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Characterization of biofilm formation by *Mycobacterium smegmatis* during different environmental stress conditions: An *in-vitro* study

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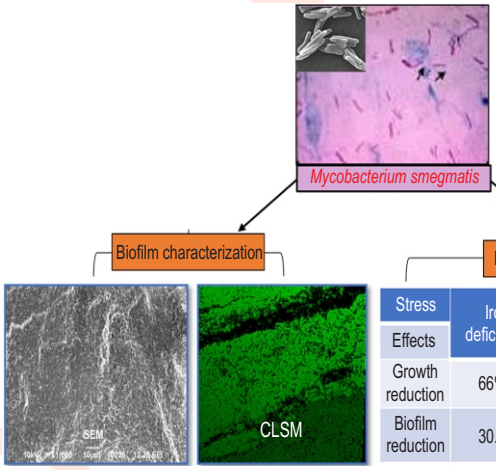
Abstract

Aim: *In-vitro* characterisation of biofilm produced by *Mycobacterium smegmatis* (*M. smegmatis*), a surrogate model for biofilm production by *Mycobacteria*, and to evaluate the impact of different environmental stress on mycobacterial growth and biofilm formation.

Methodology: *M. smegmatis* biofilms were studied using tissue culture plate and tube adherence methods. Confocal Laser scanning microscopy (CLSM) and scanning electron microscopy (SEM) were used to study the 3D structure and surface morphology, respectively. Additionally, the effect of different environmental stress, such as the absence of essential ions, exposure to harsh environmental conditions, such as acidic environment or oxidative stress, on mycobacterial biofilm formation and mycobacterial growth was assessed.



Stress / Effects	Iron deficiency	Oxidative stress	Low pH	Oxidative stress with low pH	Mg deficiency
Growth reduction	66%	98%	98%	66%	56%
Biofilm reduction	30.4%	3.8%	65.7%	10.1%	12.5%

Results: All the exposures, except for carbon supplemented media had a detrimental effect on the number of viable counts and on biofilm formation by *mycobacteria* ($p<0.001$). Growth in low pH and oxidative stress was found to be maximum showing reduction by 98% when compared with control.

The environmental stress effectively reduced the viable mycobacterial count as well as biofilm formation

Interpretation: Our findings present various environmental conditions that profoundly affect biofilm formation and thus, may find practical implications in future as effective mycobacterial control strategies having attributes of mycobacterial growth as well as biofilm inhibition.

Key words: Biofilm, Environmental stress, Multi-drug resistance, Mycobacterium

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Introduction

Despite significant efforts for the early detection and effective treatment, Tuberculosis (TB) still remains a dreaded disease that still claims around ten millions of lives annually (Chakaya *et al.,* 2021) across the world. The highest TB burden has been reported from the developing countries, especially Asia and Africa (MacNeil *et al.,* 2019). The situation is further worsened by HIV (human immunodeficiency virus) co-infection and the emergence of multi-and extensive- drug resistant (MDR and XDR) strains of the causative agent, *Mycobacterium tuberculosis* (Kwan and Ernst, 2011; Lienhardt *et al.,* 2012). The success of the causative agent, *M. tuberculosis* is based on its ability to withstand harsh conditions and adapt accordingly to allow its persistence within the host for prolonged period. In this context, biofilm formation by *M. tuberculosis* and non-tuberculous mycobacteria across different areas like hospital water tanks, dialysis water, medical equipment, and catheters, has been described as one of the crucial factors in pathogen virulence, as it shields pathogen from the bactericidal effect of antibiotics and antimicrobial compounds, thereby significantly promoting antibiotic resistance. Mycobacterial biofilm formation is reported to result in caseous necrosis and cavity formation in lung tissue (Effros *et al.,* 2005; Shpitzer *et al.,* 2007; Vega-Dominguez *et al.,* 2020) and is a major cause for delay and failure in the effective TB treatment. Inhibition of biofilm formation by Mycobacteria thus acquires significance in the wake of the WHO's End TB Strategy with a target of 90% reduction in TB cases by 2035 and TB elimination by 2050 (Uplekar *et al.,* 2015).

Several components of the mycobacterial cell wall such as glycopeptidolipids, short chain mycolic acids, monomeromycolyl diacylglycerol, etc. have been shown to play an important role in the formation of pellicle biofilms (Chakraborty and Kumar, 2019). The integrity of cell wall structure is highly significant for the *in-vivo* survival of mycobacteria. Glycopeptidolipids play major role in the biofilm formation as it promotes initial surface attachment (Chen *et al.,* 2020; Recht and Kolter, 2001). The survival of many environmental and pathogenic microbial species against antibiotics is controlled by their tendency to grow as surface-associated multicellular populations called biofilms. Numerous factors tend to promote the overall resistance of biofilm bacteria including reduced metabolic and growth rates, protection by extracellular polymeric substances and specific resistance mechanisms deliberated by the altered physiology of biofilm bacteria compared with planktonic bacteria (Davies, 2003).

Many mycobacterial strains which include *M. tuberculosis*, also have been found to make drug tolerant biofilms with the help of genetic mechanisms (Islam *et al.,* 2012). Amongst the two groups of Mycobacteria, the fast-growing mycobacteria have been reported to possess higher metabolic activity than slower counterparts, but in the process of mature biofilms this assumption isn't considered correct (Solokhina *et al.,* 2017). The detailed structure, chemistry and physiology of the biofilm differs with the nature of its resident microbes and environmental surroundings. However, an important harmony among biofilms is that their structural integrity depends upon the extracellular matrix produced by their constituent cells. Extracellular matrix plays an important role in the organisation of biofilm population (Branda *et al.,* 2005). The growth of *M. tuberculosis* along with the biofilm formation has been studied in the presence of elements such as iron and zinc, whereby the study reveals that nutrient levels determine the qualities and characteristics of biofilm (Ojha *et al.,* 2008). *M. tuberculosis* forms biofilms with specific environmental and genetic requirements distinct from those for planktonic growth, which contain an extracellular matrix rich in free mycolic acids and harbour an important drug-tolerant population that persist despite exposure to high levels of antibiotics (Bergval *et al.,* 2008; Ojha *et al.,* 2008). Biofilms have the tendency to exhibit stress resistance including a resilience against antibiotics that causes serious medical and technical problems (Serra and Hengge, 2014). Hence, keeping in view the role of environmental conditions in the formation of biofilms and the environment to which the pathogen is exposed, an attempt was made to evaluate and compare the impact of various stress conditions, such as starvation due to deprivation of some nutrients (Fe^{2+}, Mg^{2+}), acidic pH and oxidative stress simultaneously on mycobacterial growth and biofilm formation using *M. smegmatis* as an established *in-vitro* model.

Materials and Methods

***M. smegmatis* strain and growth:** *M. smegmatis* mc(2) 155 culture on Lowenstein-Jensen (LJ vial) was obtained from the Microbiology Division, CSIR-Central Drug Research Institute (CSIR-CDRI), Lucknow, and was identified by Zeihl Neelsen staining. All reagents for Sauton's media, phosphate buffer saline and crystal violet were purchased from Hi-Media laboratories, Mumbai, India. All the reagents used for preparation of media were of analytical grade. *M. smegmatis* was grown in Sauton's media consisting of Ferric ammonium chloride, L-asparagine, citric acid, magnesium sulphate, dipotassium hydrogen phosphate, sodium dihydrogen phosphate, and sodium chloride at 37°C with constant shaking at 120 rpm with an appropriate negative control.

Biofilm formation and analysis: For biofilm growth, (Malik *et al*., 2019) *M. smegmatis* was grown in 6 well microtitre plates at 37°C in Sauton's medium without Tween 80 (Pacheco *et al.,* 2013). The culture was diluted into 1:100 with fresh Sauton's medium and biofilm formation was studied by tissue culture plate method and tube adherence methods (Hassan *et al.,* 2011). In the Tissue Culture plate method, dilute culture was filled in 6 well tissue culture plates and incubated for 6 days at 37°C. Thereafter, the content of each well was removed by gentle tapping, followed by washing with distilled water to remove free-floating bacteria. Biofilm which remained adhered to the wells were fixed with 2% sodium acetate for 30 min, stained with 0.1% Crystal violet and the plates were incubated for 15 min. Excess stain was washed gently, and the plates were kept for drying. Thereafter, the biofilm in wells were treated with ethanol for 15 min at room temperature, and the optical density of stained adhered biofilm was determined at 595 nm. In the Tube Adherence method, *M. smegmatis* was

inoculated in 5 ml of Sauton's media in test tubes and incubated at 37°C for 6 days, after which the content of tubes was removed, and tubes were washed with phosphate buffer saline solution to remove the free-floating bacteria. The biofilm was stained with 0.1% Crystal violet, washed, and tubes kept inverted for drying, followed by fixation and addition of ethanol, prior to determination of OD at 570 nm.

Characterization of biofilm

Scanning Electron Microscopy: The Scanning electron microscopy was performed at Babasaheb Bhimrao Ambedkar University (BBAU), Lucknow where the biofilm was allowed to form on coverslips within tissue culture wells and was fixed in 2.5% glutaraldehyde fixative for 2 hours at 4°C. Thereafter, the biofilm was washed thrice in 0.1M Phosphate buffer, for 15 min at 4℃ and incubated with 1% osmium tetroxide for 2 hr at 4°C. The biofilm sample was again washed thrice with 0.1 M Phosphate buffer for 15 min at 4°C to remove the unreacted fixative and then dehydrated by acetone. Thereafter, the sample was air dried, followed by mounting onto the aluminium stubs with carbon tape and was coated using sputter coater (Relucenti *et al.,* 2021). Biofilm specimens were also studied under scanning electron microscopy after dehydration at the BBAU Lucknow (Abidi *et al.,* 2014).

Confocal Laser Scanning Microscopy (CLSM): Stained biofilms were examined under a confocal laser scanning microscope at the Birbal Sahni Institute of Palaeobotany (BSIP), Lucknow. Laser excitation was used at 633 nm for far red and 405 nm for blue fluorescence at a frequency of 700Hz. CLSM was performed to obtain the 3D image of the prepared biofilm sample. CLSM worked by passing a laser beam through a light source aperture which was then focused by an objective lens into a small area on the surface of biofilm sample and an image was obtained (Brenes *et al.,* 2020). *M. smegmatis* cells were washed three times with PBS, mounted on a microscopic slide and then visualized using Leica TCS SPE, CLSM (Leica Microsystems, Germany).

Impact of environmental stress on *M. smegmatis* growth and biofilm formation: To study the influence of environmental conditions on mycobacterial growth establishment and maturation of biofilms under stress conditions *in-vitro*, the quantitative tissue culture plate method was adopted. A colony of *M. smegmatis* was inoculated in Sauton's broth and incubated at 37 °C for 6 days. Then, the culture was diluted (1:100) with fresh Sauton's medium, or in respective stress media. *M. smegmatis* cultures were exposed to various stress environments such as iron deprived media (ferric ammonium citrate deficient), magnesium (magnesium sulphate) deficient media, carbon (additional sodium acetate) supplemented media, low pH (pH 5.5) media and oxidative stress (with hydrogen peroxide) in Sauton's media. A combined stress of low pH and oxidative stress was also provided to assess the impact on mycobacterial growth and biofilm formation. Uninoculated media (fresh Sauton's media) was used as a negative control and the experiments were performed in triplicates. ZN staining was performed before and after stress exposure to rule out any chances of contamination. Mycobacterial growth under stress conditions was assessed by growing serially diluted inoculum in different stress conditions followed by culturing them on Sauton's solid agar.

The growth was compared with uninoculated media which was also serially diluted for proper comparison. Only non confluent growth between 10 and 300 was considered for calculation. For biofilm formation, the diluted cultures were incubated in a sterile 96 well plate in various stress media (as mentioned above) for 7-10 days at 37°C, and thereafter the absorbance of dissolved biofilm was read at 595 nm in a plate reader. Serial dilutions were plated onto Sauton's solid agar media to determine the number of colony forming units (CFU ml^{-1}), which was compared with the number of CFUs yielded from the positive control to determine the mycobacterial survival percentage under various stress conditions.

Statistical analyses: Data represent mean±S.D. of experiments done in triplicate. Statistical analyses was performed using One-way ANOVA and post-test comparison with control was performed using Dunnett's Multiple Test. The result was considered significant at $p < 0.001$.

Results and Discussion

The present study investigated the effect of different environmental stress conditions on mycobacterial growth and biofilm formation, so as to explore which stress condition(s) can serve as effective mycobacterial control strategies having attributes of both mycobacterial growth as well as biofilm inhibition. The study of mycobacterial biofilm formation by *M. smegmatis* required an initial optimization of several factors such as slight variations in growth media composition, and incubation time. A conspicuous line was clearly observed on the inner walls of the wells of microtiter culture plates as well as tubes, clearly indicating biofilm formation by mycobacteria after incubation for 7-10 days at 37°C, which was further quantified and assessed by crystal violet staining. The biofilm samples were characterized by confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM). The CLSM images depicted the 3D structure of the biofilm exhibiting the uniform sheet of cells (Fig. 1).

The surface morphology of biofilm was observed using SEM at different magnifications, wherein a dense mat-like structure with strong mycobacterial aggregation was observed (Fig. 2) (Kumar *et al.,* 2015). The assessment of mycobacterial growth under different stress conditions revealed that in comparison to control (>3 x 10^8 CFU ml^{-1}), there was significant reduction of mean CFU ml^{-1} in different stress conditions that included iron deficiency (1.02 + 0.040 x 10^8 CFU ml^{-1}), oxidative stress (0.5 + 0.22 x 10^8 CFU ml^{-1}), low pH (0.2 + 0.026 x 108 CFU ml^{-1}), magnesium deficiency (1.3 + 0.26x 10^8 CFU ml^{-1}) and combination of oxidative stress and low pH (1.0 + 0.23 x 10^8 CFU

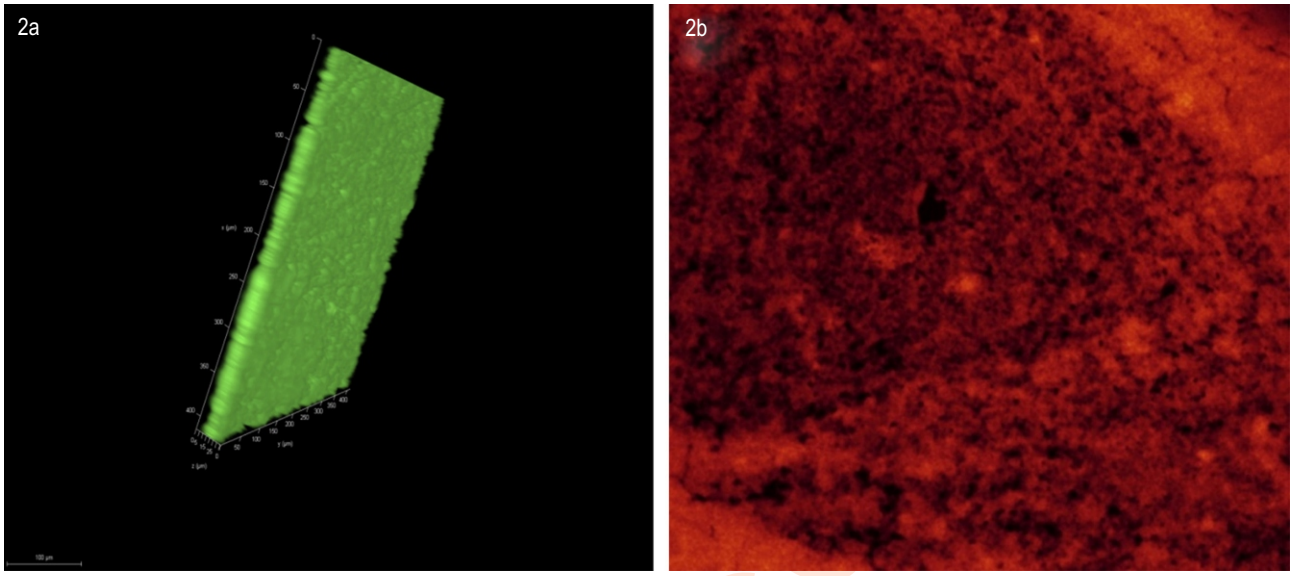

Fig. 1: Confocal microscopy images of biofilms at 10X magnification. (a) Section of biofilm and (b) three-dimensional image of adhered biofilm captured as a stack of images along z-axis and compiled into top down orthogonal and 3D reconstructions.

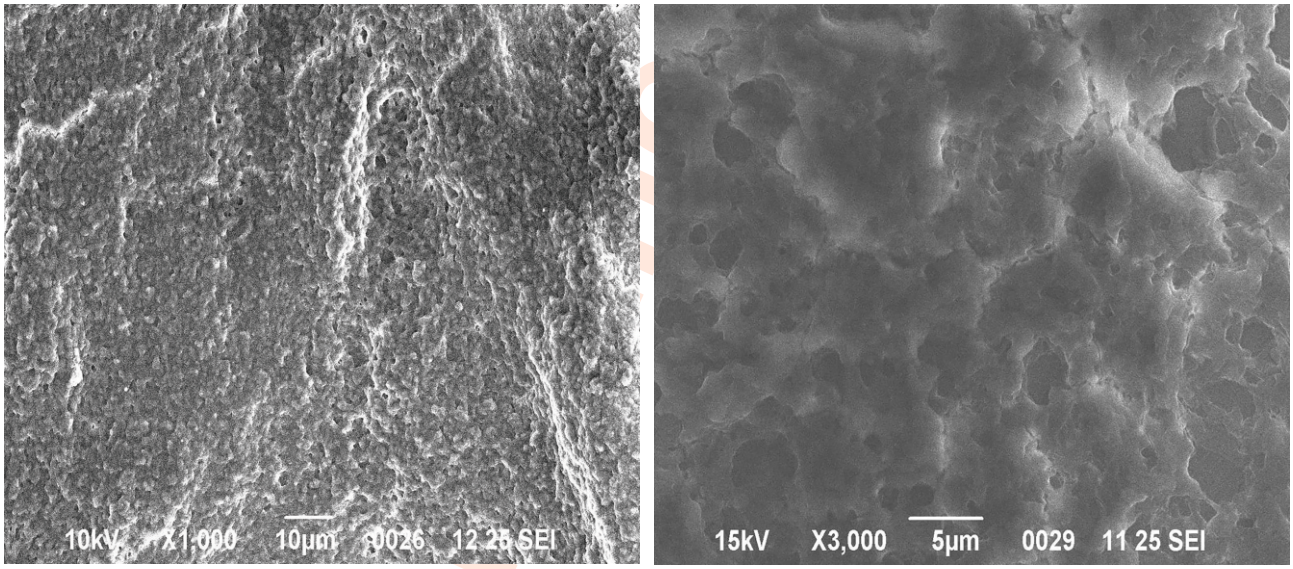

Fig. 2: Scanning electron micrographs of *M. smegmatis* biofilm at (a) 1000X and (b) 3000X magnifications.

ml^{-1}, (Fig. 3). Mycobacterial exposure to media with acidic conditions and oxidative stress conditions exhibited a maximum of 98% reduction in the viable mycobacterial counts, followed by iron deficient media, and media having combination of low pH with oxidative stress, both showing 66% reduction, while exposure to magnesium deficient media led to 56% reduction in viable mycobacteria, as compared to the control. The growth of colonies in a medium rich in sodium acetate was similar to confluent growth of control, even at lowest dilution, at which all observations were done, and thereby for ease of calculation, the CFU was taken similar to control, *i.e.,* >3 X 10^8 CFU ml^{-1} (SD=0). While the mycobacterial cells exhibited a tendency to form biofilm even in different stress conditions, the quality and quantity of nutrients, ions and several other substances/ environmental factors influenced bacterial growth (Fig. 3) and showed a significant detrimental effect on biofilm formation (Fig. 4). The study therefore, exhibited that the alteration in environmental conditions led to changes in biofilm formation as well as mycobacterial

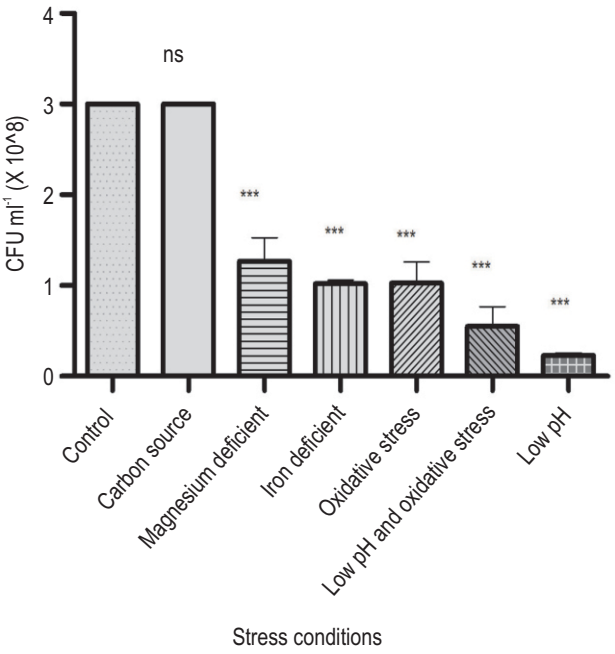

Fig. 3: In comparison to control (>3 X 10^8 CFU ml^{-1}) there was significant reduction of average CFU ml^{-1} in different stress conditions like magnesium deficiency (1.2 x 10^8 CFU ml^{-1}), iron deficiency (1.02 x 10^8 CFU ml^{-1}), oxidative stress (1.02 x 10^8 CFU ml^{-1}), low pH and oxidative stress (0.5 x 10^8 CFU ml^{-1}) and maximum reduction with low pH (0.2 x 10^8 CFU ml^{-1}); *-p<0.05; **p<0.01; ***-p<0.001; ns-non significant.

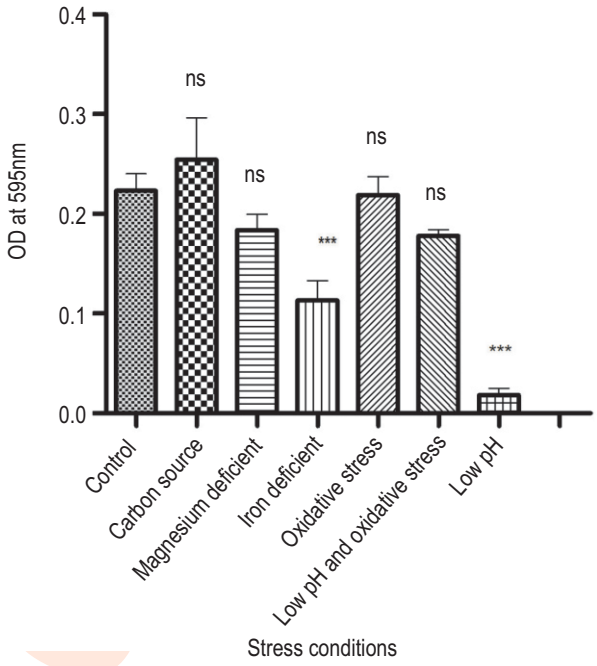

Fig. 4: Quantification of biofilm formed by Mycobacteria under different stress conditions. *M. smegmatis* biofilms were grown under static conditions in glass culture tubes and 6 well culture plate in Sautan's media at 37 °C for 7-10 days, after which cultures were gently rinsed, stained with 0.1% crystal violet and imaged. The CV was released by adding 70% ethanol and quantified by reading on a spectrometer. The samples were run in triplicate, averaged, and the error bars represent standard deviation. Dunnett's multiple comparison test (*-p<0.05; **p<0.01; ***-p<0.001; ns-nonsignificant).

growth (Table 1). Significant reduction in biofilm formation was observed on exposure to iron deficient (30.4%, p<0.001) and low pH media (65.7%, p<0.001), but media with magnesium deficiency (12.5%) and combined low pH with oxidative stress (10.1%), were seen to have much lesser efficacy in terms of mycobacterial biofilm inhibition. The study also showed that mycobacterial growth under various stress conditions appeared to be more profoundly hampered and reduced than the biofilm formation, when compared to their respective controls. CFU values were reduced in all stress conditions provided, except for the carbon supplemented medium indicating that biofilm formation is a powerful factor in providing bacteria a protective shield against different harsh environment, and possibly a reason for developing drug resistance (Islam *et al.,* 2012).

Mycobacterial biofilm formation was affected by exposure to all conditions; however, the magnitude of impairment did not correlate with the reduction in planktonic growth inhibition in all cases. Magnesium deficiency in media led to significant reduction in the biofilm formation, but not in growth of mycobacteria. Magnesium is an essential element required in many fundamental cellular activities, such as for many ATP-dependent enzymatic reactions, and in stabilizing cell membranes (Shine and Douglas, 1974). Several studies have shown that the level of magnesium can either promote or inhibit biofilm formation (Wang *et al.,* 2019). The growth of *M. smegmatis* and biofilm formation both were hindered in iron deficient Sauton's media where it was also found that iron deprivation had major effect on biofilm reduction, depicting a close association between presence of iron and formation of biofilm (Ojha and Hatfull, 2007; Pal *et al.,* 2016). Iron has been shown to be important in regulating gene expressions for attaching to surface and inducing biofilm formation. It is a critical constituent for microbial pathogenicity as it is essential for various functions like cytochrome oxidase, acts as cofactors in enzymes etc. Increased risk of death due to TB and high dietary iron overload has been seen in clinical studies in Africa (Gordeuk *et al.,* 1996; Moyo *et al.,* 1997).

The results showed that limitation in iron availability restricts mycobacterial growth and significantly decreased biofilm formation under *in-vitro* conditions. (p<0.001, ***). The addition of sodium acetate as a supplementary carbon source was recorded to enhance biofilm formation. Acetate is a substrate for acetyl-CoA production, feeding tricarboxylic acid cycle and generating intermediary metabolites to generate ATP. Thus, we can infer that acetate supplementation permits enhanced mycobacterial

Table 1: Relative inhibition of mycobacterial growth and biofilm formation under different environmental stress conditions with respect to that under control conditions.

Stress effects	Iron deficiency	Magnesium deficiency	Oxidative stress	Oxidative stress with acidic condition	Low pH
Biofilm reduction	30.4%	12.5%	3.8%	10.1%	65.7%
Growth reduction	66%	56%	98%	66%	98%

survival. Within phagosomes, the mycobacteria are also subjected to scarcity of glucose, and thus utilize fatty acids as energy source (Gordeuk *et al.,* 1996). *M. tuberculosis* has evolved to survive inside an acidic pH environment. To thrive in such harsh environment, the bacteria need to maintain its internal pH to around 7 (Booth, 1985). Amongst all stress conditions explored in this study, mycobacterial exposure to acidic media (pH 5.5) resulted in maximum reduction in growth and biofilm formation, indicating low tolerance by mycobacteria to acidic pH.

Oxidative stress, *i.e.,* exposure to hydrogen peroxide alone or in combination with acidic pH, was found to inhibit Mycobacterial growth substantially, but failed to exhibit a significant inhibition of mycobacterial biofilm formation in the study. Reduced growth and low OD were also observed upon exposure to low pH and hydrogen peroxide (Ali *et al.,* 2020). According to a study even though biofilms formed by *M. marinum* were initially resistant to destruction by hydrogen peroxide, but approximately after 120 min the biofilms were found to be as or more susceptible than its planktonic cells (Bardouniotis *et al.,* 2003). Moreover, another study reports increased killing by hydrogen peroxide upon decrease in iron concentration, perhaps due to decreased synthesis of iron containing enzymes like catalase (De Voss *et al.,* 1999). It is thus possible that prolonged exposure to hydrogen peroxide, after the tested duration (of one week), may result in further and substantial inhibition of biofilm formation by mycobacterial cells. Whether these mycobacterial spp. show biofilm inhibition upon prolonged exposure to oxidative stress or have acquired resistance needs to be tested. Further studies are planned in our lab in this direction.

Mycobacteria are known to reside and multiply within phagosomes of host macrophage (Schnappinger *et al.,* 2003), and are naturally exposed to various stress conditions, as starvation due to deprivation of some nutrients (Fe^{2+}, Mg^{2+}), acidic pH and oxidative stress. The impact of these stress factors on mycobacterial growth and biofilm formation was studied. Thus, all these *in-vitro* stress conditions including nutrient starvation, low pH and oxidative stress may mimic host environment and were selected as parameters to assess the inhibition of mycobacterial biofilm formation. This study, therefore, aimed to characterize the impact of a variety of microbicidal stresses *viz.,* acidic, oxidative, iron deprivation and nutrient starvation on their biofilm formation, using *M. smegmatis* as an established *in-vitro* model (Gordeuk *et al.,* 1996; Kumar *et al.,* 2015; Ojha and Hatfull, 2007). While the mycobacterial growth was found maximum in carbon supplemented media, the conditions involving acidic stress (low pH) as well as iron deprivation were seen to have a significant impact (***$p<0.001$) on both biofilm formation as well as the persistence of mycobacteria. Interestingly, our study also showed that the combination of oxidative stress to the already acidic broth had a synergistic and drastic inhibitory impact on mycobacterial growth, but not on biofilm formation. Since all the conditions studied (except for acetate supplementation) exhibited significant mycobacterial growth inhibition but did not show similar biofilm inhibition, it is postulated that mycobacterial growth and biofilm formation may be operating and regulated via different pathways. While mycobacterial survival and growth is essentially dependent on several pathways involving various proteins and genes (Xiong *et al.,* 2013), biofilm formation is known to be facilitated by quorum sensing as a survival strategy in bacteria, but very little information is available regarding the regulation of biofilm formation in mycobacteria (Sharma *et al.,* 2014).

This preliminary study implies that acidic stress (low pH) as well as iron deprivation have significant potential as pharmacological manipulations to restrict mycobacterial persistence and biofilm formation. Despite the limitations of this study in terms of biofilm formation by a species of soil mycobacteria under static, *in-vitro* conditions, the study is encouraging for further researchers in light of the fact that these environmental conditions may find application in future for designing drugs having attributes of mycobacterial biofilm inhibition in addition to mycobactericidal activity or in creating such stress environment(s) around the surfaces of medical devices or equipments prone to maximum biofilm formation, as a promising approach against TB and antibiotic resistance.

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Add-on Information

Authors' contribution: N. Pandey: Formal analysis, investigation, methodology, data interpretation and writing-

original draft preparation; **K. Singh:** Review and editing; **F. Ahmad:** Reviewed the language part of paper; **R. Sharma:** Conceptualization, supervision, final review and editing.

Research content: The research content of manuscript is original and has not been published elsewhere.

Ethical approval: Not applicable.

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