# Characterization of biofilm formation by slow and fast grower mycobacteria under different stress conditions

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***Abstract-*** **Attachment of mycobacteria involved in biofilm formation in the liquid air interface is a complex process, with many variables such as pH, nutrient levels, iron, oxygen, ionic strength and temperature, affecting the outcome. The standard Microtiter plate assay was used in the present study to observe the effect of pH, temperature, and in MB7H9 medium as well as Sauton's medium on non tuberculosis mycobacteria like *M. smegmatis, M. fortuitum, M avium* and *M. tuberculosis* (H37Rv). The large quantity of biofilm was produced by *M.smegmatis* at temperature 37ºC and 42 $^0$C as compared to 30$^0$C. *M. fortuitum* developed more amount of biofilm at 30ºC as compared to 37ºC and 42ºC. *M.avium* developed strong amount of biofilm at 30ºC and 42ºC as compared to 37$^0$C. *M tuberculosis* developed strong biofilm at 37 $^0$C and no biofilm at 30$^0$C and 42$^0$C in MB 7H9 media and Sauton's media.**

***Keywords*: EPS, M. tuberculosis, M avium, M. fortuitum M.smegmatis, ECM, SEM.**

## INTRODUCTION

It is now commonly established that bio films are the predominant mode of bacterial growth, reflected in the study that approximately 80% of all bacterial infections are related to biofilms (National Institutes of Health (USA)) (**Davies 2003**).Biofilms are defined as structured communities of bacterial cells covered in a self-produced polymeric matrix adherent to inert or living surfaces (**Costerton *et al.*, 1999; Donlan & Costerton, 2002)**

The growth of biofilms has been reported to form on solid substrates in contact with moisture, on soft tissue surfaces in living organisms, and at liquid air interfaces **(Costerton *et al.*, 1999).** Biofilms are composed mainly of microbial cells and EPS, (**McKenney *et al.*, 1998).** The formation of biofilms is known to be affected by a variety of environmental factors (**O'Toole *et al.*, 1998**), such as pH, iron, oxygen, ionic strength and temperature, and nutrient level. These environmental cues include a copious supply of nutrients, the availability of oxygen, and an osmotically balanced growth medium (**O'Toole *et al.*, 2000).**

*M.avium* has been reported to form biofilm in MB7H9 media with OADC enrichment and at 28 $^0$C (**Johansen *et al.*, 2009).** The low level of supplemental iron requires (**Ojha & Hatfull, 2007 ),** for *M. smegmatis* biofilm formation. The availability of CO2 plays a role in *M. tuberculosis* biofilm formation and decreasing oxygen tension or increasing concentration of organic volatile molecules – stimulate biofilm development (**Ojha *et al.*, 2008**). Mycobacteria are also capable of protecting their accompanying microbiome from toxicity by heavy metals and antibiotics. (**Sachan TK, Kumar V 2015**) Cells of a strain of *Mycobacterium scrofulaceum* that are resistant to the heavy metal mercury (Hg) can reduce Hg+2 to insoluble Hg0 that is rapidly lost from solution by volatilization (**Meissner & Falkinham, 1984**). Biofilm producing bacteria undergo a developmental program in response to environmental factors that lead to the expression of new phenotypes that distinguishes these attached cells from their planktonically growing counterparts. The composition and structure of the mycobacterial outer membrane is a major determinant of growth, physiology, ecology virulence and biofilm formation of these opportunistic pathogens (**Sachan TK et al 2015)** . The hydrophobicity of the outer cell membrane concentrate of environmental mycobacteria at air–water interfaces where organic compounds are also concentrated, providing nutrient (**Harvey & Young, 1980**).
The mycobacteria such as *M. avium, M. intracellulare* **(Stoodley and Scott., 1998),** *M. fortuitum, M. gordonae, M. abscessus,* **(Falkinham *et al.*, 2001),** *M. septicum, M. gilvum* (**September *et al.*, 2004)**, are involved in various opportunistic infection and biofilm formation (**Korber *et al.*, 1989),** including in *M .tuberculosis* H37 Rv, as recently reported (**Ojha *et al.*, 2008)**. However, the factor which affects the biofilm development is not completely understood by other investigators in slow grower and fast grower mycobacteria. Therefore, the present study has been carried out to record the extent to which a single change in growth condition affects the formation of a useful mono species biofilm, excluding possible variables such as interspecies interactions and communication which are often observed. A more comprehensive understanding of processes connected with biofilm development in different conditions will lead to new knowledge that would help in developing novel and effective control strategies for prevention of biofilms and improvement in patient management.

## MATERIALS AND METHODS

**Bacterial strains-:** The clinical isolates of mycobacterial species were taken for the study of mycobacterial biofilm. The hundred isolates of each of M. *fortuitum,* and *M. tuberculosis* H37RV *M.smegmatis* were obtained from Repository Centre of NJIL & OMD Agra and *M.avium* was obtained from Tuberculosis research centre Chennai. The Planktonic cell growths of *M. smegmatis, M. fortuitum M.avium* and *M.tuberculosis* H37RV were made in Middle brook 7H9 broth supplemented with 0.05% Tween 80 and 2% glucose. For the development of biofilm of these mycobacterial species, firstly remove the tween 80 by two to three time washing with the media. The different conditions such as pH, temperature and as well as Sauton's and MB7H9 media were analysed. *M. smegmatis,* and *M. fortuitum* as fast growers and *M.avium*, and *M. tuberculosis* H37Rv as slow growers were studied for biofilm formation under different conditions. One loopful culture of these mycobacteria from the Lowenstein Jensen media (LJ) slopes was scraped and suspended in MB7H9 media and incubated till mid log phase growth. The mid log phase culture from these bottles was centrifuged at 8000 RPM (**Sigma)** for 5 minutes at 4$^o$C. The pellets were washed with Sauton's medium and MB7H9 medium of different pH range to remove the Tween 80. These cultures were then diluted with MB7H9 and Sauton's and matched with 0.5 x McFarland standards ($10^8$ CFU⁄ml) and 1:10 serial dilutions were prepared.

**Quantification of biofilm**

Biofilm formation was determined as described previously **(O'Toole *et al.*, 2000),** by seeding 200 µl of Sauton or MB7H9 liquid media containing $1\times10^7$ bacteria in a Polystyrene plastic 96-well Microtiter plate. The assay performed to determine the ability of cells to adhere to the wells was based on the method by (**Limia *et al.*, 2001).** The plates were incubated at room temperature for one and two weeks for fast growing mycobacteria, and two and four weeks for slow growing mycobacteria.
In the present study, the effect of different factors such as pH and temperature condition on the development of biofilm of slow growers *M. tuberculosis* H37 Rv, and *M. avium* and fast growers *M.smegmatis* and *M.fortuitum* were recorded based upon the OD at 570 nm . The biofilm was classified as strong, moderate, weak or no following the protocol of (**Stepanovic *et al.*, 2000).** All tests were carried out in triplicates and the average results were calculated as (mean OD±SD). Media without bacteria incubated for similar periods as those for tests was used as a negative control. The evaluation of biofilm formation on polystyrene surfaces by four mycobacterial species were analysed in the following way.

(1). Quantification of biofilm at different pH (5.2 to 7.2 in increment of pH 1) for *M.tuberculosis* and for NTM at 4.5, to7.5 in increment of pH 1.
(2.) Quantification of biofilm of selected mycobacteria at two temperatures $30^0$C, $37^0$C $42^0$C.
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**Scanning electron microscopy (SEM)-:** The mycobacterial biofilm developed in microtiter dish as described above were fixed in a solution of 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, overnight at $4^0$C. The samples were rinsed once in the same buffer and dehydrated by increasing concentrations of ethanol (30%, 50%, 70%, 90% and 100%). The samples were dried in a fume hood were fixed on to stubs with conductive self-adhesive carbon tapes, coated with gold film sputtering and used for analysis with SEM (S3000- N). The ultrastructural picture shown by SEM described as thin, thick, and thicker of the biofilm developed in the microtiter plate and described as weak, moderate, and strong respectively.

**Statistical analysis-:** Data (Mean±SD value) showing the effect of the different factors i.e. pH, temperature and on biofilm formation were compared. The data were analysed by Student's t test with Welch's correction at 5% level of significance and was also tested with a nonparametric test where it was required. The data were plotted and analyzed with GraphPad Prism 5 software (Graph Pad Software, San Diego, CA).

**RESULTS:**

Quantification of biofilm growth developed by fast grower mycobacteria at different temperature conditions: The selected mycobacteria were incubated at different temperatures such as 30ºC and $37^0$C and $42^0$.C

**Different temperature condition**

**Biofilm formation by *M. smegmatis*-:** It was clearly observed that *M.smegmatis* developed weak biofilm at $30^0$ C and moderate biofilm at $37^0$C and $42^0$C in MB7H9 media in first week. In Sauton's media at first week *M.smegmatis* developed moderate biofilm at $30^0$C & $42^0$C and weak biofilm at $37^0$C. At second week these mycobacteria developed moderate biofilm at $30^0$C and strong biofilm at $37^0$C and more stronger biofilm at $42^0$C in MB7H9 media, but in Sauton's media these bacteria developed moderate biofilm $30^0$C and strong biofilm at $42^0$C & $37^0$C. The significant difference of *M.smegmatis* were observed at first week and second week in Sauton's media in between $37^0$ C and $30^0$ C. **Figure 1, Table-1(a,b)**

**Biofilm formation by *M. fortuitum***

*M.fortuitum* developed strong biofilm in first week in $30^0$C and moderate biofilm at $37^0$C and $42^0$C in MB7H9 media and in Sauton's media. At second week, these bacteria developed strong biofilm at $30^0$C and moderate biofilm at $37^0$C and $42^0$C in MB7H9 media and in Sautons media strong biofilm developed at $30^0$C, weak biofilm at $37^0$C, and moderate biofilm at $42^0$C. Significant difference of *M.fortuitum* were observed at first and second week in between $37^0$ and $30^0$ C. The another significant differences was also observed in between $37^0$C and $42^0$ C at second week in Sauton's media. **Figure 1, Table-2(a,b)**

**Biofilm formation by *M. avium***

For development of biofilm, we selected temperature point i.e. $30^0$C and $37^0$C, $42^0$C in Sauton media as well as MB 7H9 media. At second week *M.avium* develop moderate amount of biofilm at $37^0$C , $42^0$C, and at $30^0$C in MB7H9 media. In Sauton's a media at second weak and MB7H9 media at fourth week these bacteria developed moderate amount of biofilm at $30^0$C and $42^0$C but weak biofilm at $37^0$ C. However, in Sauton's media at fourth week moderate biofilm at $30^0$C and weak biofilm at $37^0$C and strong biofilm at $42^0$C were estimated. Statistical significant differences were observed at fourth week in MB7H9 media in between $37^0$ C and $30^0$ C and also in between $37^0$ C and $42^0$ C for *M.avium*. **Figure 2, Table -3(a,b)**

**Biofilm formation by *M tuberculosis* H37 Rv-:**

*M tuberculosis* H37Rv does not developed biofilm at $30^0$C and $42^0$C while strong biofilm was developed at $37^0$C in Sauton's as well as MB7H9 media at second week and in fourth week significantly. However, in Sauton's media these mycobacteria developed weak biofilm at $30^0$C and $42^0$ C and strong biofilm at 37ºC. However, in Sauton's media in fourth week these bacteria developed no biofilm at $30^0$C and $42^0$C and strong biofilm at $37^0$ C **Figure 2, Table -4 (a,b)**

**Biofilm formation by mycobacteria at different pH condition.** No consistence differences were observed at different pH condition for selected mycobacterial isolates. Data not shown. **(Figure 3,4,5.)**

**Ultrastructural Study-:** Scanning electron microscopy (SEM) analysis of biofilm, revealed irregular smooth colony and bacteria encased in a thick matrix of extracellular polymeric substances (EPS). These biofilm appeared to have more abundant extracellular matrix, holding the rods together, interspersed with water channels. Liquid flow occurs in these water channels, allowing diffusion of nutrients, oxygen, and even antimicrobial agents. All the figures described here were performed in triplicate in three isolates of each selected strain of mycobacteriaOne well each from isolates that were characterized as weakly adherent, moderately adherent and strongly adherent biofilm formers was examined

by Scanning electron microscopy,(SEM) after incubation. The weak biofilm former failed to colonize the majority of the surface of the polystyrene surface. Small clusters of cells were observed, but these did not aggregate to form a monolayer or a more mature biofilm structure The representative moderately adherent isolate grew in a uniform monolayer, but did not form a mature multi-layered biofilm. In the matured biofilm. Strongly adherent biofilms appeared to be more abundant, larger, and thicker in nature, and generally comprised a single morphotype, mostly rod shaped encased in a thick covering of EPS interspersed with channels., The tendency of the bacilli to become arranged together into linear cord-like formations was apparent. The SEM images supported the results achieved by crude crystal violet staining of biofilm biomass l (**Figure 6).**

**DISCUSSION:**

Biofilm development has been suggested to be a property of mycobacteria which might depend on the nutrients present in the medium **(Esteban *et al.*, 2008)**. The strength of biofilm development is reported to be dependent on various factors like contact surface, pH, temperature, humidity, nutrient availability, contact time of the bacteria with the surface, growth stage, surface hydrobhobacity and textures of surface etc. This factor affects the attachment and colonization of the bacteria for biofilm formation **(James *et al.*, 2005).** The pH, temperature **(Johnson *et al.*, 2009),** and nutrient composition **(Carter *et al.*, 2003),** are crucial factors for the growth of mycobacterial biofilm. It was observed that *M. tuberculosis* was the most restricted for growth at acidic pH. while Nontuberculosis mycobacterial species, may grow in soil or aquatic environments, which are more acidic tolerant. **(Piddington *et al.*, 2000)**. According to these observations we designed our experiments to observed the effects of different biotic and abiotic factors, temperature, pH and on the development of biofilm. In this study the selected mycobacteria developed a different amount of biofilm in different pH range and different time points such as first week and the second week for fast growers, *M. smegmatis* and *M. fortuitum* and second week and fourth week for slow growers, *M. avium* and *M.tuberculosis,* in Sauton's and MB7H9 media. In the present study fast growing mycobacterial species formed more biofilm at second week and slow growing mycobacteria at fourth week and Sauton's media are adequate for biofilm growth as compared to MB7H9 media.

In addition, surface lipids like glycopeptidolipids, mycolyldiacylglycerol, and lipooligosaccharides and mycolate are important for biofilm formation but phospholipid did not exhibit major role in biofilm formation. Further, rapidly growing cells are more susceptible to environmental stresses compared with slowly growing cells. It is important to point out the fact that because mycobacteria have only one or two ribosomal RNA (rRNA) operons (**Bercovier *et al.*, 1986**), their ability to adapt may be limited in time. There exist several examples of mycobacterial adaptation: most notably survival as a consequence of exposure to anaerobiosis (**Dick *et al.*, 1998)**, starvation (**Archuleta *et al.*, 2005**), acid (**Bodmer *et al.*, 2000**) and temperature (**Scammon *et al.*, 1964**), and elevated antibiotic and disinfectant resistance of biofilm-grown cells (**Steed and Falkinham, 2006;** A gradual reduction in oxygen concentration, generated by allowing *M. smegmatis* cells to consume oxygen in a closed culture, leads to condition where the cells are viable for long periods of time (**Dick *et al.*, 1998**). However, in other organisms, no effect on biofilm formation is seen over a range of pH. as also observed in *Pseudomonas fluorescens*, and found no effects on biofilm formation was seen in media ranging from pH 5 to 8.5 (**O'Toole *et al.*, 1998** ). Similarly, *Streptococcus gordonii* biofilm formation was unaffected in media ranging from pH 6 to 10.5 **(Loo CY 2000).** To our knowledge, this is the first study to examine the factors that influence the survival and growth of fast and slow grower mycobacteria in selected MB7H9 and Sauton's media. In addition, these studies are required using improved detection methods, the thickness of biofilm and amount of biofilm as well as the ultrastructural analysis especially *M.tuberculosis* H37Rv biofilm.

**CONCLUSION:**

The present work provides evidence that different biotic and abiotic factors affected biofilm formation is *in vitro* conditions. The nutritional and environmental conditions plays a role in biofilm development, so these observations may be useful in attempts to identify the cellular factors and molecular mechanisms involved in mycobacterial biofilm formation. Understanding critical steps involved in biofilm formation and metabolism may suggest new therapies for treatment or prevention of biofilm-related infections.

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**Tables:**

| COD | Strain | MB7H9 Media | | | Sauton's Media | | |
|---|---|---|---|---|---|---|---|
| S.No. | *M.smegmatis* | ODc | 2×ODc | 4×ODc | ODc | 2×ODc | 4×Odc |
| 1 | Temp- 30$^0$C -I,W | 0.32 | 0.64 | 1.28 | 0.35 | 0.7 | 1.4 |
| 2 | Temp-37$^0$C- I,W | 0.32 | 0.64 | 1.28 | 0.35 | 0.7 | 1.4 |
| 3 | Temp-42$^0$C-I,W | 0.32 | 0.64 | 1.28 | 0.35 | 0.7 | 1.4 |
| 4 | Temp- 30$^0$C -II,W | 0.32 | 0.64 | 1.28 | 0.35 | 0.7 | 1.4 |
| 5 | Temp-37$^0$C- II,W | 0.32 | 0.64 | 1.28 | 0.35 | 0.7 | 1.4 |
| 6 | Temp-42$^0$C-II,W | 0.32 | 0.64 | 1.28 | 0.35 | 0.7 | 1.4 |

**Table .1 (a) Cut off OD for effects of temperature on *M. smegmatis* biofilm formation**

| Week | Strain | MB7H9 Media | | Sauton's Media | |
|---|---|---|---|---|---|
| | *M.smegmatis* | Mean OD value ±S.D | Degree of formation | Mean OD value± S.D | Degree of formation |
| First | Temp-30$^0$C | 0.6317± 0.2885 | Weak | 1.054 ±0.1236 | Moderate |
| | Temp-37$^0$C | 0.9098 ±0.07783 | Moderate | 0.6009 ±0.07436 | Weak |
| | Temp- 42$^0$C | 0.9733 ±0.06787 | Moderate | 1.108 ±0.1663 | Moderate |
| Second | Temp-30$^0$C | 1.134 ±0.1719 | Moderate | 1.340 ±0.1396 | Moderate |
| | Temp- 37$^0$C | 1.611 ±0.4016 | Strong | 1.838 ±0.1645 | Strong |
| | Temp- 42$^0$C | 1.853 ±0.06926 | Strong | 2.033 ±0.1405 | Strong |

**Table .1 (b) Effects of temperature on *M. smegmatis* biofilm formation**

| C OD | Strain | MB7H9 Media | | | Sauton's Media | | |
|---|---|---|---|---|---|---|---|
| S.No. | *M.fortuitum* | ODc | 2×ODc | 4×ODc | ODc | 2×ODc | 4×ODc |
| 1 | Temp- $30^0$C - I,W | 0.32 | 0.64 | 1.28 | 0.35 | 0.7 | 1.4 |
| 2 | Temp-$37^0$C- I,W | 0.32 | 0.64 | 1.28 | 0.35 | 0.7 | 1.4 |
| 3 | Temp-$42^0$C-I,W | 0.32 | 0.64 | 1.28 | 0.35 | 0.7 | 1.4 |
| 4 | Temp- $30^0$C - II,W | 0.32 | 0.64 | 1.28 | 0.35 | 0.7 | 1.4 |
| 5 | Temp-$37^0$C- II,W | 0.32 | 0.64 | 1.28 | 0.35 | 0.7 | 1.4 |
| 6 | Temp-$42^0$C- II,W | 0.32 | 0.64 | 1.28 | 0.35 | 0.7 | 1.4 |

**Table. 2(a) Cut off OD for effects of temperature on *M. fortuitum* biofilm formation**

| Week | Strain | MB7H9 Media | | Sauton's Media | |
|---|---|---|---|---|---|
| | *M.fortuitum* | Mean OD value ±S.D | Degree of formation | Mean OD value±S.D | Degree of formation |
| First | Temp-$30^0$C | 1.283 ±0.2606 | Strong | 1.458 ±0.2207 | Strong |
| | Temp-$37^0$C | 0.6689 ±0.1227 | Moderate | 0.7820 ±0.1415 | Moderate |
| | Temp- $42^0$C | 0.7267 ±0.09552 | Moderatre | 0.8240 ±0.1303 | Moderate |
| Second | Temp-$30^0$C | 1.351 ±0.2724 | Strong | 1.995 ±0.1205 | Strong |
| | Temp- $37^0$C | 1.224 ±0.2296 | Moderatre | 0.6402 ±0.1228 | Weak |
| | Temp- $42^0$C | 1.047 ±0.2277 | Moderate | 1.326 ±0.1423 | Moderate |

**Table. 2(b) Effects of temperature on *M. fortuitum* biofilm formation**

| Cut Off OD | Strain | MB7H9 Media | | | Sauton's Media | | |
|---|---|---|---|---|---|---|---|
| S.No. | *M.avium* | ODc | 2×ODc | 4×ODc | ODc | 2×ODc | 4×ODc |
| 1 | Temp- $30^0$C –II W | 0.25 | 0.5 | 1 | 0.28 | 0.56 | 1.12 |
| 2 | Temp-$37^0$C- II W | 0.25 | 0.5 | 1 | 0.28 | 0.56 | 1.12 |
| 3 | Temp-$42^0$C-II W | 0.25 | 0.5 | 1 | 0.28 | 0.56 | 1.12 |
| 4 | Temp- $30^0$C –IV W | 0.23 | 0.46 | 0.92 | 0.26 | 0.52 | 1.04 |
| 5 | Temp-$37^0$C- IV W | 0.23 | 0.46 | 0.92 | 0.26 | 0.52 | 1.04 |
| 6 | Temp-$42^0$C-IV W | 0.23 | 0.46 | 0.92 | 0.26 | 0.52 | 1.04 |

**Table. 3(a) Cut off OD for effects of temperature on *M. avium* biofilm formation**

**Table. 3 (b) Effects of temperature on *M. avium* biofilm formation**

| Week | Strain | MB7H9 Media | | Sauton's's Media | |
|---|---|---|---|---|---|
| | *M.avium* | Mean OD value ±S.D | Degree of formation | Mean OD value ± S.D | Degree of formation |
| Second | Temp-$30^0$C | 0.6359 ±0.1322 | Moderate | 0.7021 ±0.2023 | Moderate |
| | Temp-$37^0$C | 0.5292 ±0.06492 | Moderate | 0.5076 ±0.2066 | Weak |
| | Temp- $42^0$C | 0.7007 ±0.2091 | Moderate | 0.9050 ±0.2641 | Moderate |
| Fourth | Temp-$30^0$C | 0.9348 ±0.07881 | Moderate | 1.073 ±0.1361 | Moderate |
| | Temp- $37^0$C | 0.6868 ±0.07933 | Weak | 0.6358 ±0.3070 | Weak |
| | Temp- $42^0$C | 0.9883 ±0.06819 | Moderate | 1.156 ±0.06865 | Strong |

| Cut Off OD | Strain | MB7H9 Media | | | Sauton's Media | | |
|---|---|---|---|---|---|---|---|
| S.No. | *M.tuberculosis* | ODc | 2×ODc | 4×ODc | ODc | 2×ODc | 4×ODc |
| 1 | Temp- 30$^0$C –II W | 0.344 | 0.6881 | 1.376 | 0.26 | 0.52 | 1.04 |
| 2 | Temp-37$^0$C- II W | 0.344 | 0.6881 | 1.376 | 0.26 | 0.52 | 1.04 |
| 3 | Temp-42$^0$C-II W | 0.344 | 0.6881 | 1.376 | 0.26 | 0.52 | 1.04 |
| 4 | Temp- 30$^0$C –IV W | 0.43 | 0.86 | 1.72 | 0.43 | 0.86 | 1.72 |
| 5 | Temp-37$^0$C- IV W | 0.43 | 0.86 | 1.72 | 0.43 | 0.86 | 1.72 |
| 6 | Temp-42$^0$C-IV W | 0.43 | 0.86 | 1.72 | 0.43 | 0.86 | 1.72 |

**Table. 4 (a) Cut off OD for effects of temperature on *M. tuberculosis* biofilm formation**

| Week | Strain | MB7H9 Media | | Sauton's Media | |
|---|---|---|---|---|---|
| | *M.tuberculosis* | Mean OD value ±S.D | Degree of formation | Mean OD value±S.D | Degree of formation |
| Second | Temp- 30$^0$C – II W | 0.2833 ±0.1834 | No | 0.3867 ±0.2875 | Weak |
| | Temp-37$^0$C- II W | 1.775 ±0.5617 | Strong | 1.491 ±0.5861 | Strong |
| | Temp-42$^0$C-II W | 0.3067 ±0.1607 | No | 0.3067 ±0.1662 | Weak |
| Fourth | Temp- 30$^0$C – IV W | 0.2617 ±0.1429 | No | 0.3543 ±0.3427 | No |
| | Temp-37$^0$C- IV W | 2.874 ±0.5351 | Strong | 2.576 ±1.078 | Strong |
| | Temp-42$^0$C-IV W | 0.1800 ±0.06083 | No | 0.4203 ±0.1476 | No |

**Table. 4 (b) Effects of temperature on *M. tuberculosis* biofilm formation**

FIGURES

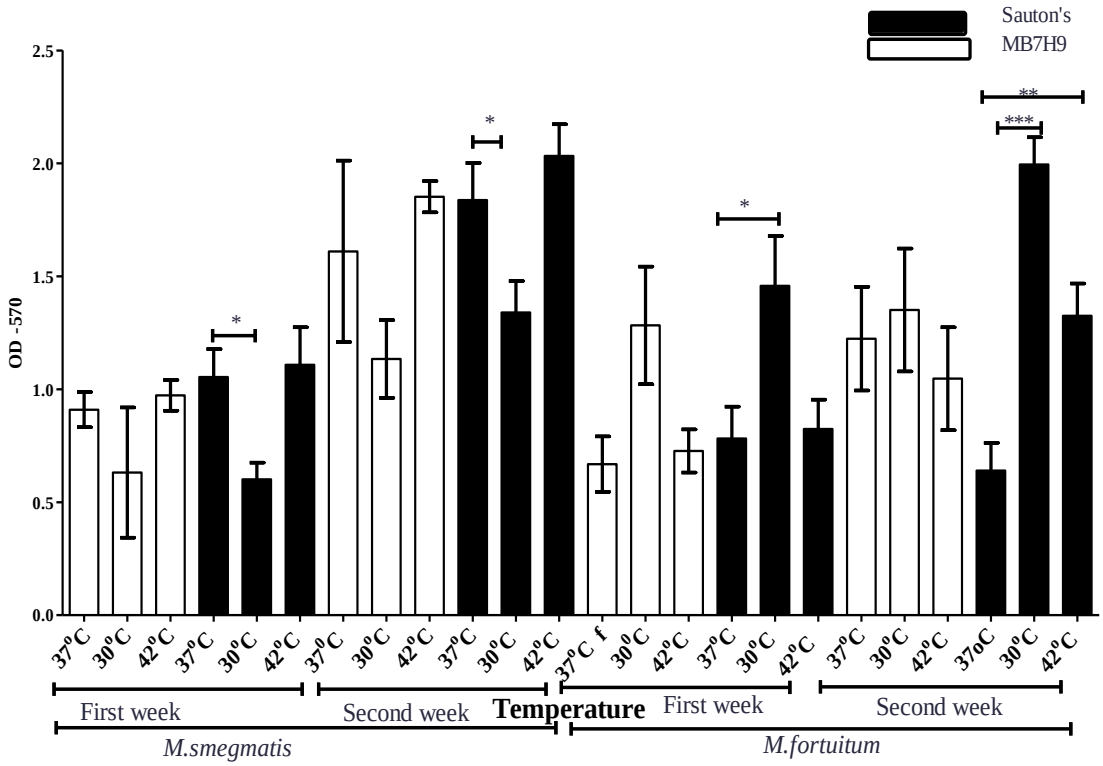



**Fig .1 Effect of temperature on development of biofilm of *M.smegmatis* and *M.fortuitum***

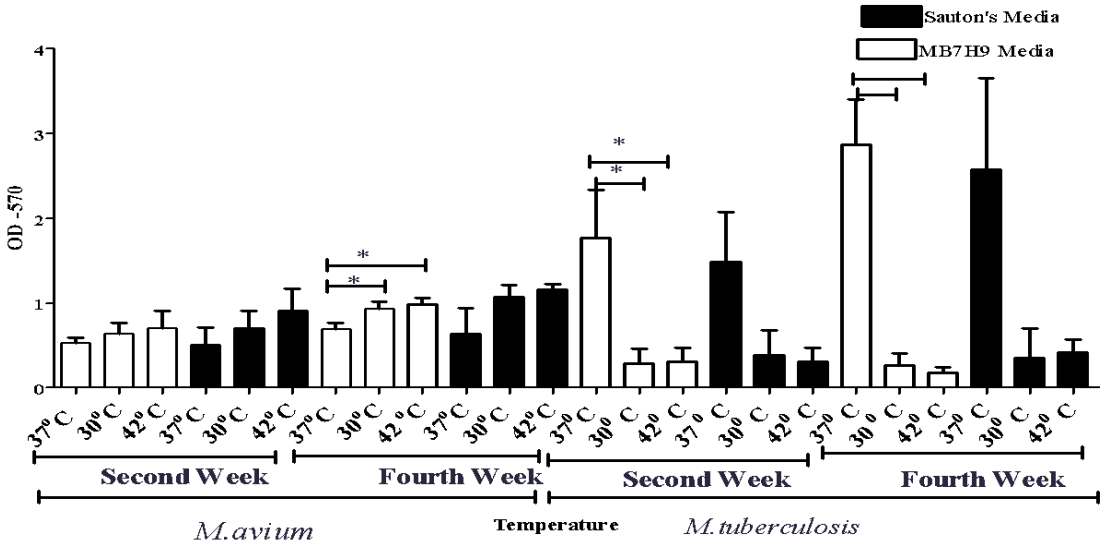



**Fig.2 Effect of temperture on development of biofilm of *M.avium* and *M.tuberculosis* H37Rv**

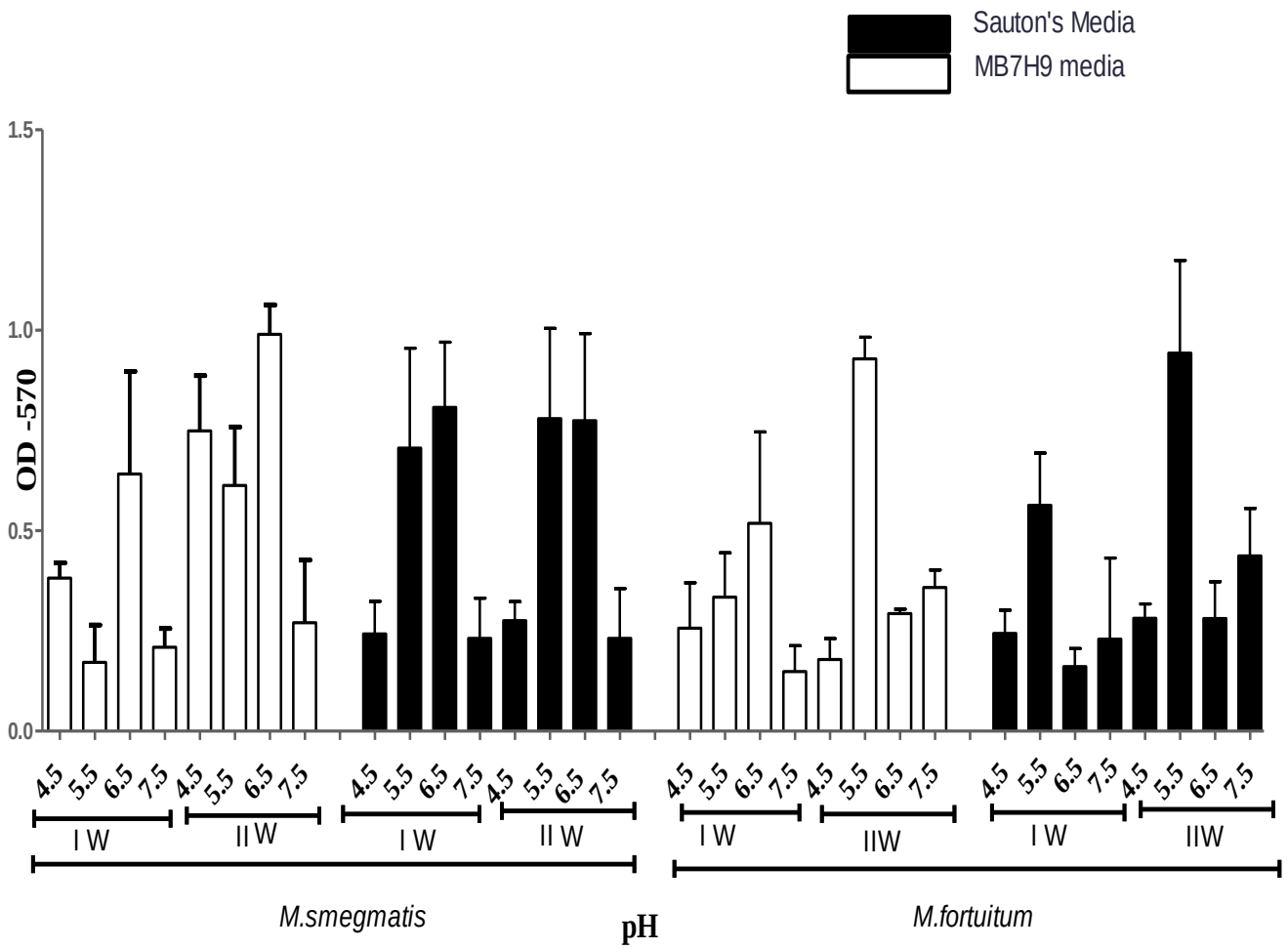



Fig.3 Effect of pH on development of biofilm of *M.smegmatis* and *M.fortuitum*

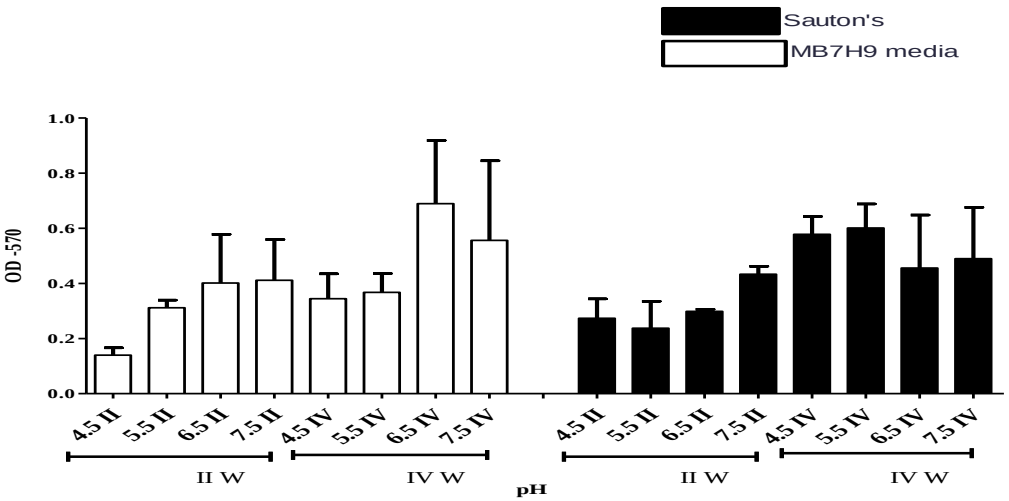



Fig. 4. Effect of pH on development of biofilm of *M.avium at* second week and fourth week.

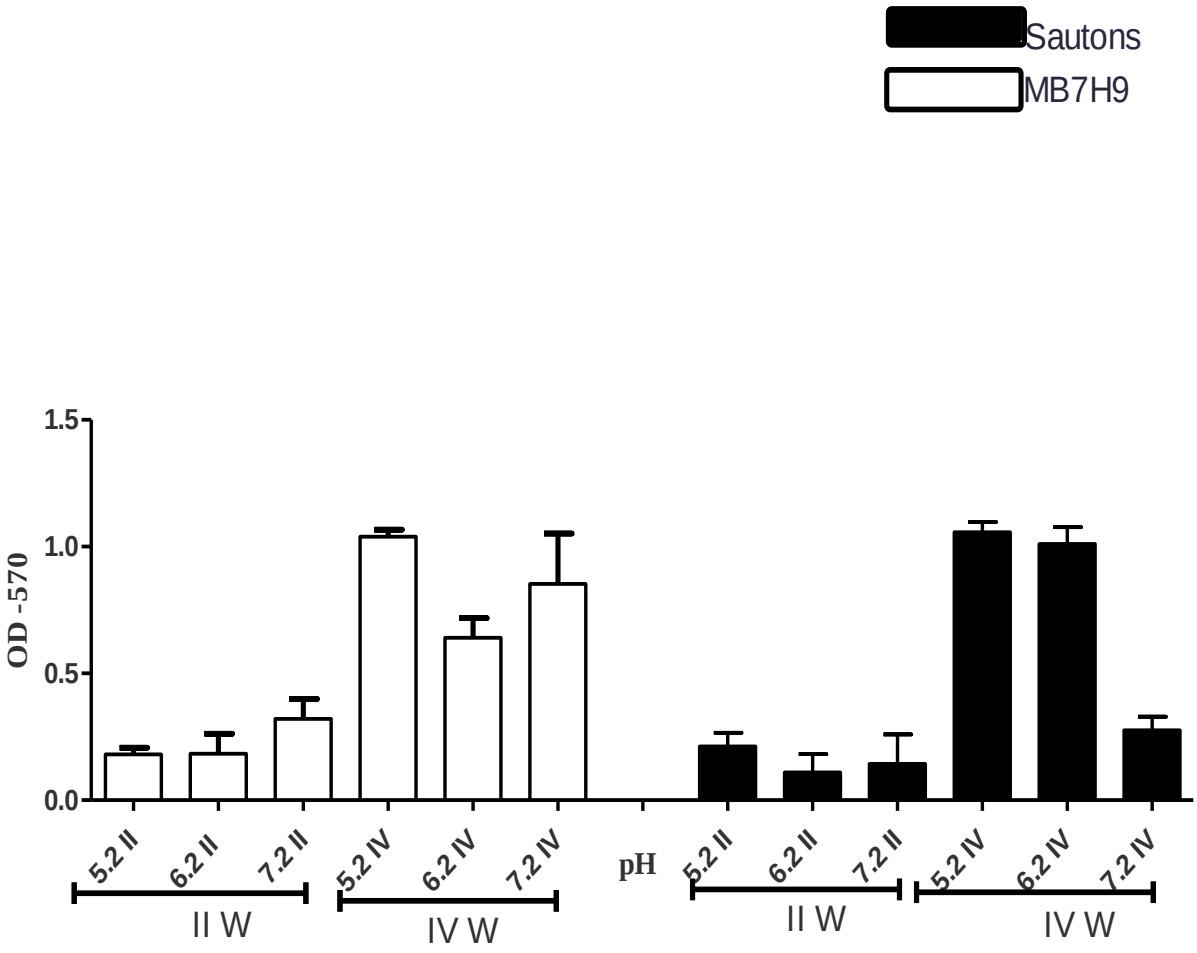
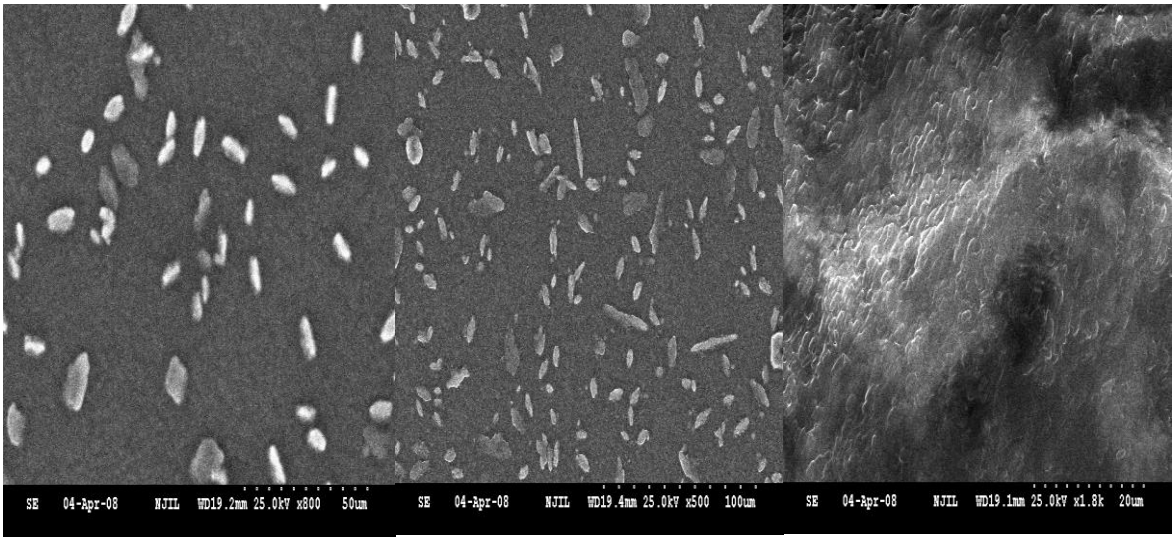



Fig. 5 Effect of pH on development of *M.tuberculosis* at second week and fourth week.



Weak Moderate Strong

Figure 6 - Ultrastructural picture for thickness of mycobacterial biofilm