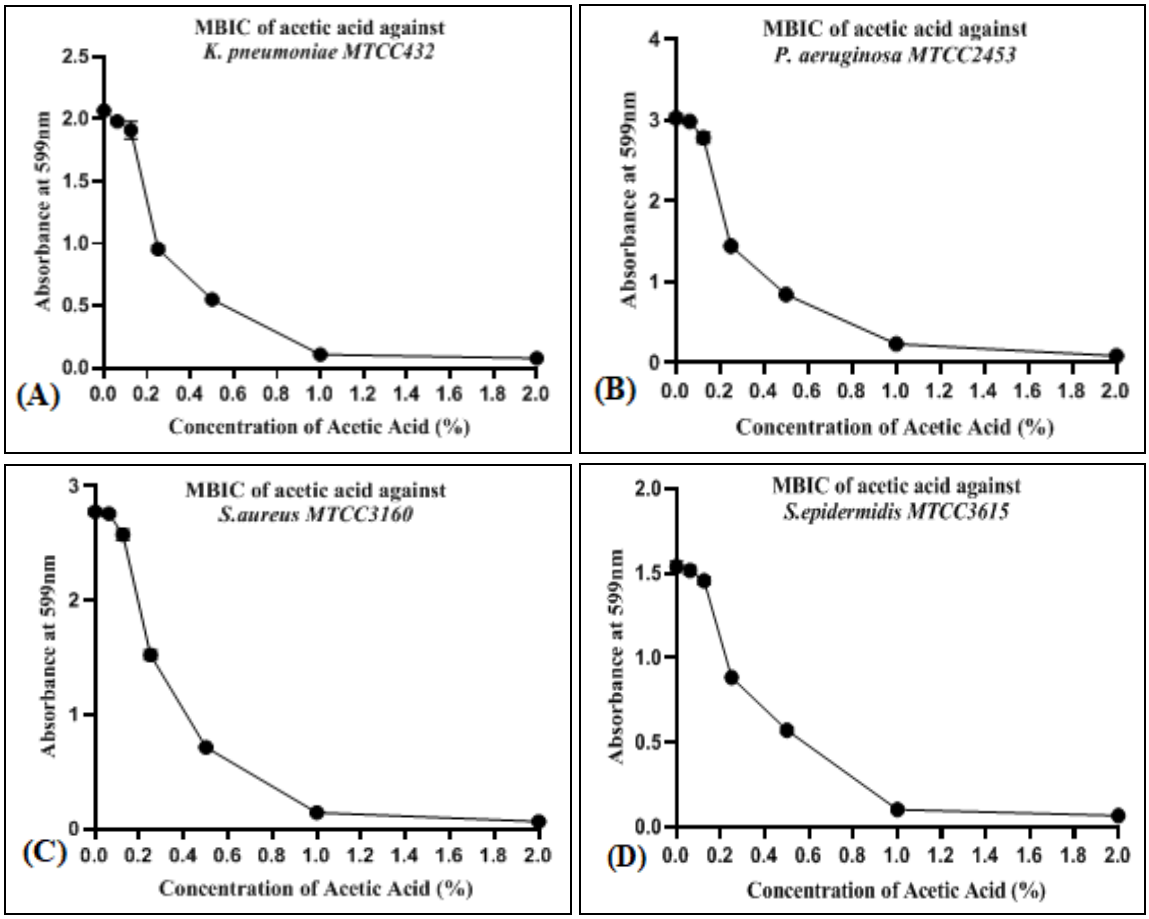
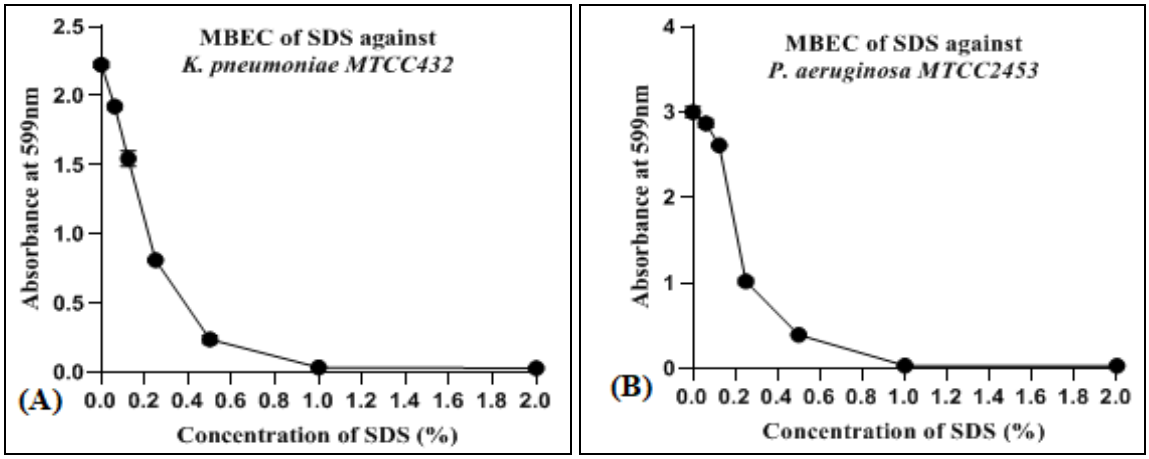


FIG. 1: DETERMINATION OF MINIMUM BIOFILM INHIBITORY CONCENTRATION OF ACETIC ACID AGAINST (A) *KLEBSIELLA PNEUMONIAE* MTCC 432, (B) *PSEUDOMONAS AERUGINOSA* MTCC 2453, (C) *STAPHYLOCOCCUS AUREUS* MTCC 3160 AND (D) *STAPHYLOCOCCUS EPIDERMIDIS* MTCC 3615

Determination of Minimum Biofilm Eradication Concentration (MBEC): The minimum biofilm eradication concentration of sodium dodecyl sulphate was found to be 1% against *Klebsiella pneumoniae* MTCC 432, *Pseudomonas aeruginosa* MTCC 2453, 0.5% *Staphylococcus epidermidis* MTCC 3615 and 0.25% against *Staphylococcus aureus MTCC 3160*. The data was statistically analyzed using non-linear regression (Inhibitor vs response curve). The R^2 values for the assay against *Klebsiella pneumoniae* MTCC 432, *Pseudomonas aeruginosa* MTCC 2453, *Staphylococcus aureus* MTCC 3160 and *Staphylococcus epidermidis* MTCC 3615 was found to be 0.9928, 0.9950, 0.9970, 0.9627 respectively.



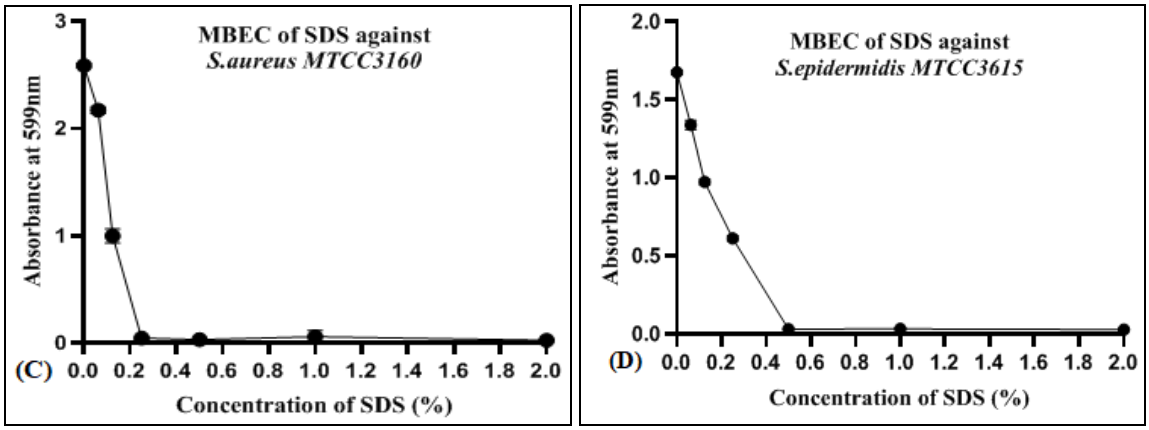


FIG. 2: DETERMINATION OF MINIMUM BIOFILM ERADICATION CONCENTRATION OF SDS AGAINST (A) *KLEBSIELLA PNEUMONIAE* MTCC 432, (B) *PSEUDOMONAS AERUGINOSA* MTCC 2453, (C) *STAPHYLOCOCCUS AUREUS* MTCC 3160 AND (D) *STAPHYLOCOCCUS EPIDERMIDIS* MTCC 3615

Determination of Dispersal of Cells from the Biofilm: Using the TTC assay it was found that the optimal concentration of SDS required for the dispersion of cells from the biofilms varied as per the strain used in the study. The data was subjected to one way ANOVA. The p values for all the assays were found to be <0.0001. The concentration of SDS for dispersion was found to be 0.125% against *Klebsiella pneumoniae* MTCC 432, 0.25% against *Pseudomonas aeruginosa* MTCC 2453, 0.0625% against *Staphylococcus epidermidis* MTCC 3615 and *Staphylococcus aureus* MTCC 3160. At higher concentrations the absorbance is found to be low as the dispersed cells are killed at higher concentrations of SDS and TTC detects only viable cells. Hence the dispersion rate of cells may increase at higher concentration but will not be detectable using TTC assay.

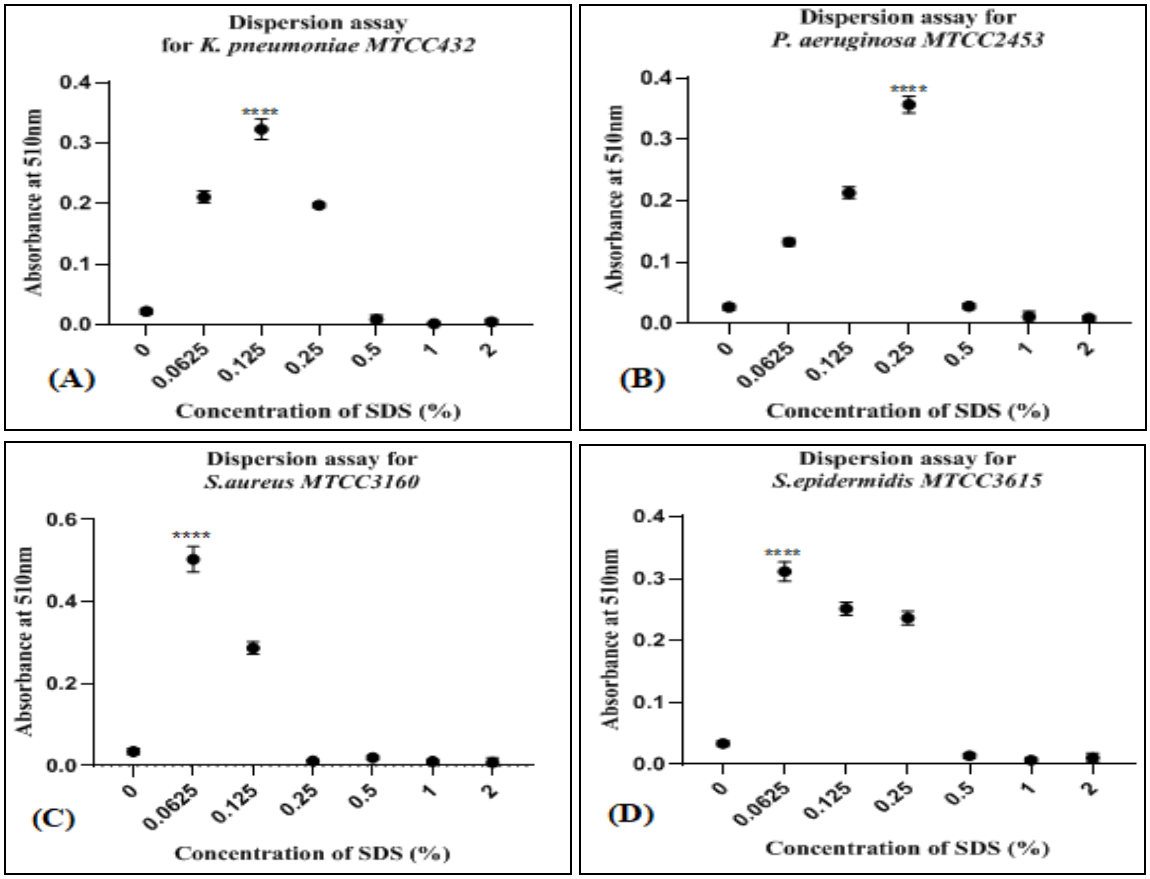


FIG. 3: DISPERSION ASSAY USING SDS AGAINST (A) *KLEBSIELLA PNEUMONIAE* MTCC 432, (B) *PSEUDOMONAS AERUGINOSA* MTCC 2453, (C) *STAPHYLOCOCCUS AUREUS* MTCC 3160 AND (D) *STAPHYLOCOCCUS EPIDERMIDIS* MTCC 3615

Determination of Viability of Cells in the Biofilm Post Treatment with Antibiofilm agent: The control and treated group for every strain was subjected to unpaired t test for statistical analysis. It was found that the p values for assays for *Klebsiella pneumoniae* MTCC 432, *Pseudomonas aeruginosa* MTCC 2453, *Staphylococcus aureus* MTCC 3160 and *Staphylococcus epidermidis* MTCC 3615 are 0.0004, 0.0014, 0.0039 and 0.0006 respectively. The graphs in **Fig. 4** indicate 100% reduction in viability of the cells in the biofilm post treatment.

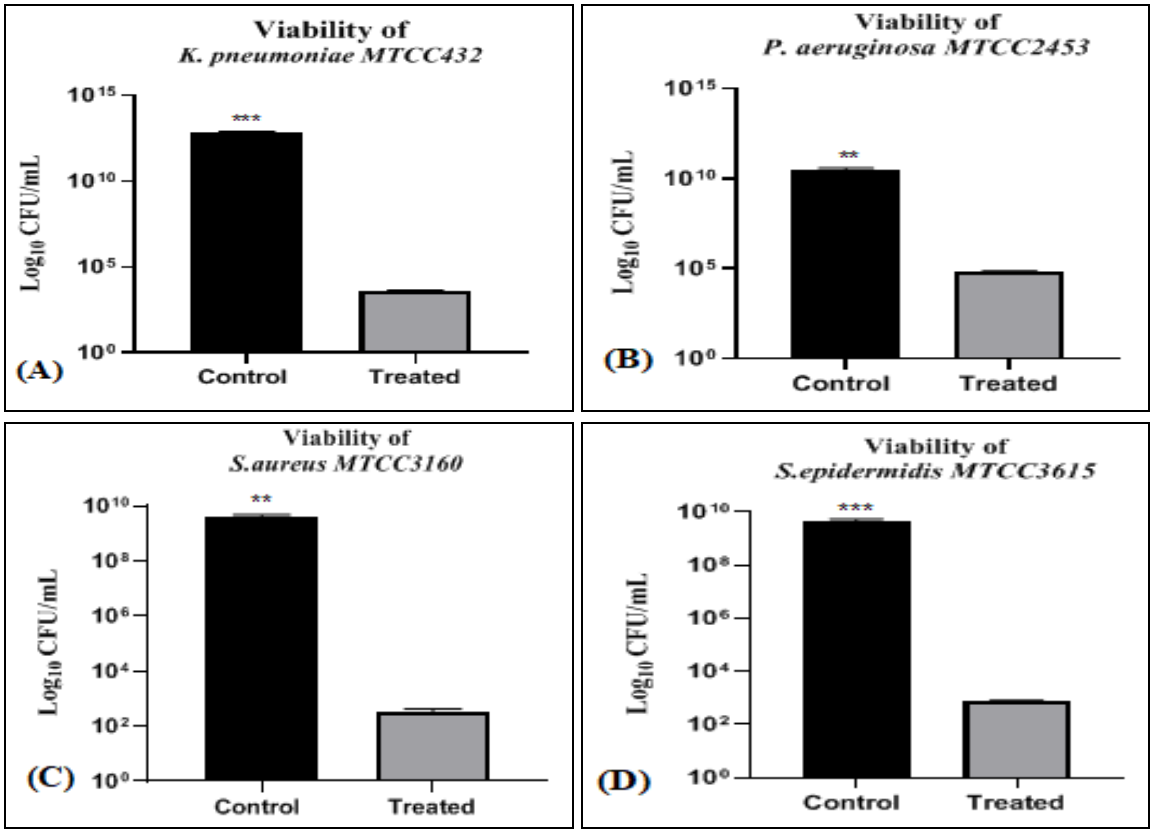


FIG. 4: VIABILITY OF CELLS IN THE BIOFILM POST TREATMENT WITH 1% SDS FOR 6 HOURS (A) *KLEBSIELLA PNEUMONIAE* MTCC 432, (B) *PSEUDOMONAS AERUGINOSA* MTCC 2453, (C) *STAPHYLOCOCCUS AUREUS* MTCC 3160 AND (D) *STAPHYLOCOCCUS EPIDERMIDIS* MTCC 3615

Qualitative Detection of Extracellular DNA: It was observed that the presence of eDNA in the supernatant drastically reduced in the presence of acetic acid as compared to the control. This could be due to the growth inhibition by acetic acid and the basal reading in treated samples could be due to the killing of cells in the inoculum. The data was analyzed by two-way ANOVA and the p value was found to be <0.0001.

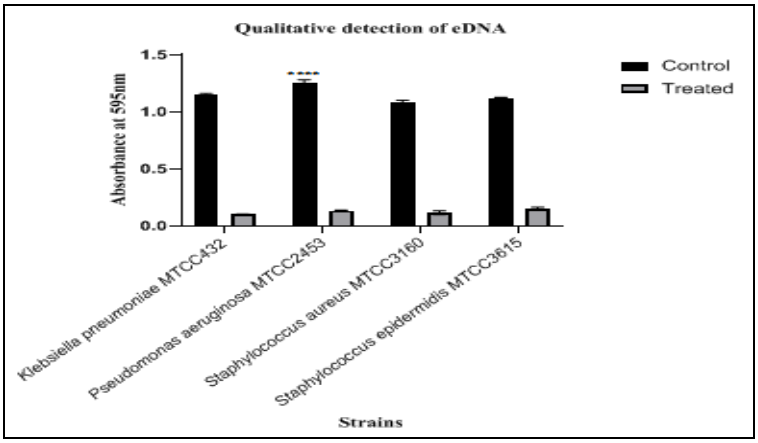


FIG. 5: EFFECT OF 1% ACETIC ACID ON THE PRESENCE OF EXTRACELLULAR DNA (EDNA)

Determination of Effect of Antibiofilm agent on Production of Exopolysaccharide: It was observed that in the presence of 0.2% acetic acid, there was a reduction in the EPS content produced by the biofilm forming strains. The reduction in EPS content observed for *Klebsiella pneumoniae* MTCC 432, *Pseudomonas aeruginosa* MTCC 2453, *Staphylococcus aureus* MTCC 3160 and *Staphylococcus epidermidis* MTCC 3615 is 47%, 48%, 46% and 45% respectively. The data was analyzed by two-way ANOVA and the p value was found to be <0.0001.

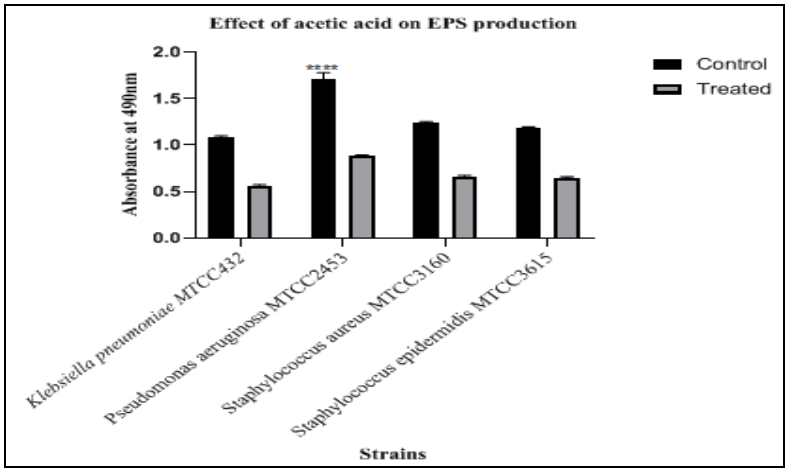


FIG. 6: EFFECT OF 0.2% ACETIC ACID ON THE PRODUCTION OF EPS

Determination of Effect of Antibiofilm agent on Emulsification of Exopolysaccharide: It was observed that 1% sodium dodecyl sulphate was able to emulsify EPS of the biofilm. The reduction in EPS content observed for *Klebsiella pneumoniae* MTCC 432, *Pseudomonas aeruginosa* MTCC 2453, *Staphylococcus aureus* MTCC 3160 and *Staphylococcus epidermidis* MTCC 3615 is 73%, 81%, 81% and 83% respectively. The data was analyzed by two-way ANOVA and the p value was found to be <0.0001.

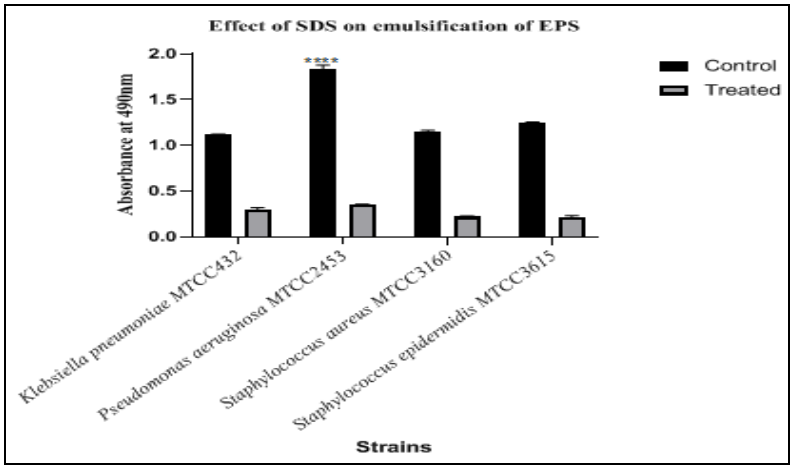


FIG. 7: EFFECT OF 1% SODIUM DODECYL SULPHATE ON THE EMULSIFICATION OF EPS

DISCUSSION: Biofilm associated infections pose a significant clinical challenge due to their resistance to antimicrobial agents and their role in persistence and dissemination of antimicrobial resistance. This study was carried out with an aim to optimize and standardize a set of *in-vitro* assays for evaluation of efficacy of antibiofilm agents using acetic acid and sodium dodecyl sulphate as model agents against clinically relevant biofilm forming bacterial strains. This study optimizes methods for assessing biofilm inhibition, eradication, dispersion, cell viability and presence of key matrix components like extracellular DNA and exopolysaccharides [2]. The determination of MBIC revealed that 1% acetic acid consistently inhibited biofilm formation across all tested strains. The high R^2 values obtained from non-linear regression analysis indicate assay performance and

reproducibility. MBEC values for SDS varied among the test organisms indicating the species-specific differences in biofilm composition and the ability of SDS in emulsifying their matrix for effective disruption of biofilms. The gram-positive strains used in the study exhibited lower MBEC values as compared to the gram-negative strains. This could be likely due to differences in EPS density and outer membrane complexity [4].

Biofilm dispersal assays indicated that sub-eradication concentrations of SDS were sufficient to induce dispersal of cells from mature biofilms, though the optimal concentrations for dispersal varied among strains. The TTC based viability detection revealed a critical point that higher concentrations of the antibiofilm agent may increase the dispersal of cells but will give rise to reduced detectable signal as these agents at higher concentrations may be lethal for the cells. This emphasizes the need to carefully select the concentration of the agent and the detection methods to assess dispersion in order to avoid misinterpretation of the antibiofilm activity [18].

Viability assays performed using plate counts confirmed loss of viability in biofilm associated cells with the treatment of 1% SDS for 6 hours. This may not be the case for all antibiofilm agents as majority of the agents act as emulsifying agents and help in disruption of biofilm matrix leading to dispersal of cells without affecting the viability of cells.

A significant reduction in eDNA was observed in the presence of acetic acid. This may be due to growth inhibition activity of acetic acid. The basal readings observed could be due to the lysis of the cells in the inoculum. Similarly, acetic acid significantly reduced the EPS production across all strains [19] while SDS emulsified and disrupted the preformed EPS which confirms the surfactant mediated disruption activity of SDS. For these assays to work effectively, it is also important to minimize the variability in assays that can be introduced by inoculum density, incubation conditions, treatment duration etc [8, 20, 21]. Use of these optimized methods will ensure reduced variability and enhanced inter laboratory comparability of data and will improve screening accuracy of antibiofilm agents.

The limitation of the study is that the interpretations are based only on crystal violet assay and TTC which measures metabolic activity. Further microscopic evaluation of the treated biofilms is needed to understand the structural changes and also needs inter laboratory validation.

The study focuses mainly on acetic acid and sodium dodecyl sulphate as antibiofilm agents, use of other coloured antibiofilm agents, reducing or precipitating agents will require assay-specific controls as they may interfere with the assays used in this study.

CONCLUSION: The present study successfully optimized and standardized *in-vitro* methods to evaluate antibiofilm efficacy. The methods optimized include assessment of biofilm inhibition, eradication, dispersion, cell viability and presence of eDNA and EPS.

The findings demonstrate that antibiofilm agents can be screened for their ability to disrupt biofilms or inhibit its formation in a reproducible manner. It also indicates the need to assess the agent against different biofilm forming strains as the potential of the agent may be species specific. Overall, the standardized methods minimize experimental variability and reduce the risk of over or under estimating antibiofilm efficacy. These methods can be used for screening and comparing novel antibiofilm agents and also increases the potential of translational relevance of in vitro biofilm research.

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CONFLICTS OF INTEREST: Nil

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