

MBDS Solvent: An Improved Method for Assessment of Biofilms

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ABSTRACT

Biofilms are recognised as an important contributor to bacterial resistance towards traditional antimicrobial treatments. Assessment of biofilm formation currently relies on a 96 well microtitre plate assay, which usually involves the colourimetric detection of stain (typically crystal violet) removed from previously stained biofilm. The amount of crystal violet released is then used as a quantitative indicator of the amount of biofilm formed. Currently, this is achieved by solubilisation of the stain by ethanol which results in partial decolourisation of the crystal violet stained biofilm which impacts the accuracy and reproducibility of this method. Herein, we describe a modified biofilm dissolving solution (MBDS) which produces a more uniform and reproducible colour release from stained biofilm through solubilisation of the biofilm architecture itself. Here we use crystal violet stained biofilms of *P. aeruginosa* strain PA0-1, to demonstrate an approximate two fold increase in crystal violet release by MBDS, as compared to ethanol treatment. In addition, when ethanol decolourised biofilms were treated again with MBDS, an almost equal amount of remnant crystal violet was recovered by dissolving the biofilm and the stain trapped within it. These results were reflected in microscopic analysis of ethanol treated and MBDS treated biofilm. Similar results were obtained when MBDS was used to decolourise and dissolve the biofilms of a number of other bacterial species highlighting the advantages of MBDS as a universal solvent for the colour detection of biofilm.

Keywords: Crystal Violet; *Pseudomonas aeruginosa*; Microtitre Plate Assay

1. Introduction

Biofilms are a mode of surface associated microbial growth which bacteria frequently employ in order to better deal with environmental stresses [1]. Biofilms also play an important role in the infection processes of a number of organisms by contributing strongly to factors such as antimicrobial resistance. *Pseudomonas aeruginosa* is a bacterium well documented to form biofilms on moist surfaces, medical instruments and in chronic respiratory infections, particularly in cystic fibrosis patients [2-4]. Biofilms of *Streptococci* and *Staphylococci* species have also been implicated in many nosocomial infections, such as encrustation of catheters and cases of endocarditis [5,6].

One of the main characteristics of bacterial biofilms is a complex extracellular matrix secreted around the adherent cells [7]. This extracellular matrix consists of a number of long chained polymeric molecules [8]. The general composition of these molecules can vary between

species [1] but are primarily comprised of exopolysaccharides and lipopolysaccharides [7]. Within this matrix, bacteria are able to survive for a significantly longer time than their planktonic counterparts [9] due to the protection from adverse environments [10] including the protection from phagocytes and components of the host immune system [11].

Due to the importance of biofilms to the infective pathways of a number of organisms, there is a need to be able to accurately and effectively quantitate biofilm formation and to study the effects of agents which may be able to inhibit the processes of biofilm formation. Currently, many assays rely on the spectrophotometric quantitation of ethanol decolourisation of crystal violet stained biofilm [12,13]. This method results in a widely varying data due to ineffective decolourisation and solubilisation of the stained biofilm. Here, we describe an alternative method which relies on solubilisation of stained biofilm matrix, providing increased reliability and sensitivity.

2. Materials and Methods

2.1. Biofilm Formation

The *P. aeruginosa* strain PA0-1 was grown aerobically in liquid Luria Bertani media for 12 hours. A number of other bacterial species were used; *Klebsiella pneumoniae* strain GH-KP1, *Enterobacter cloaca* strain GH-EC1, *Serratia marscescens* strain GH-SM1, *Clostridium perfringens* strain GH-CP1 and *Staphylococcus aureus* strain GH-SA1. These bacterial strains form part of the Griffith University Culture Collection and were isolated from clinical sources from the Gold Coast Hospital. These bacterial strains were grown aerobically overnight on Columbia blood agar (Oxoid) supplemented with 5% defibrinated horse blood. Bacterial cultures were then diluted with fresh Luria Bertani broth (Oxoid) an optical density of 0.5 as assessed at 600 nm. 100 μ L of this bacterial suspension was then individually inoculated into a minimum of 3 rows of 8 wells in polystyrene 96-well microtitre plates (Nunc). Bacterial suspension was also used to inoculate 24-well plates containing glass cover slips. The plates were incubated aerophilically at 37°C for 24 hours as outlined [13].

2.2. Crystal Violet Staining and Solubilisation

The 96-well plates containing formed biofilm were then washed with distilled water three times to remove planktonic cells and media. A 1% crystal violet solution (Sigma) was then used to stain the remaining biofilm and allowed to penetrate for 15 minutes. The wells were then washed a further 3 times with distilled water until runoff was clear.

Four solutions were then assessed for the ability to extract crystal violet from the stained biofilm; 95% ethanol (Chem Supply) (a standard solution for crystal violet decolourisation of 96-well plate biofilm assays (12), a 5% sodium dodecyl sulphate (SDS, Amresco) solution, a modified biofilm dissolving solution (SDS dissolved to a final concentration of 10% with 80% ethanol in H₂O) and distilled H₂O. It was hypothesised that when combined with ethanol, SDS would be more able to break down the lipid rich architecture of the biofilm, liberating more crystal violet and providing a more complete assessment of biofilm formed.

Following staining of the biofilm in the 96-well plates, crystal violet was solubilised with 100 μ L of solvent and mixed by pipetting and removed into a clean plate. The plate containing solubilised crystal violet was then read with a Victor X multilabel plate reader at a wavelength of 600 nm. A student's t-test *P* value of ≤ 0.05 was used as a cut-off when testing for significant difference between absorbance readings.

2.3. Microscopic Analysis

The cover slips containing formed biofilm were removed from the 24-well plates and washed three times to remove planktonic cells. A 1% crystal violet solution was used to stain biofilm containing cover slips. These were then treated with either ethanol or MBDS. The cover slips were then re-stained for a further 15 minutes with crystal violet, dried and mounted onto glass microscope slides. The slides were then examined using a Nikon Eclipse E600 light microscope and images were taken using a Nikon DXM-1200C digital camera and Act-1 visualisation software.

3. Results and Discussion

3.1. Assessment of SDS Based Decolourises of Stained *P. aeruginosa* Biofilm

Following biofilm formation, each series of wells was stained with a 1% crystal violet solution. One of either ethanol (95%), MBDS, SDS solution (5%) or distilled H₂O was then applied to determine the effectiveness in solubilising crystal violet from stained biofilm.

Upon treatment with each respective agent, MBDS and 5% SDS showed an almost 2 fold increase (*p* value > 0.05) in biofilm recovery compared to traditional ethanol treatment (Figure 1). In addition, a number of other differences were observed in the method of solubilisation. The 5% SDS solution showed a uniform colour, however, substantial foaming was produced, impacting on the accuracy of the spectrophotometric assessment of dissolved crystal violet. The MBDS demonstrated similar level of decolourisation to 5% SDS, however, the foaming of the solution was substantially reduced. Ethanol solubilisation was not effective in completely solubilising crystal violet, with many insoluble clumps of crystal violet stained material being present during treatment. The H₂O control shows a negligible OD600 spectrophotometer reading of ≤ 0.05 suggesting that it is ineffective at liberating crystal violet from stained biofilm and was used as a control.

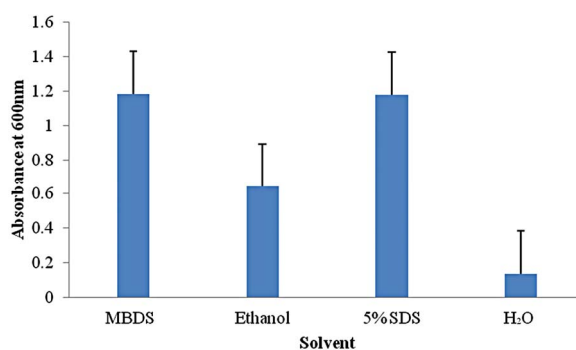


Figure 1. Spectrophotometry values quantifying the initial recovery of crystal violet from stained biofilm by different solvents.

Following the initial treatment with solvents, the remaining biofilm was again washed with water and a MBDS treatment was then applied to all wells to determine whether any remnant crystal violet could be extracted from the remnant of stained biofilm. Both MBDS and SDS showed similar recovery of stain following subsequent MBDS treatment (**Figure 2**). Stained biofilm previously treated with ethanol yielded further crystal violet recovery following the MBDS treatment equal to that detected by the initial spectrophotometer reading, suggesting that ethanol was only able to dissolve approximately 50% of the crystal violet penetrating the biofilm. The control H₂O treated wells showed that almost none of the crystal violet was dissolved in the initial H₂O treatment as resolubilisation by the MBDS showed a spectrophotometer reading similar to those shown in **Figure 1** for initial solubilisation by the MBDS.

Following spectrophotometric assays, microscopic analysis was conducted in order to determine the effect of ethanol and MBDS treatment on formed *P. aeruginosa* biofilm. Prior to treatment, stained biofilm shows that much of the slide contains large aggregates of bacterial biofilm covering the microscope slide. Ethanol treatment of biofilm shows that large aggregates have detached, however, many of the smaller clusters of biofilm remain. Treatment of biofilm with the MBDS results in solubilisation of the majority of formed biofilm and little remains adhered (**Figure 3**). This was reflected with secondary MBDS treatment which demonstrates a marked decrease in stained biofilm of previously ethanol treated biofilms. This was not seen with secondary ethanol treatment of formed biofilm. This further demonstrates the solubilisation action of the MBDS. Although large aggregates can easily detach following ethanol treatment, much of the crystal violet remains trapped in smaller clusters which are solubilised following treatment with the MBDS.

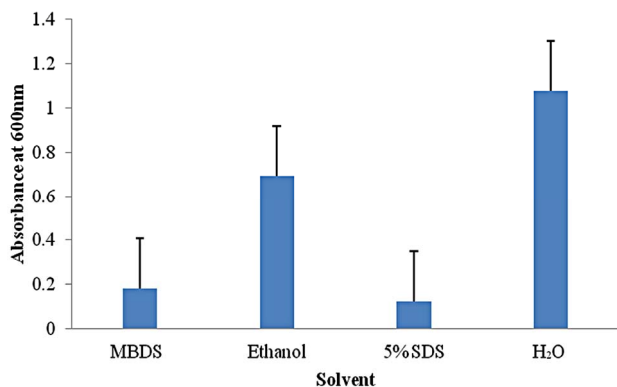


Figure 2. Spectrophotometry readings showing crystal violet recovery by secondary MBDS treatment of previously solvent treated biofilm.

3.2. Effectiveness of MBDS on Biofilms of Other Bacterial Species

Similar assays were conducted to evaluate the MBDS solvent for its ability to quantitate biofilms of a number of other Gram negative and Gram positive bacterial species. Formed biofilm was stained with crystal violet and either ethanol, MBDS, SDS or H₂O were used to decolourise stained biofilm of four Gram negative organisms (*S. marscescens*, *K. pneumoniae*, *E. cloacae* and *C. perfringens*) and a Gram positive organism (*S. aureus*) (**Figure 4**). Gram negative organisms tested showed similar results to *P. aeruginosa* biofilm with an approximate two fold increase in the amount of stained biofilm recovered by MBDS when compared to ethanol (all *p* value > 0.05). The Gram positive organism *S. aureus* showed only a slightly increased OD600 when treated with MBDS (*p* value 0.3).

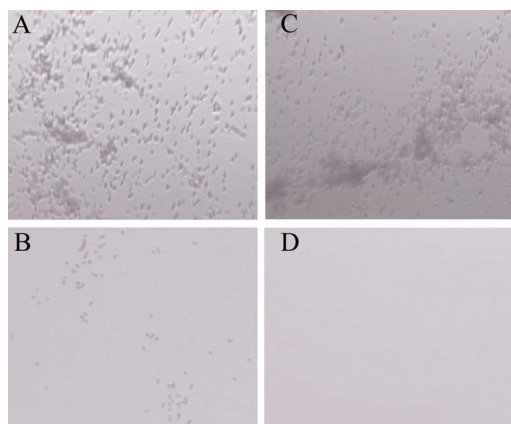


Figure 3. Comparisons of formed biofilm with ethanol or MBDS treatment. (A) Pre-ethanol treated biofilm; (B) Post-ethanol treated biofilm; (C) Pre-MBDS treated biofilm; (D) Post-MBDS treated biofilm. All images taken at 400× magnification.

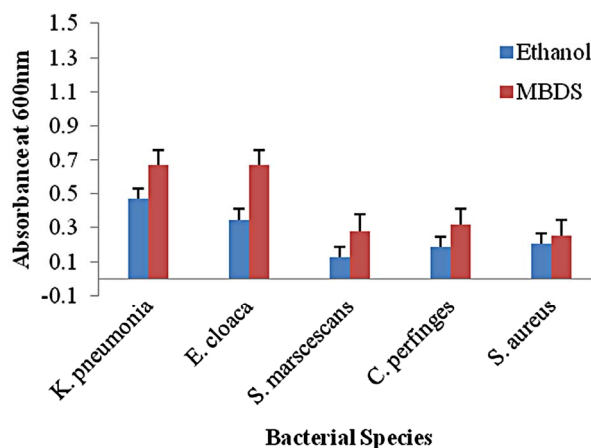


Figure 4. Comparisons of the ability of MBDS and ethanol to recover crystal violet from stained biofilms of a number of bacterial species.

3.3. MBDS Dissolves Biofilm Rather than Decolourises

Ethanol is widely accepted to decolourise stained biofilm, leaving biofilm at least partially intact whilst extracting crystal violet. However, crystal violet stained portions of the biofilm, which may not be accessible by ethanol, remain. The MBDS appears to work through solubilisation of the biofilm itself. In order to confirm this, stained biofilm was treated and re-stained to determine whether there was any residual biofilm remaining in the wells of the microtitre tray.

When subjected to a secondary treatment, the MBDS shows that there is significantly less recoverable crystal violet upon re-staining and decolourising when compared to the initial stain and treatment (p value ≤ 0.05) then following the traditional ethanol treatment, suggesting that the biofilm itself may have been solubilised (Figure 5). Ethanol treatment following re-staining shows that the amount of crystal violet recovered is similar to that released by the initial treatment (p value > 0.05), suggesting that much of the original biofilm remains.

A large portion of the biofilm architecture of most organisms consists of lipid and glycan based polymeric molecules such as lipopolysaccharides [7]. Although widely used [12,13], traditional biofilm quantitation that involved staining the biofilm with crystal violet and then destaining it with 95% ethanol, is often unreliable due to partial decolourisation of the stain and presence of insoluble fragments of stained biofilm in solution. In contrast, the SDS component of the alternative solvent, MBDS, described in this study, appears to be able to solubilise the most of the biofilm matrix, as compared to traditional ethanol treatment, allowing more complete liberation of crystal violet from stained biofilm. In addition to providing greater recovery of crystal violet from biofilm, MBDS produces a more uniform colour, dissolving ethanol insoluble clumps of biofilm. This uniform colour provides a more accurate assessment via spectrophotometry. The dissolution of formed biofilm

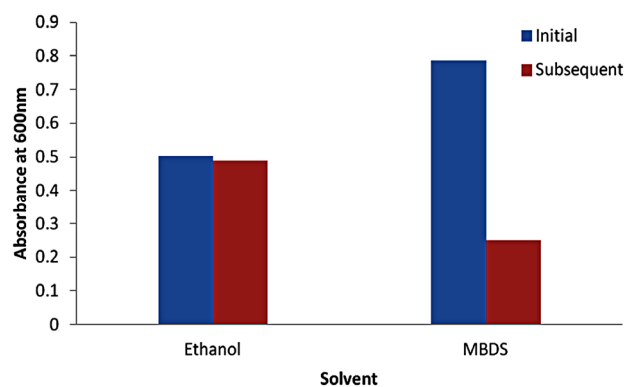


Figure 5. Spectrophotometer values showing recovered crystal violet from initial treatment and re-stained biofilm.

also provides a more efficient and accurate assessment of the amount of biofilm formed. Whilst the SDS component is responsible for the action of MBDS, when used alone as a solution, substantial foaming by the detergent is produced and affect the ability of traditional 96-well plate readers to accurately assess the optical density.

When MBDS was used to quantitate the biofilms of other Gram negative organisms, it yielded the same degree of biofilm solubilisation as for *P. aeruginosa* biofilm, whereas the recovery of stain from the biofilm of Gram positive *S. aureus* resulted in less significant difference to that of the traditional ethanol solubilisation. This may be due to dissimilarity in the components of Gram negative and Gram positive biofilm architecture. Gram positive organisms do not possess an extensive lipid profile when compared to Gram negative bacterium. In addition, Gram positive organisms have a decreased capacity to synthesise lipid molecules. This reduced lipid content compared to Gram negative organisms will enable ethanol to more effectively penetrate the biofilm leading to only a slight increase in MBDS recovered biofilm stain.

Biofilms are an important component involved in infections of many bacterial organisms and the need to find agents inhibiting biofilm formation will enable the prevention of many nosocomial infections such as those of *P. aeruginosa*. Novel antimicrobial agents are continuously being investigated for potential anti-biofilm activity and usually rely on the colourimetric microtitre plate method for assessment of biofilm formation. The use of an MBDS solution for assessment of biofilms, particularly when levels of the biofilm may be reduced, will provide a more accurate and reliable measurements to be achieved.

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