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Targeting Bio-Film in Myco-Bacteria

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Abstract

The current project is based on the experimental work of Dr, Ashwani Kumar. Drug tolerance and resistance are the major proponents of any bacterial disease like TB. These mechanisms are achieved through unique phenomenons like the formation of a biofilm. The current project focuses on the formation of biofilms in *Mycobacterium smegmatis* (Msm) cultures. These biofilms are in the form of pellicles. The principal work done in the current project is the induction of biofilm using DTT at a range of concentrations in a 96 well plate. The films were then visualised using the Crystal Violet assay. The entire project focuses more on the process of biofilm formation and the mechanisms. The performed work can be further used for screening drugs and exploring ways to combat AMR.

Keywords: Biofilm, DTT, AMR, Drug Resistance, Mycobacterium Smegmatis (Msm), Mycobacterium Tuberculosis (Mtb).

INTRODUCTION

My previous project in last year was based on the Latent Tuberculosis Infection and how it promotes cardiovascular diseases in its patients. The research on LTBI infection was centred around the genus 'Mycobacterium' and mainly focused on the bacteria '*Mycobacterium tuberculosis*' with special emphasis on how the bacteria cause the tuberculosis infection and enter the stage of latency as well as the mechanisms they use, to maintain the state of latency. Several mechanisms were explored throughout the process of writing the systematic review, one of them being biofilm production. (EH Tobin *et al*.,)

The current project, is a lab based work that focuses on the production of biofilm using the bacteria '*Mycobacteria smegmatis'* as the model for observing biofilm production in a thiol reductive stress based environment induced using DTT (Dithiothreitol) at a range of concentrations to check which one of them is the most suitable for the formation of stable biofilms in the model pathogen (Kumar A. *et al*.,).

As per Tumulu *et al*., (1), Mycobacteria have had a very long association with the immune systems of humans. This long term association has encouraged the human immune systems to adapt and enhance its response due to long term exposure to the pathogen in the environment. Amongst the genus Mycobacteria, *M. leprae* is responsible for causing leprosy, *M.bovis* is famous for being a notorious pathogen amongst animals and the 'tuberculosis complex' is known to contain several pathogenic species that cause tuberculosis like Mtb. The nontuberculous mycobacteria commonly referred to as 'NTM' mostly acquire the environmental zones or habitats along with habitating the plumbing zones and shower heads. They are omnipresent and hence, have coevolved with the humans.

Tuberculous and non tuberculous bacteria are capable of inducing allergies and paradoxically they reduce the risk of development of allergic disorders as well. This paradox exists due to immunomodulation and the activation of regulatory immune pathways.

Experimental studies have demonstrated how exposure to environmental mycobacteria reduces the risk of developing allergies in allergic asthma mouse models. A randomised control trial was conducted with 24 males having mild/moderate asthma. The trial showed a decrease in IL-5 and IgE post a single intra-dermal injection of the heat killed version of *Mycobacterium vaccae*, an environmental mycobacterium. The paper has also stated that the bacteria are also responsible for reducing the risk of other infections like atopic dermatitis but these conclusions vary as per the dosage, timing and administration routes. This phenomenon is called heterologous protection (protection against other pathogens). The paper has aimed at exploring mutualism between humans and mycobacteria but the results turn out to be mixed. (Tumulu *et al.*, (1))

As per Brennan *et al*., (2), Mycobacteria have cell walls of low permeability, that helps them develop resistance to therapeutics. The cell walls generally have several C60-C90 fatty acids and mycolic acids that have covalent bonds with arabinogalactan. The hydrocarbon chains of the extractable lipids present in their cell wall, form an exceptionally thick asymmetric bilayer. Differences in the structure of mycolic acids affect the permeability and fluidity of this bilayer.

According to Cook G.M. *et al*., Mycobacteria are aerobic and have a wax- like envelope. The genus has nearly 100 closely related species, including *M. smegmatis* which is an environmental species of the genus. The bilayer includes peptidoglycan-arabinan based on several clinically significant species that have a much slower growth rate in vitro. The genome size is approximately 3.27 Mb (*M. leprae*) to 6.99 Mb (*M. smegmatis)*. Mycobacteria have at least one or two ribosomal RNA operons. They have highly developed inner membrane transporters for substrates like carbohydrates, lipids, phosphates, sulfates, nitrogen compounds etc.

Mycobacteria enter hypoxic states within the granulomas and exhibit non-replicating persistence (NRP). NDH-2 is important for species like Mtb and *M.smegmatis* during respiration for NADH oxidation along with the linked proton gradient. Under the hypoxic state, the genes coding for fumarate reductase, nitrate transport, and other hydrogenases are activated.

The current project focuses on the species, *M.smegmatis* more which has one of the largest mycobacterial genomes and still is a fast growing, non-pathogenic, environmental mycobacterium. It has a growth rate of 0.23 per hour (generation time is nearly 3 hours). Its ecoindex is very close to that of the metabolically active form of E.coli. The cells have a slender rod shape, with the length typically depending on the age of the culture. Phylogenetic analysis of the species, shows that the ancestral strains have had 2 operons, rrnA and rrnB (Cook G.M. *et al*.,).

As per Cook G.M. *et al*., the unique feature of these bacteria, unlike other species, is that the inactivation of any of the 2 operons does not impact its specific growth rate. In its outer membrane and the cell envelope, the MspA improves permeability and forms a water filled channel (about 9.6 nm long). M. smegmatis. Also has a wider range of transporters like ABC, MFS, PTS, MIP and SSS families. It has an efficient respiratory chain, comprising NDH-2 and 1, menaquinone, succinate dehydrogenase, cytochrome bd along with the bc1-aa3 branch. It is capable of growing on several carbon sources, like glycerol, glucose, fatty acids and cholesterol.

The following table represents the basic features of the selected model bacteria, *M. smeg*.

Table 1.1: Features of *M. smegmatis* (As per Cook *et al*.,)

Feature	**M. smegmatismc²155**
Basic nature	non-infectious, NTM (environmental saprophyte), a prominent model mycobacterium
Size of the genome	6.99 Mb (largest amongst the entire genus)
Growth parameters (growth rate)	0.23 h^{-1} (generation time ≈ 3 h) in laboratory media.
Value of Ecoindex	Nearly 0.74, (close to that of E.coli)
Rate of DNA replication	For S phase it is approximately, 2.0×10^6 bp h^{-1}/ replication fork; Duration of the S-phase: 1.75 hours.
Types of operons	rrnA and rrn B
Size of the cells and their shape	Diameter: 0.3–0.5 µm; Rod shaped cells
Dominant outer membrane porin	MspA
Function of MspA	Its deletion significantly decreases the uptake of phosphates and sugars. This shows that fast growth occurs due to this porin
Uptake of Phosphates	It has at least three high affinity systems meant for uptake including PstSCAB and PhnDCE, along with an unidentified third system.
Respiratory enzyme (ATP synthase)	Essential atpBEFHAGDC (F_1F_0-ATP synthase)
Value of the proton motive force	180 mV
Sources of carbon	glycerol and other carbon sources mentioned in the paper

The write up now shifts on to the topic of 'Biofilms', the major aspect of the current work.

Most of the mycobacterial species including the environmental and pathogenic ones, treat biofilm formation as a part of their default lifestyle.

Biofilms consist of a group of mycobacterial cells that can be adhered or just existing as a group of cells. As per Kumar A. *et al*., they can adhere to a surface or to each other, self- produce an extracellular matrix and then the cells get enclosed within the extracellular matrix. After getting enclosed, the cells then enter a persistent yet slow growth stage characterised by a high grade tolerance to drugs like antibiotics and other environmental stressors. The paper states that biofilms are their primary mode of growth in their various habitats including human hosts.

Mycobacteria uniquely form hydrophobic cell walls that exhibit strong adhesion combined with clumping. The ECM is lipid rich and these biofilms are robust even in water systems. They also have an extra cellular DNA that helps in providing structural integrity, drug/antibiotic tolerance and a solid architectural backbone for the biofilm. The biofilms can be in the form of pellicles as seen in Mtb and or chosen model bacteria, Msm. These biofilms of Mtb contain drug tolerant and resistant bacilli. The other type of biofilms are surface attached biofilms as per the paper (Kumar A. *et al*.,).

Generally, important cell wall components in mycobacteria are mycolic acids, trehalose dimycolate, glycopeptidolipids and phthiocerol dimycocerosates. These components help in surface attachment, aggregation and microcolony formation. In Msm, polysaccharides, most importantly extracellular polysaccharides, form a significant component of the biofilms. The paper also states and has experimentally concluded that the Msm biofilms like Mtb also contain cellulose as their main component (Kumar A. *et al*.,).

A basic sequence of biofilm formation in mycobacteria first the stage of aggregation and surface attachment followed by microcolony formation along with the formation of serpentine rope-like bundles (cording). Whenever, there is an increase in the environmental stress or when there is a sudden change in the nutrients, the biofilm disperses releasing the highly infectious cells. Due to the biofilm entrenchment, culturing becomes very difficult (Kumar A. *et al*.,).

As per Yang Z. *et al*., for biofilm formation, there are various in vitro models like the pellicle biofilm model that shows floating biofilms at the interface of air and liquid, surface attached biofilms show biofilms formed on solid surfaces, clumping models show mycobacterial cell clumps behaving as micro-biofilms, the flow cell model depicts biofilms grown under continuous liquid flow. In vivo models involve granuloma associated biofilms, implant infection models meant for NTM, high throughput microtiter plate model, solid media wrinkle model/colony biofilms.

According to R. A. Muzamil *et al*., there are several mechanisms of biofilm formation. These mechanisms have been briefly discussed through the previously discussed paper. The bacteria first make weak, reversible adherence through hydrophobic/electrostatic interactions. After this, the attachment becomes irreversible with the help of adhesive proteins, pili or fimbriae, altered genetic expression, or lipids. The formation occurs generally when the stress response gets activated. Post adhesion, microcolonies form through clonal expansion. During this stage, extracellular polymeric substances are produced. These form the backbone of the biofilm. As cells come together and form a biofilm, they begin communicating with each other through a process called 'Quorum sensing'. The secondary messengers also get activated during this process like c-di-GMP, promoting sessile growth conditions, cell adhesion with respect to each other and the surface. As the amount of the secondary messengers decreases, the cells disperse and later the biofilm disintegrates. QS also induces the RND pumps, ABC transporters and MFS pumps. It also helps in the production of antioxidant enzymes. Antibiotic resistant enzymes like beta lactamases and proteins are present in the biofilm matrix. c-di-GMP also gets regulated through diguanylate cyclases (increase the amount) and phosphodiestrases (decrease the amount).

The biofilm promotes drug resistance which is multi faceted. The film acts as a physical barrier blocking antibiotics as well as neutralising other drugs along with slowing down the drug diffusion process. These mechanisms involve the upregulation of efflux pumps and various ways of horizontal gene transfer allowing the spread of AMR (antimicrobial resistance) genes. The adaptive environment of a biofilm allows selection for only the antimicrobial resistant cells.

The paper also states the several types of QS molecules like, N-acyl homoserine lactones (gram-negative bacteria), autoinducing peptides (gram positive bacteria), Pseudomonas Quinolone Signal and AI-2 (autoinducer) (R. A. Muzamil *et al*.,).

The biofilm of *Pseudomonas aeruginosa* has four QS systems, like las, rhl, PQS and IQS. las induces rhl, rhl then activates PQS which then further activates IQS. AI-2 is important for biofilm formation in *Staphylococcus epidermidis* (R. A. Muzamil *et al*.,).

However, biofilms generally exhibit drug tolerance more than resistance. But resistance and

tolerance go hand in hand and one can convert into the other. e-DNA is very crucial for conferring antibiotic resistance.

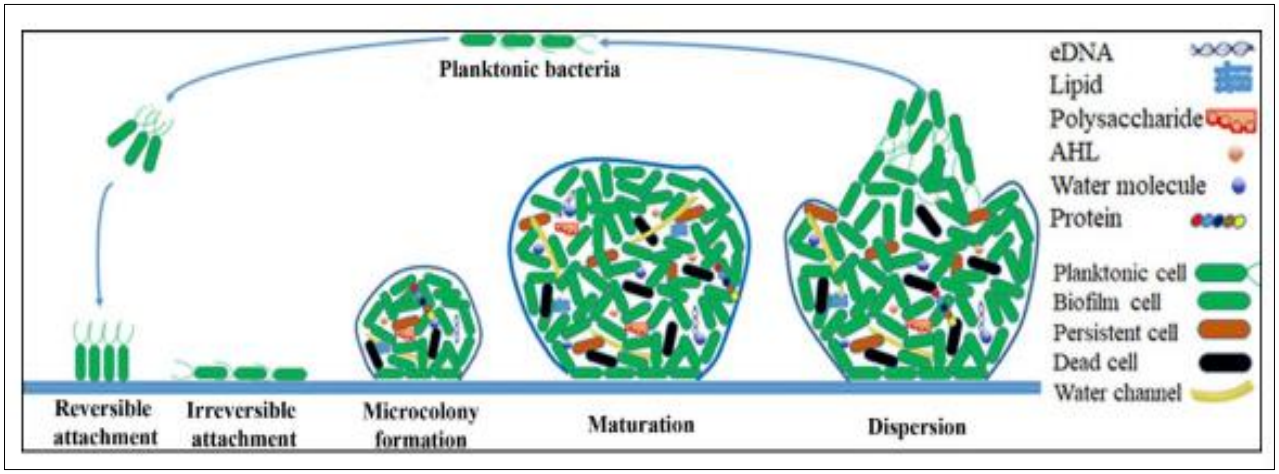

Figure 1.1: Process of Biofilm formation (R. A. Muzamil et.al)

During the attachment stage, glycolysis genes downregulate while the ones for gluconeogenesis upregulate. The opposite occurs during the stage of maturation. Signalling pathways like cAMP-PKA, MAPK and transcription factors like Phd1, Sok2 etc. are also involved (R. A. Muzamil *et al*.,).

In *A.fumigatus*, adhesion is regulated by MAP kinase pathways involving SakA, MpkA and MpkC. In *K. Pneumoniae*, the PTS enzyme second complex is crucial. *Candida albicans,* ARO1 gene is crucial for the biofilm synthesis. sRNA plays an important role in *E.coli, Salmonella, aeruginosa and V. cholerae* (R. A. Muzamil *et al*.,).

MATERIALS AND METHODS (Kumar A. *et al*.,)

Equipments and materials/chemicals used:

Chemicals: ADC, Glycerol, 7H9, milliQ water, DTT, Msm culture, 1XPBS, ethanol.

Equipment: Nanodrop, spectrophotometer, autoclave, shaking incubator, biosafety cabinet, culturing tubes, eppendorf tubes, spatula, beaker, pipettes, burner, 96 well plate.

The current work is based on the experimental research of Dr. Ashwani Kumar and his team. His work established that a Thiol Reductive Stress environment created using DTT (dithiothreitol) induces the formation of mycobacterial biofilms in Mtb and Msm. Media components like BSA were observed to check whether it plays any role in biofilm formation. The composition of the biofilms was also analysed (Kumar A. *et al*.,).

Only a section of the entire reference research has been performed here. The project work involves the induction of biofilm formation in Msm using DTT. The reference research states that DTT has a very high reduction potential of -0.33 V at a pH value of 7.2. This potential was observed to reduce the disulfide bonds at a range of millimolar concentrations. This procedure has been performed in the lab. BSA is a crucial component of the supplements that are used for the mycobacterial cultures. BSA is a protein of size 66 kDa with nearly 17 disulfide bonds along with an unpaired cysteine molecule. This structure makes it highly prone to reductive factors. The reduction of BSA leads to its aggregation, during which amorphous aggregates form through disulfide bonds that are formed randomly (Kumar A. e*t al*.,).

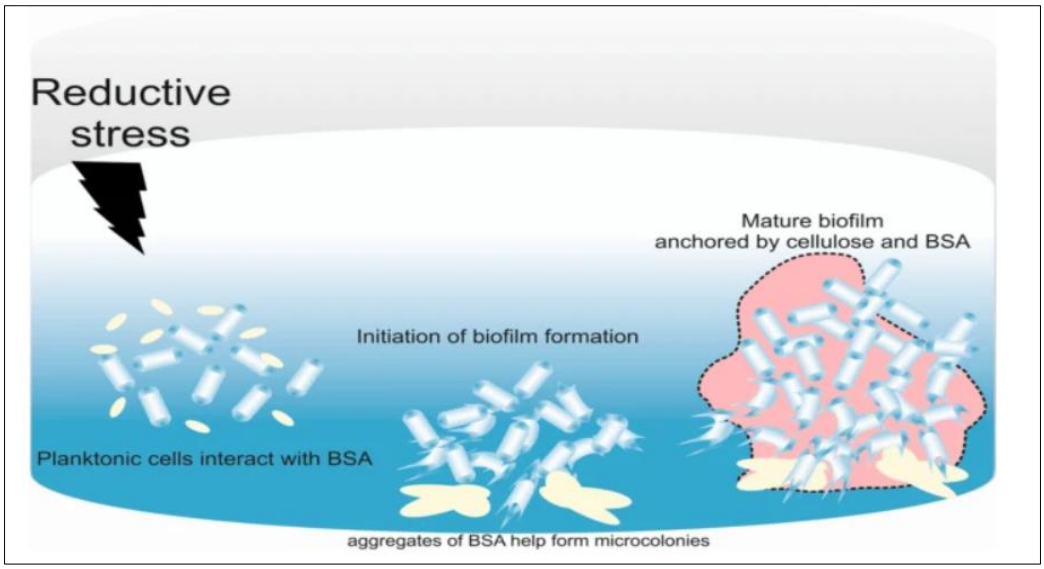

Figure 1.2: Induction of Biofilm using DTT (Kumar A. *et al*.,)

This aggregation had been observed in the lab as well. As per the paper, a 4mM concentration is enough to induce biofilm formation and 6mM concentration shows the highest amount of biofilm formation (Kumar A. *et al*.,).

First, for the culture, 200 ml of the media was prepared. For this, Middlebrook 7H9 medium was used which was supplemented with 10 percent ADS along with 0.2 % glycerol and 0.05 % Tween 80 and milliQ water. This entire mixture was then autoclaved at 121℃,15 psi for 15 minutes. After the mixture cooled down, it was stored and ADS was added only before the time of use. (Kumar A. *et al*.,).

After the media was prepared, primary inoculation was performed, for which 10 ml of the media was used, followed by the addition of 10 percent ADS. It was then aliquoted into 5 ml media for control and 5 ml for the culture. From the stalk, about 10 ul of the culture was added into the falcon tube for inoculation. This entire process was carried out in a biosafety cabinet (Kumar A. *et al*.,).

After the inoculation, the tubes were taken out and incubated at 37℃ at 220 rpm in a shaking incubator. The primary culture was then left overnight in the incubator. The next day the secondary culture (subculturing) along with the DTT assay was performed on a 96 well plate. For the assay, 1 M stock solution of DTT was prepared. (Kumar A. *et al*.,).

For the double dilution method which involved the DTT assay as well, one technical plate and duplicates were prepared Three controls were kept, one with the media and DTT, the other with just the bacterial culture and the third was that of the media only. The total volume to be maintained was 200 microlitre and the initial concentration in the first well was kept as 12 mM. The technical plate was prepared in the same way, as the duplicates. Only a slight change was present in the layout of the plate (Kumar A. *et al*.,).

In the technical plate, starting from B to G, second lane was, meant for the combination of media + culture+ DTT, the third lane for the combination of media and DTT (control 1), on lane 4 and 5, B4 and B5 wells were meant for the media control only (control 2) and the C4 and C5 for the bacterial culture (control 3) (Kumar A. *et al*.,).

Before the assay was performed, the OD of the culture was set at 0.4 so that through dilution, the OD turns out to be 0.2 using a spectrophotometer (Kumar A. *et al*.,).

First, 197.6 microlitre of the media was added and 2.4 microlitre DTT in the first well (B2). After this, 100 microlitre of the media was added in the consecutive wells until G2 which was then serially diluted. After this, 100 microlitre of the culture was added into the first well and the same process of dilution. The same method was applied to the wells from B3-G3 with the DTT control. The only difference here, was that instead of the culture, media was used for the second time of dilution. In the fourth and the fifth column, in B4 and B5, 100 microlitre of the media was added in each well and in C4 and C5, 100 microlitre of the culture was added in each well (Kumar A. *et al*.,)

The same procedure was performed for the duplicates, where column 2 and 3 was meant for the first combination, 4 and 5 for the DTT control and then in column 7 and 8, in B7 and B8, 100 microlitre each had the culture and in D7 and D8, 100 microlitre each had the media control. The assay was then incubated for nearly 56 hours, instead of the standard protocol of 29 hours (Kumar A. *et al*.,).

The initial and final readings of the OD of the bacterial culture were taken after the CV assay. This was done to calculate the inhibition percentage of DTT on the bacterial culture for the double dilution procedure, the final range of the concentrations came out to be, 6mM, 3mM, 1.5mM, 0.75mM, 0.375mM and 0.1875 mM for the respective wells, B, C, D, E, F and G (Kumar A. *et al*.,).

For the CV assay, 1 X crystal violet solution was prepared. After the formation of the biofilm, the media was removed by tapping the plate and was washed with 1X PBS. After washing, CV was added to the plate. The plate was incubated for 15 minutes in the shaking incubator at 37 degree celsius. After the incubation, the plate was then washed thrice with PBS (Kumar A. *et al*.,).

After this, the plate was again kept for a 10-minute incubation. The bound CV was then extracted using 200 microlitre of 95 percent ethanol. The absorbance readings were then taken at 595 nm using the nanodrop (Kumar A. *et al*.,).

RESULTS

The induction of the mycobacterial cells with the help of DTT was successful and the formed biofilms were clearly visible in the developed plates of the DTT assay. The biofilms were in the form of pellicles and could be seen attached to the surface of the 96 well plate. The CV assay performed also showed proper binding and differences in the amount of the bound crystal violet, the darker the well, the greater the intensity of the biofilm formed. 6mM concentration was observed to be relevant for the formation of the film. The highest intensity of the film was observed at this concentration only.

Figure 1.3: DTT assay plate of the duplicates showing the developed biofilms

In Figure 1.3, the developed biofilms can be very clearly seen in the DTT assay plate post the incubation period of 56 hours. Column 2 (B2-G2) and Column 3 (B3-G3) have the combination media plus bacterial culture plus DTT. Column 4 (B4-G4) and 5 (B5-G5) have the combination media plus DTT. B7,8 and D7,8 have bacteria and media control respectively.

Table 1.2: Layout of the DTT assay plate of the duplicates

Concentration (mM)	Media+bacteria+DTT (Column 2 and 3)	DTT+ media (DTT control) (Column 4 and 5)	Bacterial control(B7.8) and media control (D7.8)
6	B2,B3	B4,B5	-
3	C2,C3	C4,C5	-
1.5	D2,D3	D4,D5	-
0.75	E2,E3	E4,E5	-
0.375	F2,F3	F4,F5	-
0.1875	G2,G3	G4,G5	-

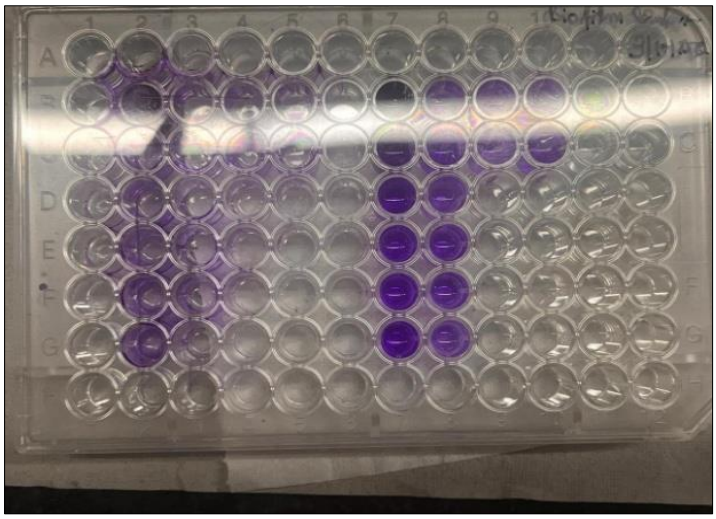

Figure 1.4: First technical CV assay plate

Figure 1.4 shows the CV assay plate for the first replicate. As discussed in the methods section, Column 7 (B7-G7) is for the combination media plus bacterial culture plus DTT. Column 8 (B8-G8) is for DTT control (DTT plus media. Column 9 and 10 have the media and bacterial culture control in the wells B9,10 and C9,10 respectively.

Table 1.3: Layout of the CV assay of the first technical plate

Concentration (mM)	Media + Bacteria +DTT (Column 2)	DTT + Media (Column 3)	Media control (B4,5) and bacteria control (C4.5)
6	B7	B8	-
3	C7	C9	-
1.5	D7	D9	-
0.75	E7	E9	-
0.375	F7	F9	-
0.1875	G7	G9	-

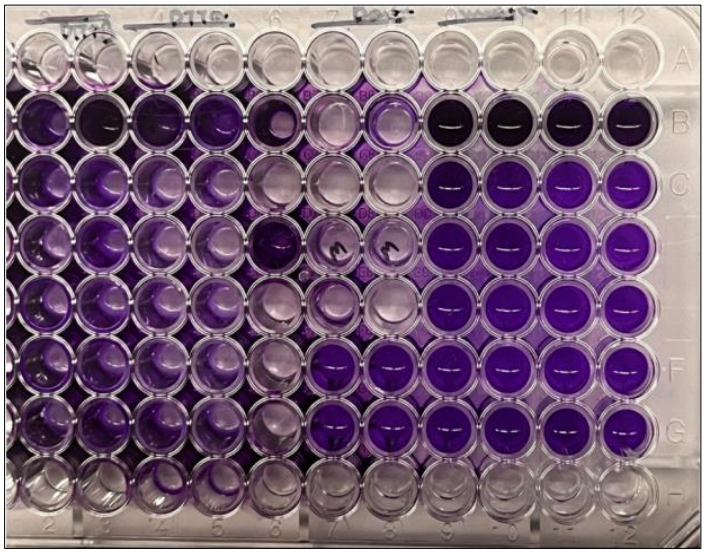

Figure 1.5: CV assay plate of the duplicates

Figure 1.5 shows the CV assay plate of the duplicates. Column 9 (B9-G9) and Column 10 (B10-G10) have the combination of media plus bacterial culture plus DTT. Lane Column 11(B11-G11) and Column 12(B12-G12) have the combination, media plus DTT (DTT control). F7,8 have the bacterial controls and G7,8 have the media controls.

Table 1.4: Layout of the CV assay of the Duplicates

Concentration (mM)	Media+bacteria+DTT (Column 9 and 10)	DTT+ media (DTT control) (Column 11and 12)	Bacterial control (F7.8) and media control (G7.8)
6	B9, B10	B11, B12	-
3	C9, C10	C11, C12	-
1.5	D9, D10	D11, D12	-
0.75	E9, E10	E11, E12	-
0.375	F9, F10	F11, F12	-
0.1875	G9, G10	G11, G12	-

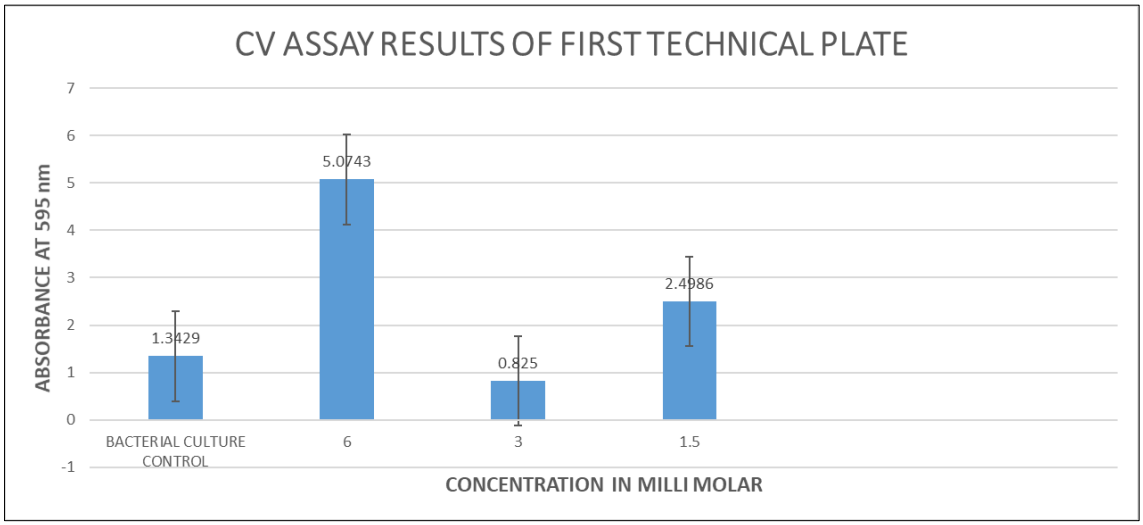

Figure 1.6: CV assay results of the first technical plate (Graph of Absorbance readings of the CV assay versus concentrations of DTT)

Figure 1.6 shows the CV assay results of the first technical plate. From these results, it can be very clearly observed that 6mM concentration is the most suitable for inducing biofilm formation. The readings with respect to other concentrations like 3 and 1.5 mM decrease but an unusual spike can be observed between the absorbance values at concentration 3 and 1.5 mM. This spike can be observed due to handling and pipetting errors. The culture control shows a lesser but significant absorbance value as the bacteria itself is capable of producing a biofilm along with the pipetting errors that were observed.

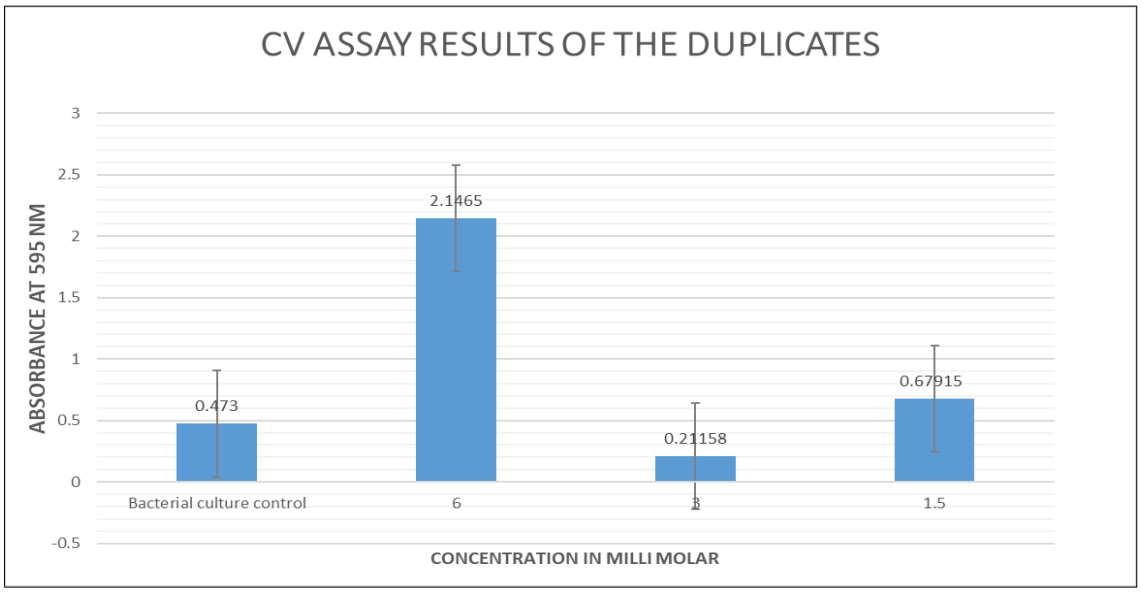

Figure 1.7: CV assay results of the duplicates (Graph of absorbance readings of the CV assay versus the concentrations of DTT)

Figure 1.7 shows the absorbance readings of the CV assay of the duplicates. These readings show that 6mM concentration is the most relevant for inducing biofilm formation at the highest intensity. An unusual spike in the readings can be observed here between concentrations 3mM and 1.5 mM. This is due to handling and pipetting errors. A very low amount of absorbance has been recorded in the bacterial culture control. This is a much more reliable value as compared to the first technical plate as the absorbance value should be comparatively lower as some amount of biofilm the bacteria does form but it does a stress induced environment to accelerate the process.

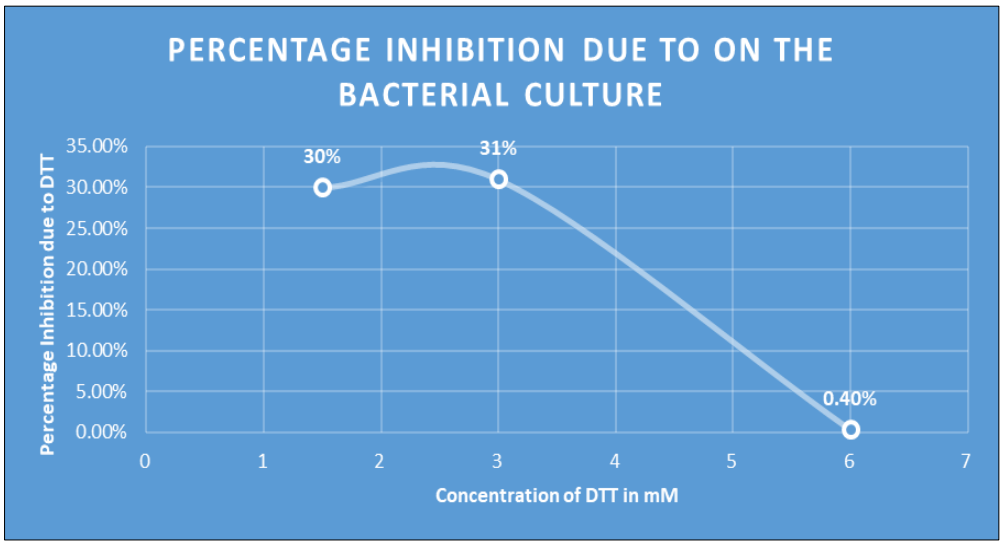

Figure 1.8: Graph of Percentage inhibition due to DTT on the bacteria versus concentrations of DTT

In figure 1.8, the graph represents the amount of inhibition caused by DTT on the bacterial culture. From the graph, it can be very easily observed that the lowest amount of inhibition due to DTT can be observed at 6mM concentration. The amount of inhibition increases on the bacterial culture in terms of biofilm formation, i.e. the amount of biofilm formation decreases with the decrease in the concentration of DTT, as the intensity of the reductive environment provided by DTT decreases. The results again confirm that 6mM concentration of DTT is the most reliable concentration level to induce biofilm formation.

DISCUSSION

The entire experiment shows that at a range of concentrations of DTT, biofilm formation could be observed. There are some irregularities in the results due to contamination and pipetting errors but most of the results indicate that the reductive environment produced by DTT is conducive for formation of biofilm on Msm cultures.

Msm is fast-growing and has a much shorter doubling time of three hours. As per the results, DTT inhibition is much lower at the 6mM concentration and rises with its decreasing concentration. This pattern confirms that the CV assay results were successful and correct. The results can further be used to understand the stages of latency, biofilm formation mechanism, its composition as well as how they can be disintegrated.

CONCLUSION AND FUTURE DIRECTIONS (EH Tobin *et al*.,)

As per the results, it can be concluded that DTT is efficiently capable of inducing biofilm formation in Msm cultures. It can be established that 6mM concentration is suitable for inducing biofilm formation.

With respect to a future perspective, the current project can be further used to characterise biofilms and screen drugs, to gain a better understanding of how the biofilms can be targeted and destroyed. This understanding can help develop models that can be used to understand the new ways of adapting to the hostile environment by the bacteria. This study is typically crucial to the exploration and cure of tuberculosis disease.

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