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BACTERIAL CONDITIONED MEDIA: AN EFFECTIVE AGENT AGAINST *MYCOBACTERIUM SMEGMATIS* BIOFILM

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ABSTRACT

Biofilm plays a major role in the pathogenesis of *Mycobacterium tuberculosis*. Due to the presence of this polymeric matrix, anti-mycobacterial agents fail to exert their activity and thus *Mycobacterium* exhibits drug resistance phenotypes. Considering this, the current study investigated anti-biofilm activity of several conditioned media obtained after different bacterial culture using *Mycobacterium smegmatis* as model organism. For this purpose, logarithmically growing *Mycobacterium smegmatis* cells were treated with 11 different conditioned media prepared from different soil bacteria. Among these, five of the conditioned media exhibited considerable activity against biofilm. Thereafter, these five conditioned media were added to biofilm encapsulated mycobacterial cells to verify their ability to disperse preformed biofilm. The result showed that all these five conditioned media have ability to disperse preformed biofilm. Consistent with this, when any of these five conditioned media was added along with either isoniazid or rifampicin to biofilm encapsulated mycobacterial cells, cell viability was reduced drastically indicating the fact that these five conditioned media augment anti-mycobacterial activity of isoniazid and rifampicin. Thus the present study identified five bacterial strains, the conditioned medium of which exhibited anti-mycobacterial biofilm activity and augmented the activity of isoniazid and rifampicin against biofilm encapsulated cells. Thus, the current study provides agents that have the potential to be used in anti-mycobacterial therapy and may help in public health management.

KEYWORDS: Tuberculosis, Mycobacterium, Biofilm, Conditioned medium.

INTRODUCTION

The enormous numbers of infectious diseases in human beings are mostly caused by a plethora of pathogenic microorganisms that colonize various tissues as well as organs of human hosts and are metabolically dependent on the human hosts. Within the host body, most pathogenic microorganisms such as the species of *Pseudomonas, Mycobacterium, Escherichia* etc., survive within a stable self-produced matrix of extracellular polymeric substances known as biofilms that provide a stable microenvironment for their survival.[1] This stable microenvironment confers several advantages to the microorganism such as suitable resource utilization from the host, exhibition of virulence and also provides resistance to a wide array of antimicrobial agents.[2] Therefore, disassembly of biofilms often times makes the non-biofilm planktonic counterpart susceptible to a wide array of antimicrobial agents and antibiotics. Thus, physical and chemical agents that are capable of dispersing biofilm are considered to be effective strategy for antimicrobial therapy.[3] Tuberculosis is one such infectious disease that is mediated by the microorganism *Mycobacterium tuberculosis*, which is capable of extensive biofilm formation.[4] In the epidemiological map of world infectious disease, tuberculosis (TB) is the most dreaded disease and causes enumerable death worldwide. It is estimated that one third of the global population is infected with this disease.[5] The African and South-East Asian countries are the most affected.

M. tuberculosis forms biofilm during their colonization and pathogenesis and unlike conventional biofilm, mycobacterial biofilm is composed of eDNA and short chain mycolic acids that makes the biofilm hydrophobic.[6] Because of this biofilm barrier, commonly used mycobacterial drugs fail to penetrate the coats and thus biofilm encapsulated bacteria exhibit drug resistance phenotype.[7] Therefore, agent or agents that are capable of dispersing biofilm may make the drug resistance mycobacterium again susceptible to respective drugs.[8] To this end previous studies reported the use of various small molecules, conditioned media obtained after growth of various bacteria exhibited anti biofilm property.[9], [10], [11], [12]

In this context, current study was aimed at identifying some natural products from bacterial origin that have anti-biofilm property. To this end, the current study investigated the anti-mycobacterial biofilm activity of different bacterial conditioned medium.

MATERIALS AND METHOD

Bacterial strains and media

Mycobacterium smegmatis mc^2155 strains were grown in Middlebrook7H9 media (Himedia) supplemented with 0.02% glycerol, 0.025% BSA and 0.001% tween 20. Subculture of *M. smegmatis* was done on Middlebrook7H9 agar base medium supplemented with 0.02% glycerol every 15 days interval. All other chemicals were purchased from Merck.

Soil sample collection

For the isolation of different soil microorganisms, especially bacteria, the soil samples were collected from various sites of Kolkata municipal solid waste dumping ground DHAPA. After the collection, soils were sieved through strainer to remove particles, plastics and plant roots. Thereafter soil was stored in 4°C.

Isolation and characterization of soil bacteria

Soil bacteria were isolated by the standard serial dilution plate technique. 1 g of each soil sample was weighed and soaked in 10 ml of sterile PBS. The samples were then serially diluted. 100 µl from each dilution (10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) of each sample were plated on LB (Luria-Bertani growth medium) agar plates. Based upon the morphology and colour, different colonies were picked up and streaked on LB agar plate separately in order to obtain pure isolated colonies. Pure culture was stored at 4°C for subsequent studies.

Isolated bacterial strains were identified by ribotyping. Briefly, 3 ml of overnight culture of each bacterium was centrifuged and then the pellet was dissolved with 0.5 ml of sterile nuclease free water and then heated at 95°C for 15 minutes. Thereafter, the suspension was centrifuged to collect the supernatant. Then the supernatant was used as template for PCR amplification of 16S rDNA by using two universal primers namely 27F and 1492R. Thereafter the PCR products were purified and sequenced. Subsequently 16S rRNA gene sequences were exported to ‘‘Basic Local Alignment Search Tool” (BLAST) (NCBI-http://www.ncbi.nlm.nih.-gov) to identify matches with existing characterized reference sequences.

Preparation of conditioned medium

For the preparation of bacterial conditioned medium, individual organism was inoculated into 50 ml of sterile LB and was incubated at 30°C for 10 days with shaking condition. Thereafter the suspension was centrifuged at 8000 g for 15 minutes at 4°C to eliminate bacterial biomass. Then the supernatant was filter sterilized by passing through 0.22 µm syringe filter.

Biofilm assay

6-well polystyrene coated plates were used in this assay. Each well was poured with 3 ml of Middlebrook 7H9 medium supplemented with 0.02% glycerol and inoculated with 20 µl of overnight grown *M. smegmatis* mc^2155. Plate was incubated at 37°C for 4 days to allow biofilm formation in presence or absence of the conditioned medium. Thereafter, liquid medium were aspirated carefully and the adhered biofilms was stained with 1% crystal violet solution followed by extraction of stain by 30% acetic acid. The absorbance of the extracted stain was measured at 550 nm.

For pre-formed biofilm assay, first the biofilm was allowed to grow (by incubating at 37°C for 4 days). Thereafter, planktonic cells were aspirated carefully and the fresh medium was added. Subsequently, 50 µL of different conditioned medium was added alone or in combination with known anti-tubercular drugs and were incubated at 37°C for 4 days. Then the crystal violet assay (CV assay) was performed to quantify the biofilm.

Bacterial viability assay

Bacterial viability was performed by plate count method. 100 µl aliquot from each well was taken and then serially diluted by using PBS. Thereafter, 200 µl of the diluted sample was plated on Middlebrook 7H9 -Agar plate and then the plates were incubated at 37°C for 3 to 4 days. Thereafter the viability was determined by counting the number of colonies on the agar plates.

RESULTS AND DISCUSSION

Anti- mycobacterial biofilm activity profile of the conditioned media obtained after several bacterial growth

Conditioned media (CM) from 11 different bacteria isolated from soil as described in methods and materials have been examined for their anti-mycobacterial biofilm activity. Ribotyping was performed to identify these isolates. The names of identified bacteria are summarized in Table 1. To examine the anti-mycobacterial activity, *M. smegmatis* cells were inoculated in presence and absence of these condition medium obtained from different bacterial cultures and incubated at 37°C for four days. After the incubation, a mesh like structure was appeared in control well (well no. 1), wherein no conditioned medium was added. However, wells, wherein CM was added, exhibited variation in the morphological appearance of the biofilm mesh. While biofilm mesh remained present in wells 7 to 12 wherein CM of AKS N4, AKS N5, AKS N6, AKS N9, AKS N12 and AKS N15 were added respectively, the mesh like structure was completely disappeared in wells 2 to 6 wherein CM of AKS 31, AKS 37, AKS 44, AKS N1 and AKS N2 were added respectively (Fig. 1A upper panel). To further validate, crystal violet (CV) assay was performed. Consistent with the above observation, the absorbance at 550 nm was drastically reduced in presence of CM of AKS 31, AKS 37, AKS 44, AKS N1 and AKS N2 compared to the control. On the other hand there was no significant change in absorbance wherein CM from AKS N4, AKS N5, AKS N6, AKS N9, AKS N12 and AKS N15 was used compared to the control (Fig. 1B). These results documented that the CM obtained from of AKS 31, AKS 37, AKS 44, AKS N1 and AKS N2 inhibit the formation of biofilm by *M. smegmatis*.

Mycobacterial biofilm dispersal profile of the conditioned media

In continuation with the previous observations, further analysis was performed to verify whether the conditioned media (CM) that exhibited anti biofilm activity are able to disperse preformed mycobacterial biofilm or not. For this purpose, mycobacterial culture was allowed to grow for four days in 6-well polystyrene coated plates. The biofilm mesh was appeared in each well and the CM was added to the well and again incubated for four more days. After the incubation the mesh like structure remained intact in control cell where no CM was added. On the other hand, wherein CM was added a granular appearance was observed instead of the mesh like structure (Fig. 2A, upper panel; well nos. 5, 6, 7, 8 and 9). The appearance of granular structure indicated the dispersal of biofilm. Two known mycobacterial drugs namely isoniazid and rifampicin have also been used in this experiment. In contrast to the conditioned media, the biofilm mesh appeared similar in presence of either isoniazid or rifampicin compared to controls (wells 1 and 2). This result is in agreement with previously published report (Fig. 2A). DMSO (5%) has been used as negative control as it was used as solvent for isoniazid and rifampicin (Fig. 2A, well no. 2). Crystal violet assay was also performed to confirm this observation. In agreement with previous observation, OD value was markedly reduced for the wells 5, 6, 7, 8 and 9 compared to the control well 1. The wells 5 and 7 exhibited maximum reduction in their OD. On the other hand again the presence of isoniazid and rifampicin caused a little reduction in OD, indicating that these two agents do not have much effect on preformed biofilm (Figure 2B). No difference in OD was found in between control well 1 and well 2 containing DMSO. It indicated that DMSO does not affect preformed biofilm. Moreover, the biofilm mesh was undisturbed in well 10 wherein CM of AKS N4 was added. Taken all these results together it can be concluded that CM of AKS 31, AKS 37, AKS 44, AKS N1, and AKS N2 respectively have the ability to disperse preformed mycobacterial biofilm.

Conditioned medium augment the activity of known anti-tubercular drugs

Rifampicin and isoniazid cannot kill biofilm encapsulated mycobacterial cells. Since CM of AKS 31, AKS 37, AKS 44, AKS N1 and AKS N2 can disperse mycobacterial biofilm, it is likely that the treatment of biofilm encapsulated mycobacterial cells with either isoniazid or rifampicin in combination with any of the above mentioned CM result in killing of the mycobacterial cells. To verify this, either isoniazid or rifampicin was added along with the different conditioned medium to performed mycobacterial biofilm. After 4 days of incubation, cell viability was assayed. Consistent with our expectation, neither the addition of different CM nor the addition of isoniazid and rifampicin resulted in reduced cellular viability (Fig. 3). In contrast, when the CM was added along with either isoniazid or rifampicin the viable count was drastically reduced compared to the control wherein no such agents were added. Interestingly, in this assay rifampicin exhibited better activity than isoniazid by reducing higher number of viable cells (Fig. 3).

Thermal stability profile of the conditioned media

To examine the thermal stability, the conditioned media were heated at 100°C for 15 minutes and centrifuged. The supernatants were collected and examined for their activity on mycobacterial biofilm development by adding heat treated and untreated CM. After four days of incubation of mycobacterial culture, mesh like structure was appeared in control well, wherein only sterile LB broth was added in extra indicating the development of mycobacterial biofilm (Fig. 4A and B). On the other hand, consistent with the expectation, the mesh like structure was disappeared when untreated CM of AKS 31 was added (Fig. 4A and B). However, the same CM when added after heat treatment, anti-biofilm activity was reduced significantly and biofilm mesh was appeared (Fig. 4A and B). Similar result was also observed for the CM of AKS 44. While heat untreated AKS 44 CM exerted anti biofilm activity, the same CM after the heat treatment exhibited significantly reduced anti-biofilm activity. On the other hand, CM obtained from AKS 37, AKS N1 and AKS N2 exhibited similar anti-biofilm activity even after heat treatment (Fig. 4A and B) compare upper panel with lower panel). Taken together, it can be concluded that the component(s) responsible for anti-biofilm activity of AKS 37, AKS N1 and AKS N2 are heat resistance but the component(s) responsible for anti-biofilm activity of CM of AKS31 and AKS44 are heat sensitive.

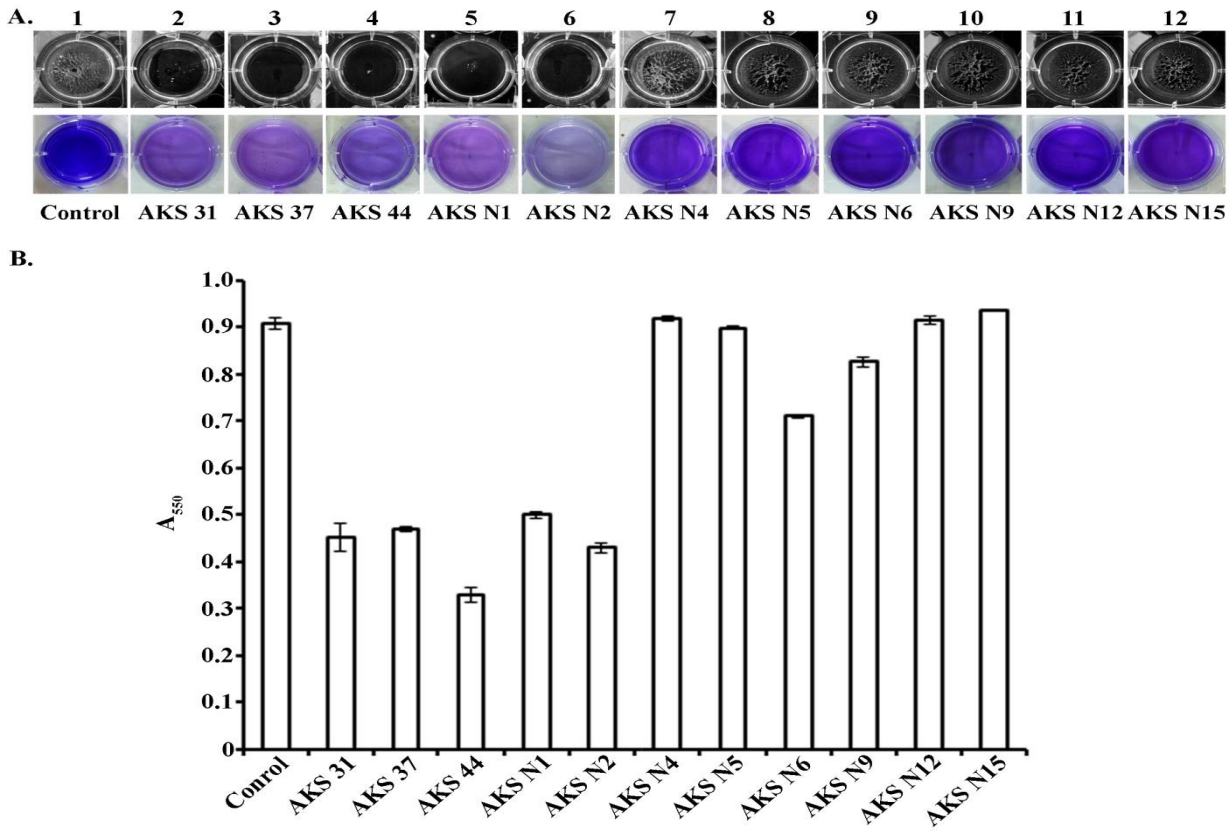

Figure 1: Anti- mycobacterial biofilm activity profile of the conditioned media. *M. smegmatis* mc^{2}155 was grown in 6 well tissue culture plate in presence or absence of different conditioned media for 4 days. Thereafter CV assay was performed. A. Photograph of the wells before (upper panel) and after (lower panel) CV staining. B. Graphical representation of CV assay result.

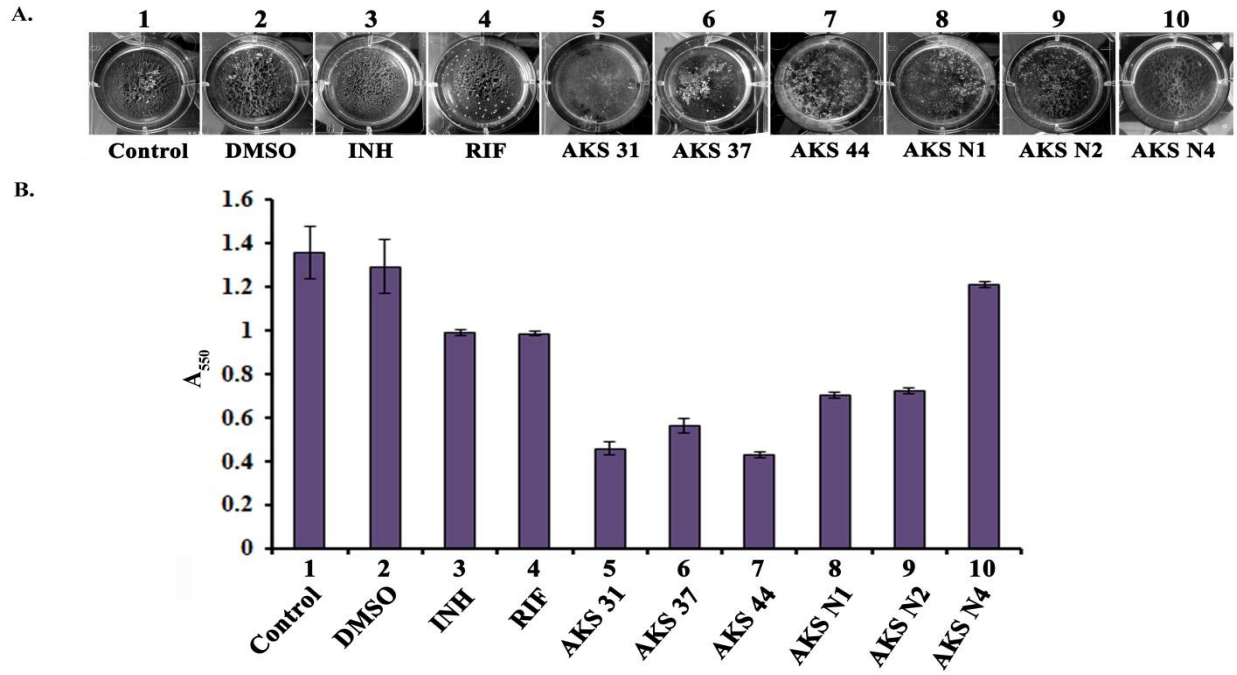

Figure 2: Dispersal of pre-formed mycobacterial biofilm by conditioned media. *M. smegmatis* mc^{2}155 was allowed to grow for 4 days in 6 well tissue culture plates. Thereafter, different types of conditioned media were added as indicated and incubated for 4 more days. Thereafter the cells were stained with crystal violet and CV assay was performed. A. Photograph of the wells before B. Graphical representation of CV assay result.

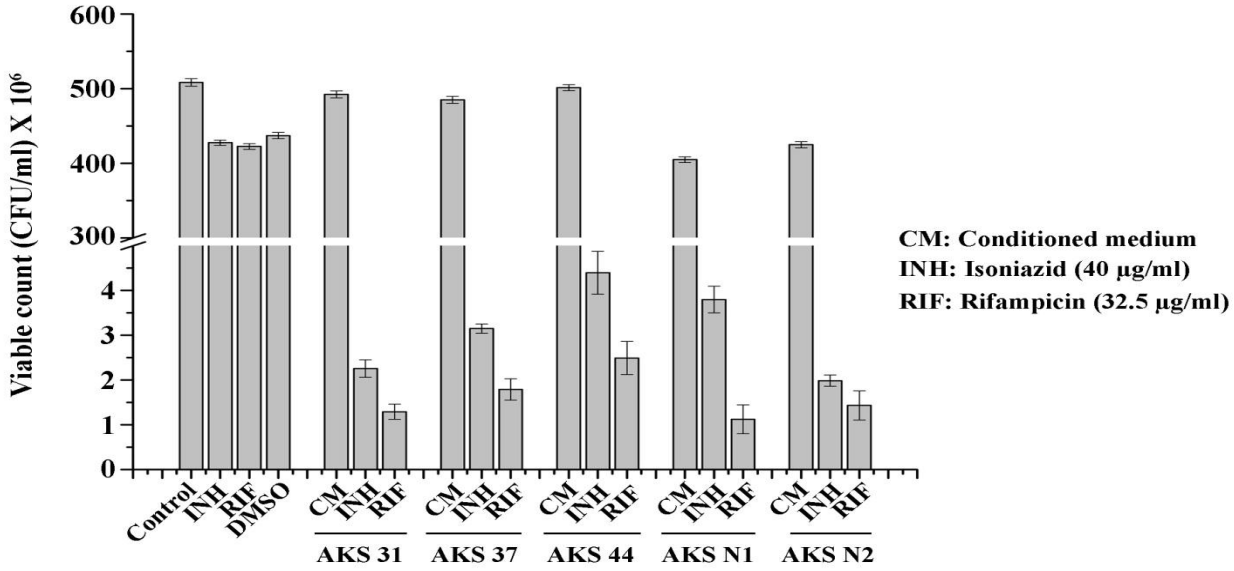

Figure 3: Bacterial conditioned media augment the activity of isoniazid and rifampicin. *M. smegmatis* mc^{2}155 was allowed to grow for 3 days in 6 well tissue culture plates. Thereafter, different conditioned media were added along with isoniazid (40 µg/mL) and rifampicin (32.5 µg/mL) as indicated. The plates were incubated for 4 more days and cell viability (CFU/mL) was assessed. The bar diagram represents the viable counts of *M. smegmatis* differentially treated with conditioned media, isoniazid and rifampicin.

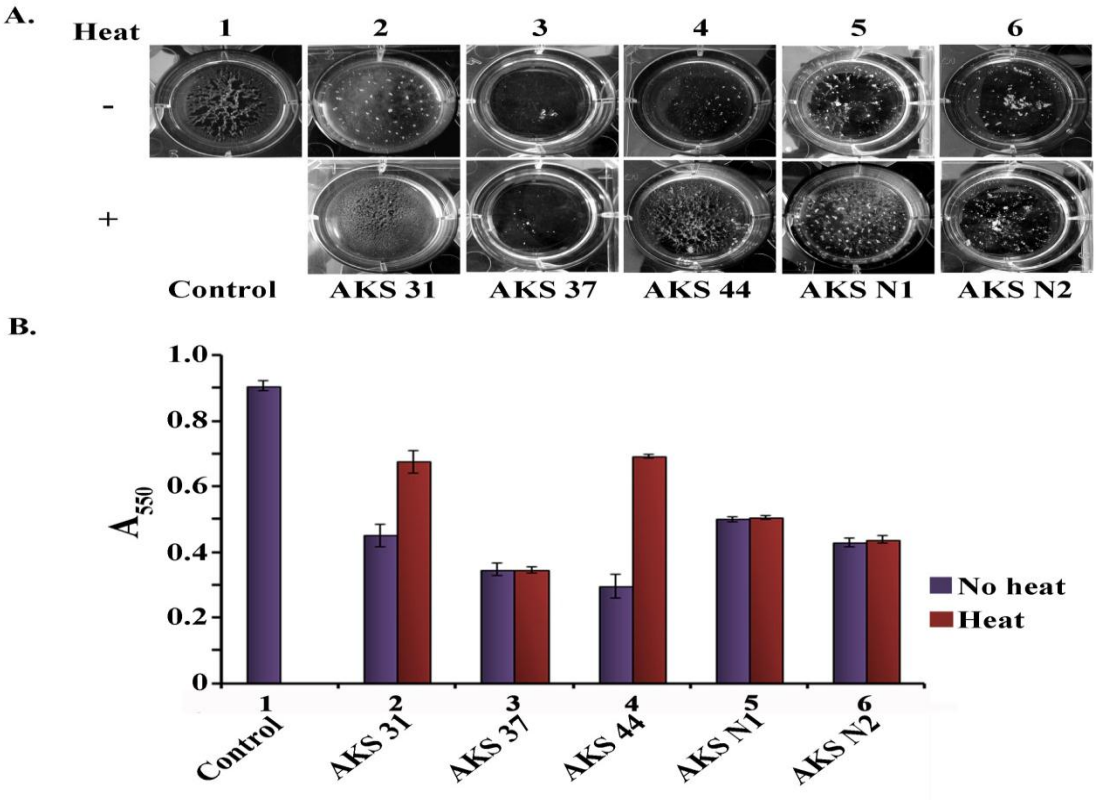

Figure 4: Heat tolerance profile. *M. smegmatis* mc^{2}155 was allowed to grow for 3 days in 6 well tissue culture plates. To each well different types of heat treated or untreated conditioned media added as indicated and incubated for 4 more days. Thereafter the cells were stained with crystal violet and CV assay was performed. A. Photograph of the wells before (upper panel) and after (lower panel) heat treatment of conditioned media. B. Graphical representation of CV assay result.

Table 1: Isolated soil microorganisms.

Sl. No	Organism
1.	***Pseudomonas sp.* AKS 31**
2.	***Pseudomonas sp.* AKS 37**
3.	***Brodetella sp.* AKS 44**
4.	***Exiguobacterium sp.* AKS N1**
5.	***Kacuria sp.* AKS N2**
6.	***Bacillus sp.* AKS N4**
7.	***Kacuria sp.* AKS N5**
8.	***Kacuria palustris* AKS N6**
9.	***Microbacterium sp.*AKS N9**
10.	***Exiguobacterium auranticum* AKS N12**
11.	***Staphylococcus warneri* AKS N15**

CONCLUSION

In conclusion, the present study identified five bacteria namely *Pseudomonas sp.* AKS 31, *Pseudomonas sp.* AKS 37, *Brodetella sp.* AKS 44, *Exiguobacterium sp.* AKS N1 and *Kacuria sp.* AKS N2 that secrete some metabolite(s) in the growth media having activity against mycobacterial biofilm. These conditioned media are not only active against the development of biofilm but also they are able to disperse pre-formed mycobacterial biofilm. Consistent with this, these conditioned media, augment the activity of known anti-tubercular drugs namely isoniazid and rifampicin that are known to be ineffective against preformed biofilm. Furthermore, among these five, CM from AKS37, AKSN1 and AKSN2 are heat resistant indicating their higher shelf life. Thus these CM hold promise to be good therapeutic agents in combination with known anti-tubercular drugs. In this context further studies are required to identify the active component(s) present in the conditioned medium that have the anti-biofilm property.

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COMPETING INTEREST

Authors declare no competing interest.

REFERENCES

1. Blanchette, K. A., & Orihuela, C. J. (2012). Future perspective on host–pathogen interactions during bacterial biofilm formation within the nasopharynx. *Future microbiology*, *7*(2): 227-239.
2. Esteban, J., & García-Coca, M. (2018). Mycobacterium biofilms. *Frontiers in microbiology*, *8:* 2651.
3. Romero, D., & Kolter, R. (2011). Will biofilm disassembly agents make it to market?. *Trends in microbiology*, *19*(7): 304-306.
4. Brennan, M. J. (2017). Biofilms and Mycobacterium tuberculosis. *Infection and immunity*, *85*(10): e00411-17.
5. World Health Organization ed., 2018. *Global tuberculosis report 2018*. World Health Organization.
6. Richards, J. P., & Ojha, A. K. (2014). Mycobacterial biofilms. *Molecular Genetics of Mycobacteria, Second Edition*, American Society of Microbiology, 773-784.
7. Hall, C. W., & Mah, T. F. (2017). Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria. *FEMS microbiology reviews*, *41*(3): 276-301.
8. Roizman, D., Vidaillac, C., Givskov, M., & Yang, L. (2017). In vitro evaluation of biofilm dispersal as a therapeutic strategy to restore antimicrobial efficacy. *Antimicrobial agents and chemotherapy*, *61*(10): e01088-17.
9. Molina, L., Constantinescu, F., Michel, L., Reimmann, C., Duffy, B., & Défago, G. (2003). Degradation of pathogen quorum-sensing molecules by soil bacteria: a preventive and curative biological control mechanism. *FEMS Microbiology Ecology*, *45*(1): 71-81.
10. Worthington, R. J., Richards, J. J., & Melander, C. (2012). Small molecule control of bacterial biofilms. *Organic & biomolecular chemistry*, *10*(37): 7457-7474.
11. Oppenheimer-Shaanan, Y., Steinberg, N., & Kolodkin-Gal, I. (2013). Small molecules are natural triggers for the disassembly of biofilms. *Trends in microbiology*, *21*(11): 594-601.
12. Parrino, B., Schillaci, D., Carnevale, I., Giovannetti, E., Diana, P., Cirrincione, G., & Cascioferro, S. (2019). Synthetic small molecules as anti-biofilm agents in the struggle against antibiotic resistance. *European journal of medicinal chemistry*, *161:* 154-178.