ANALYSIS OF BIOFILM FORMATION AND IDENTIFICATION OF GENE(S) INVOLVED IN BIOFILM FORMATION IN *MYCOBACTERIUM FORTUITUM*

Project Thesis submitted in partial fulfillment of the requirement for the degree of

Bachelor of Technology

in

Biotechnology

By

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Under the Guidance of

Dr. Rahul Shrivastava

to

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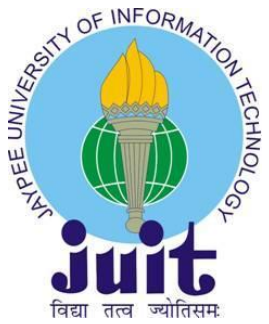
DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY,

WAKNAGHAT, SOLAN, HIMACHAL PRADESH -173234.



CERTIFICATE

This is to certify that the work titled **"Analysis of biofilm formation and identification of gene(s) involved in biofilm formation in *Mycobacterium fortuitum*"**, submitted by **"Kinam Gupta (133803)"** in partial fulfillment for the award of degree of B. Tech in Biotechnology of Jaypee University of Information Technology, Solan has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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CANDIDATE'S DECLARATION

I hereby declare that the work presented in this report entitled **"Analysis of biofilm formation and identification of gene(s) involved in biofilm formation in *Mycobacterium fortuitum*"** in partial fulfilment of the requirements for the award of the degree of **Bachelor of Technology** in **Biotechnology** submitted in the Department of Biotechnology and Bioinformatics**,** Jaypee University of Information Technology Waknaghat is an authentic record of my own work carried out over a period from August 2016 to April 2017 under the supervision of **Dr. Rahul Shrivastava** (Assistant Professor (Senior Grade)) Department of Biotechnology and Bioinformatics.

The matter embodied in the report has not been submitted for the award of any other degree or diploma.

Kinam Gupta, 133803

This is to certify that the above statement made by the candidate is true to the best of my knowledge.

Dr. Rahul Shrivastava

Assistant Professor (Senior Grade)

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ACKNOWLEDGEMENT

It is indeed a great pleasure to thank all those individuals who have, directly or indirectly, contributed and extended their valuable assistance in the progress and completion of this project.

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ABSTRACT

Mycobacterium fortuitum is a non tuberculous, rapidly growing mycobacterium which is present ubiquitously in nature. *M. fortuitum* is an emergent pathogen attaining relevance in human health as it is one of the leading causes of opportunistic infections such as local cutaneous diseases, osteomyelitis, joint infections, ocular disease, post surgical infections etc. An obstruction is imposed on the way of its treatment as it is evolving resistance to the currently prescribed drugs. Growing incidence of nosocomial infections caused by *M. fortuitum* may be correlated with its ability to assemble biofilm on implantable devices and other surgical instruments. Biofilm formation plays an important in the pathogenesis of *M. fortuitum,* thus, the present study aims to identify genes responsible for biofilm formation in *M. fortuitum* by random mutagenesis.

As the genomic sequence of *M. fortuitum* is not known, random mutagenesis of wild type *M. fortuitum* (ATCC 6841) was done by electroporation of vector pRT291 containing transposon Tn5 with kanamycin resistance. *M. fortuitum* mutants were screened on NAT plates containing Kanamycin, Cycloheximide, X-gal, IPTG and 5% glucose.

Borrowed library containing 120 mutants was screened for biofilm formation using crystal violet assay, out of which 50 mutants showing minimum optical density were selected. These mutants were further examined for biofilm formation by carbol fuschin staining method to shortlist 5 mutants with attenuation in biofilm formation for further experiments.

The mutants were individually subjected to the standardised biofilm forming protocol, to check for any deviation in the amount of biofilm formed. Genomic analysis followed by homology study of mutant was done, which revealed a hypothetical gene i.e. “Anthranilate phosphoribosyl transferase”, which may be involved in biofilm formation in *M. fortuitum*. Gene identified by this study can serve as potential drug targets for development of novel drugs or other intervention strategies.

Kinam Gupta (133803) **Dr. Rahul Shrivastava**

LIST OF FIGURES

LIST OF SYMBOLS AND ACRONYMS

AFM	Atomic Force Microscopy
ALS	Alkaline Lysis Buffer
CAPD	Continuous Ambulatory Peritoneal Dialysis
^{0}C	Degree Celsius
ECM	Extra Cellular Matrix
EPS	Extracellular Polymeric Substances
GTE	Glucose Tris EDTA
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KAN^R	Kanamycin Resistance
LB	Luria Bertani Broth
LBGT	Luria Bertani Broth Glycerol Tween
µF	Micro-farad
µL	Micro-liter
µg/mL	Microgram per Milliliter
mg/mL	Milligram per Milliliter
Nm	Nanometer
NAT	Nutrient Agar Tween
NTM	Non Tuberculous Mycobacteria
OD	Optical Density
PGL	Phenolic Glycolipid
Pks	Polyketide Synthase
RPM	Revolution Per Minute
Sec.	Seconds
SDS	Sodium Dodecyl Sulfate
SEM	Scanning Electron Microscope
TAE	Tris Acetate EDTA
TEM	Transmission Electron Microscope
TES	2-[Tris(hydroxymethyl)-methylamino]-ethanesulfonic acid
UV	Ultra-Violet
X-GAL	X –galactosidase
%	Percentage

Chapter 1

Introduction

1. INTRODUCTION

Mycobacterium fortuitum is an emergent pathogen capable of infecting with variable clinical manifestations to normal as well as immuno-compromised individuals with varying degree; accounting for many asymptomatic infections [5]. *Mycobacterium fortuitum*, belonging to phylum actinobacteria, is one of the many species of non-tuberculous mycobacteria (NTM) that are commonly found in the environment [6]. *M. fortuitum* is a commonly isolated organism from respiratory specimens in clinical laboratories in many countries. *Mycobacterium fortuitum* is a fast-growing species that can cause infections. The term "fast growing" is a reference to a growth rate of 3 or 4 days, when compared to other Mycobacteria that may take weeks to grow out on laboratory media. Optimal temperature for the growth of *M. fortuitum* is 30-37^{0}C. Pulmonary infections of *M. fortuitum* are uncommon, but *Mycobacterium fortuitum* can cause local skin disease, osteomyelitis (inflammation of the bone), joint infections and infections of the eye after trauma. *Mycobacterium fortuitum* has a worldwide distribution and can be found in natural and processed water, sewage, and dirt [5].

Bacteria classified as *Mycobacteria*, include the causative agents for tuberculosis and leprosy. *Mycobacteria* are sometimes referred to as "acid-fast bacteria," a term referencing their response to a laboratory staining technique. This simply means that when microscopic slides of these bacteria are rinsed with an acidic solution, they retain a red dye. *Mycobacterium fortuitum* is one of the many species of Non Tuberculous Mycobacteria (NTM) that are commonly found in the environment. These are not involved in tuberculosis. This does not mean, however, that they will not cause an infection in the right circumstances.

Mycobacterium tuberculosis was identified by Robert Koch in 1882, and *M. fortuitum* was identified soon afterwards. It was classified as a Non Tuberculous Mycobacterium (NTM) and was not studied until 1954. Edward Runyon was the scientist to categorize all the NTMs. *M. fortuitum* is now classified as Runyon Group IV, which means, among other lab culture characteristics, it is a rapid grower.

Infection in healthy humans caused by *Mycobacterium fortuitum* is rare, but exposure to large and repeated amounts of the organism can activate the immune system and cause disease. Infections most likely occur in immunocompromised patients, making *M.*

fortuitum an opportunistic pathogen. Diseases and infections caused vary from each other and involve almost every organ and tissue system. The most common infections involve NTM lung disease, local cutaneous disease, disseminated disease, surgical site and injection site infections. Most surgical site and injection site infections occur due to the ability of *M. fortuitum* to persist as biofilm within healthcare units.

Mortality is very rare, but death may come from extensive pulmonary or disseminated disease in immunocompromised patients. The morbidity depends on the site of infection. Localized skin lesions can heal without antibiotics or surgical excision. The average antibiotic treatment for all infections is six months. If no progress is made within six months, surgical excision of the lesion or removal of the device (ex. catheter) is required.

In a study, over 115 people in California were reported to be infected with *M. fortuitum* after being exposed to it in whirlpool footbaths at a spa. Even though the footbaths were cleaned with bleach between each use, the water filter where the water entered the footbath was not cleaned, and the bacteria colonized behind it. Everyone developed lesions on their lower legs, some requiring antibiotics and excision and some not. It was suggested that bacteria was able to colonize the water filter due to its ability to assemble biofilm.

Above study indicates that biofilm formation plays a key role in pathogenesis of bacteria. Biofilms are difficult to eradicate with common decontamination practices and are relatively resistant to standard disinfectants [7, 8]. Biofilms are also resistant to high concentrations of antimicrobial drugs and are able to modulate the host immune system [9]. This high resistance is mainly due to the virulence enhancing caused by biofilms. The self-produced matrix is also considered important in enhancing bacteria virulence. The matrix builds a barrier that can inactivate antibiotics, delaying or preventing antibiotic penetration within the biofilm and recognition of their targets [10]. Thus, there is a need to find potential drug targets which can result in reduction of biofilm formation ability of *M. fortuitum*.

Chapter 2

Review of Literature

2. REVIEW OF LITERATURE

2.1 Introduction

Mycobacterium fortuitum, a gram-positive and rod-shaped bacterium, is a nontuberculous mycobacterium. Generation time of *M. fortuitum* is 4 hours. It can be found in natural and processed water, sewage and dirt [5]. It is an emergent pathogen whose incidence of infection is increasing worldwide. It is the cause of many hospital associated infections. It is an opportunistic pathogen which causes disease mainly in immuno-compromised and immuno-competent patients. It is the major cause of nosocomial infections because of its ability to assemble biofilm on implantable devices and surgical instruments [11]. *M. fortuitum* has the ability to form biofilm which plays a major role in it pathogenesis. During biofilm development, bacteria undergo several changes in their phenotypic state forming a heterogeneous, dynamic, and differentiated community. They are part of a successful bacterial survival strategy in severe environments, since biofilm provides protection against environmental stressors [12]. For this reason, biofilm formation by *M. fortuitum* is an important research topic in mycobacteria pathogenesis.

2.2 Epidemiology of *M. fortuitum*

According to the guidelines, it is not necessary to report NTM infections [13]; therefore, it is impossible to determine the exact estimates of disease prevalence and incidence. NTM is distributed worldwide; however due to difference in the environmental nature of microorganisms, significant geographic differences in terms of species incidence are experienced by NTM. Most European Union (EU) and western countries are predominated by bacteria present in the *Mycobacterium avium* complex (MAC), followed by *M. xenopi* and *M. gordonae* [14]. The next most frequently isolated NTM in EU countries is the rapid grower *M. fortuitum*. The major species isolated from Saudi Arabia (Middle East) include *M. abscessus*, *M. intracellulare* and *M. fortuitum*, followed by *M. gordonae*, *M. kansasii*, and *M. avium* [15]. In India, the same pattern is observed where the most commonly isolated NTM is *M. fortuitum* [16]. As it has been reported, different geographic spots have distinct etiological agents responsible for the infection, thus, different regions require completely dissimilar therapeutic approaches. Hence, this atypical aspect of NTM geographical distribution represents a challenge to manage infectious disease.

2.3 Clinical Manifestation of *M. fortuitum*

M. fortuitum infection is responsible for causing various clinical syndromes. It is an atypical cause of NTM lung disease. Ocular disease (eg, corneal ulcers, keratitis), joint infections, local cutaneous disease, and osteomyelitis may occur after trauma. *M. fortuitum* infection is a rare cause of isolated lymphadenitis. Disseminated disease, usually with disseminated skin lesions and soft tissue lesions, occurs almost exclusively in the immunocompromised individuals, especially AIDS patients. Endocarditis has also been reported.

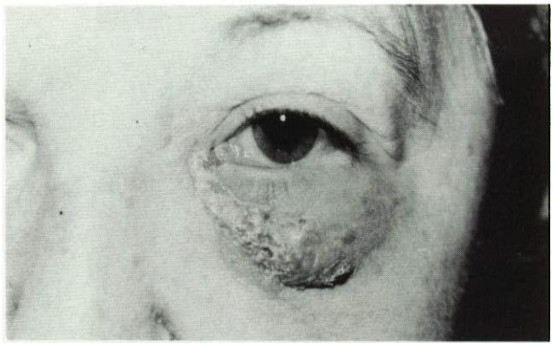


***Fig. 1**: Patient with Dacryocystitis caused by M. fortuitum* [1].

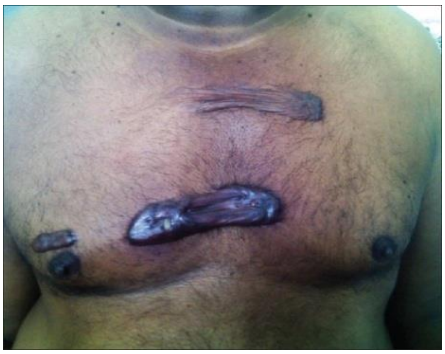


***Fig.2**. Multifocal keloids associated with M. fortuitum following intralesional steroid therapy* [4].

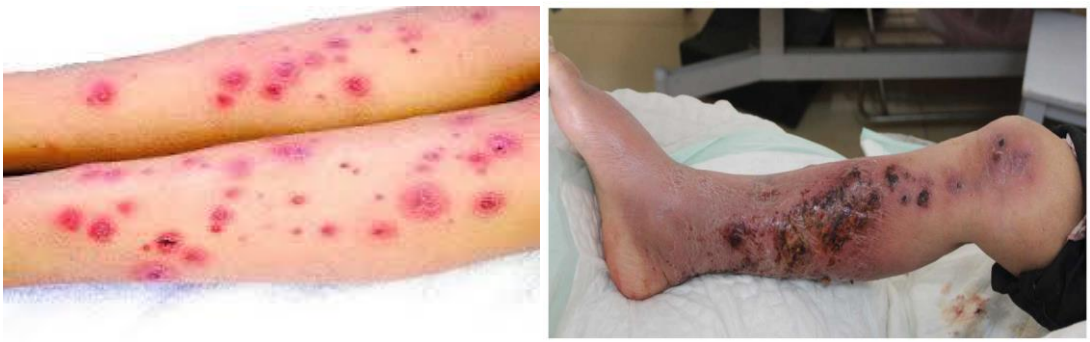


***Fig. 3 and Fig. 4**: Skin lesions caused by M. fortuitum* [3].

Surgical-site infections due to *M. fortuitum* infection are well-documented, especially in association with cardiothoracic surgery. The source is frequently contamination of the wound, directly or indirectly, with colonized tap water. Other nosocomial infections with this organism include infections of implanted devices (eg, catheters) and injection-site abscesses. Pseudo-outbreaks have been associated with contaminated endoscopes. Recent outbreaks have also been described in immunocompetent hosts after use of contaminated

whirlpool footbaths in nail salons. One case of meningitis due to *M. fortuitum* has been reported in association with a puncture wound and foreign body, while another has been reported with AIDS [39].

In a study, *Mycobacterium fortuitum* was documented to be the cause of peritonitis in patients who received continuous ambulatory peritoneal dialysis. According to that report, from past several years *Mycobacterium fortuitum* is being recognized as a potential pathogen. Moreover now, it is being more commonly isolated from soft tissue and skin infections. Through their study they emphasized on the fact that the patients with CAPD peritonitis in which conventional culturing repeatedly yields no organisms, the culturing for organisms such as mycobacteria should be done [17].

2.4 Biofilm Formation

2.4.1 What is Biofilm?

Bacteria have a natural propensity to grow as sessile, matrix-encapsulated, multicellular communities called biofilms [18]. Biofilms are structurally complex, dynamic systems with characteristics of both multifaceted ecosystems and primordial multicellular organisms [19]. Biofilm forming microorganisms have the ability to produce a matrix of extracellular polymeric substances (EPS) which is composed of exopolysaccharides, proteins and nucleic acids [20]. Although, not much is known about the mechanical properties of intact biofilms, but biochemical analysis of the EPS slime matrix shows that biofilms are HYDROGELS. Different species, even strains, have significantly distinct Extra Cellular Matrix (ECM) molecular. It has been observed that biofilm formation is significantly influenced by both the environment and the genome. Formation of biofilms proceeds through genetically programmed, distinct developmental stages signaled by intricate networks of communication among the constituent population and their environment. Thus, Quorum Sensing plays a major role in biofilm formation and also controls biofilm differentiation. Fossil records show that biofilm formation is an ancient and integral component of the prokaryotic life cycle, and is a key factor which helps in survival of microorganisms in diverse environments [19].

2.4.2 Composition of Biofilm

Biofilms mainly consists of microbial cells and Extracellular Polymeric Substance (EPS), which accounts for approximately 50.0-90.0% of the total organic carbon of biofilms. The

instant conditions of life of biofilm cells are determined by EPS. Generally, bacterial EPS comprises of polysaccharides, lipids, proteins and extracellular DNA. However, mycobacterial EPS consists of mycolic acids and lipids rather than polysaccharides, which makes the composition of mycobacterial biofilms significantly different from that of other bacteria. Mycobacterial biofilms comprises of glycopeptidolipid (GPLs), which is a class of glycolipid produced by several NTM [12].

2.4.3 Steps involved in Biofilm formation

Using confocal laser microscopy, it has been observed that development of biofilm is not a simple and uniform process, but rather more complex and differentiated [18]. Proteomic studies have revealed that biofilm formation proceeds as a regulated developmental sequence, and distinct stages include:

- Surface Attachment: Ideal environment for the attachment and growth of microorganisms is the solid-liquid interface between a surface and an aqueous medium (e.g., water, blood) [12]. Quorum-sensing (QS) also known as bacteria cell-to-cell communication plays a regulatory role in this process [21].

- Sessile Growth: After the bacteria attaches to the inert surface/living tissue, the association becomes stable for microcolony formation. Bacteria emit chemical signals so as to communicate with other bacterial cells and begin to multiply to form microcolony.

- Matrix Synthesis (Maturation): Exopolysaccharide production is activated when the signal intensity exceeds a certain threshold level. This leads to the formation of extra cellular matrix and water-filled channels. These channels act as primitive circulatory systems, which helps to deliver nutrients to and remove waste products from the communities of cells present in the microcolonies.

- Dispersal: This stage is characterized by the shedding or sloughing of biofilm cells. Detachment can be caused by external perturbations, such as increased fluid shear or by internal biofilm processes, such as quorum sensing, endogenous enzymatic degradation or by the release of EPS or

surface-binding proteins. Dispersal is termed as an active process in some species, as it is assumed to allow colonization of new niches. Three different biofilm dispersal strategies have been reported: 'swarming/seeding dispersal', in which individual cells from a microcolony are released into the bulk fluid or the surrounding substratum; 'clumping dispersal', in which aggregates of cells are shed as clumps or emboli; and 'surface dispersal', in which biofilm structures move across surfaces [19].

Each stage appears to be associated with distinct sets of genetic factors, expressions of which are regulated through master regulators and signalling molecules [18].

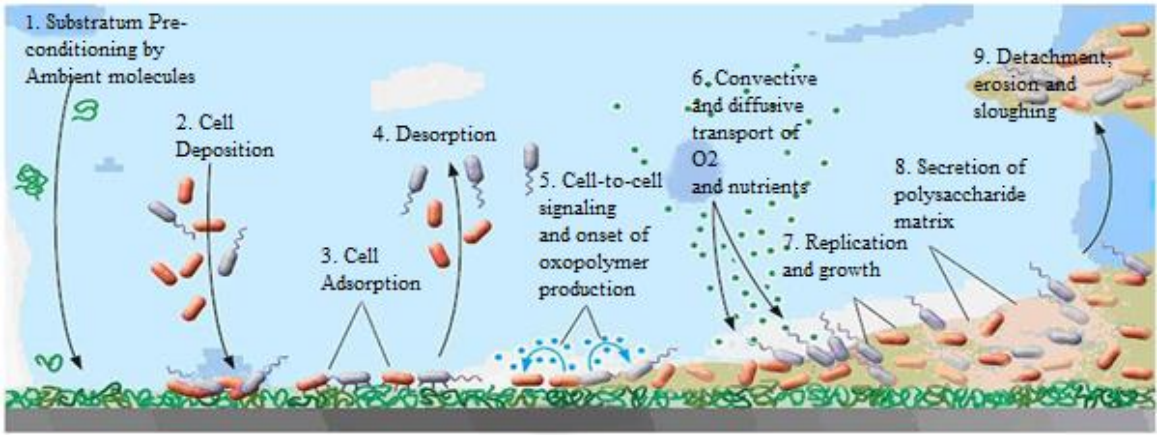


Fig.5*: Steps involved in biofilm formation* [12].

2.4.4 Importance of Biofilm Formation for bacteria

- Cells growing in a biofilm are more stable as surfaces provide a degree of stability in the growth environment and might have catalytic functions through localizing cells in close proximity.

- Biofilm formation protects bacterial cells from a wide range of environmental challenges, such as metal toxicity, UV exposure, acid exposure, dehydration and salinity, several antibiotics and antimicrobial agents and phagocytosis [19]. Hypothesis which explain biofilm resistance mechanisms are:

    a) Diffusional Limitation due to Slime Matrix- It has been hypothesized that multiple layers of cells and EPS may form a

compact structure within which it might be difficult for biocides to penetrate and reach internal layers, thus hampering their efficacy. The barrier properties of the EPS hydrogel might also protect against UV light and dehydration, and might localize enzymatic activity [22]. Potential interactions between biocides and biofilm components are impaired due to the presence of organic matter such as proteins, nucleic acids or carbohydrates Thus, transport limitations may be a mechanism that contributes to the resistance of biofilms to antimicrobial agents. This seems to be related mainly to physicochemical interactions between the biocide and EPS or bacterial cells rather than steric hindrance inside the biofilm [20].

b) Phenotypic Adaptations of Biofilm Cells- The transport limitation can result in low penetration of biocides in deeper regions. Thus, there is a possibility that biofilm cells can develop adaptive responses to sublethal concentrations of the biocides. The adaptive responses include protein biosynthesis, upregulation of specific proteins, adaptation and detoxification. Another possible mechanism of resistance may be the up-regulation of genes which codes for multidrug efflux pumps in biofilms. Cells present in the internal layers of biofilm have poor nutrient microenvironment as compared to cells present at the periphery; thus, chemical gradients are present within the biofilm [23, 24]. This leads to the onset of physiological heterogeneity. Since, the mechanism of action of most of the antimicrobials involve disruption of a microbial process, thus, presence of stationary phase dormant zones in biofilms seems to play a major role in the resistance of biofilm populations. It has also been suggested that lateral gene transfer confers specific traits through the exchange of genetic sequences including transposons or integrons, plasmids, resulting in virulence expression and antimicrobial resistance [20].

c) Presence of resistant subpopulation (Persisters)- They are a small fraction of the bacterial cells population which may enter a highly protected state displaying resistance [25, 26]. These cells have also

been identified in planktonic cells indicating that they are phenotypic variants and not genetic mutants. It is presumed that more persisters develop in a biofilm than in a planktonic culture due to the presence of specific environmental conditions prevailing within the structure, and which may contribute to better biocide protection in the biofilm [20].

d) Pathogen protection in multispecies biofilms- It is confirmed that biofilms are complex mixtures of different species in their natural environments, rather than the biostructures of single species [27, 28]. Species interaction in these complex consortia can lead to the emergence of specific biofilm phenotypes. It has been hypothesized that more viscous matrix is formed due to chemical interactions between the polymers produced by different species, leading to reduction in the permeation of biocides. Similarly, because a biocide can be inactivated in a biofilm matrix by enzymes, the enzymes produced by the different species may act synergistically against toxic compounds benefiting the non-productive species from the association through enzyme complementation. Another explanation is that because of the specific spatial arrangement of certain bacterial species within a biofilm, some strains may be protected from a biocide by their aggregation with others within the 3-dimensional structure [20].

2.4.5 Biofilm formation in Mycobacteria

Environmental mycobacterial pathogens can be categorized into two groups based on growth rate; fast growing mycobacteria which include *M. smegmatis, M. fortuitum* and the slowly growing species such as *M. avium, M. xenopi, M. kansasii, M. intracellulare,* and *M. marinum*. The first colonizers of natural and engineered surfaces are mycobacteria. Silastic rubber and high density polyethylene surfaces are used to form biofilm by mycobacteria like *M. fortuitum, Mycobacterium chelonae, M. kansasii, and M. phlei*. Probably, the biofilm formation is contributed by the high cell surface hydrophobicity of mycobacteria. *M. tuberculosis* and numerous other species of mycobacteria, including *M. smegmatis*, *M. marinum* and *M. fortuitum* form biofilm on

liquid air interface. In a report, the SEM image of biofilm of *M. fortuitum, M. chubuense, M. vaccae, M. gilvum* and *M. obuense* showed curved structures arranged in a definite order and voids were clearly visible with long fibre and short fibre. Mycobacterial biofilm development depends on the nutrient composition of the medium. The crucial factors for the growth of mycobacterial biofilm include temperature, pH and nutrient composition [12].

2.4.6 Biofilm formation in *Mycobacterium tuberculosis*

Biofilm formation also plays a major role in pathogenesis of *Mycobacterium tuberculosis* as it helps the mycobacterium to persist against the challenges of antibiotics and competent host immune system. However, specific conditions like limited exchange of air with atmosphere and 9:1 ratio of headspace: media is required for the maturation of *M. tuberculosis* biofilm. These specific requirements can be explained by the fact that *M. tuberculosis* is an obligate human pathogen and thus, has adapted to tissue environments. *M. tuberculosis* show crystalline and globular structure when morphology and ultrastructure of its biofilm is resolved using SEM. A number of genes have been identified in *M. tuberculosis* which contributes in its biofilm formation. The genes include:

- *Pks1*: Gene *Pks15/1* produces *M. tuberculosis* protein polyketide synthase (Pks15/1) which is necessary for the production of an immunomodulatory lipid virulence factor, that is, phenolic glycolipid (PGL). Pks15/1 is a multidomain protein. Depending on the mycobacterial strain, Pks1 and Pks15 units occur as one or two open reading frames (ORFs). The five domains of Pks1 are dehydrogenase (DH), acyl transferase (AT), ketoreductase (KR), acyl carrier protein (ACP), and enoyl reductase (ER), and the single domain of Pks15 is a keto acyl synthase (KS). This protein forms p-hydroxyphenylalkanoic acid intermediate of the PGL backbone. In a study, it was reported that the pks1 mutant showed attenuation for biofilm formation. It was observed that biofilms made by the pks1 mutant did not thicken or develop cords which are the characteristics of mature wild-type biofilms after 5 weeks. When pks1 was expressed in the mutant through complementation, the biofilm phenotype was restored. Thus, these findings indicated that Pks1 contributes to biofilm maturation [29].

- Gene *Rv1013:* This gene encodes a putative acyl-CoA synthase and, although the gene and its product are not well-characterized, it is reported to be upregulated about 2-fold after 24 h starvation. Mutation in this gene leads to severe biofilm defect. Complementation with *Rv1013* restores the biofilm phenotype [30].

- *helY*: This gene belongs to helicase family and encodes for a probable helicase in *Mycobacterium tuberculosis*. It has been reported that mutant with defective *helY* gene shows a similar biofilm defective phenotype as that of mutant with defect in gene *Rv1013* [31].

- *groEL1:* This Gene encodes for GroEL-1 which modulates the biosynsthesis of mycolic acid by directly interacting with the fatty acid synthase complex II (FAS II) during maturation of pellicles. This induces synthesis of abundant extracellular lipid, that is, free mycolic acids (FM) in the later stages of biofilms [32]. FM can be considered as the component of matrix due to its abundance and extracellular location as well as its association with the matured architecture of biofilms. Interestingly, FM is also an abundant extracellular lipid of M. tuberculosis pellicles. Thus, it has been observed that a Δ*groEL1* mutant is defective in maturation as it fails to make these lipids and hence, has a defective biofilm phenotype [18].

2.4.7 Biofilm formation in Non-Tuberculous Mycobacteria

Non-tuberculous mycobacteria (NTM), comprises of more than 65 species, are those mycobacteria which are not part of the *Mycobacterium tuberculosis* complex. NTM are becoming pathogens and gaining importance in human health as they are being recognized to cause opportunistic infections in HIV patients. Biofilm formation plays a major role in pathogenesis of NTM. Most studied NTM with respect to biofilm formation is *M. avium.* This *Mycobacterium* has the ability to assemble biofilms even when incubated only with water explaining its presence on water distribution systems, showerheads, and clinical settings. It has been reported that NTM are resistant to disinfectants, such as chlorine which helps NTM to easily colonize, persist and replicate within drinking water distribution system. In a study, it was observed that resistance of *M.*

avium to antimicrobial agents and antibiotics increases transiently when it is organised within biofilms. However, resistance provided by biofilm formation is adaptive rather than genetic in NTM as bacteria lose resistance in a short period of time when it again becomes planktonic cell. Although EPS composition is not well known even for the most studied NTM, but it has been observed that EPS composition varies among different species. It has been discovered that biofilm formation and GPL biosynthesis are closely connected in *M. smegmatis* and *M. avium* as some genes responsible for GPL biosynthesis are upregulated during the biofilm formation and its establishment. In addition to lipids, biofilm formation, its structure and matrix composition is also affected by the presence of other factors such as iron, protein kinase, and GroEL1, or the lack of factors, for example, polyphosphate deficiency [33].

2.4.8 Biofilm formation in *Mycobacterium fortuitum*

The rapidly-growing *M. fortuitum* has been recognised as an opportunistic NTM which causes several hospital associated infections involving hospital water systems, hospital instruments, and peritoneal catheters. It has also been isolated from the samples of ponds, lakes, drinking water, and distilled water.

In 1998, Hall-Stoodley and his team through their experiments confirmed that *M. fortuitum* has the ability to colonise surfaces. They used modified Robbins device to grow and sample biofilms. They choose silastic rubber as the test surface as it is commonly used in the medical environment. In their study, *M. fortuitum* formed dense biofilms within 48 h. They found high numbers of sessile organisms suggesting that *M. fortuitum* readily forms biofilms. Their observations also suggest that M. *fortuitum* may be the primary colonising organism as it did not require presence of other organisms for colonisation [2].

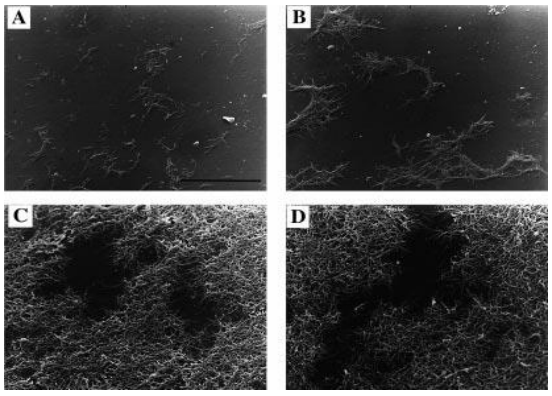


***Fig.6**: Initial report of biofilm formation by M. fortuitum on silastic rubber after (A) 2 h; (B) 12 h; (C) 24 h; and (D) 48 h in 1998 [2].*

It has been suggested that antimicrobial resistance and mode of transmission can be a result of biofilm formation in *M. fortuitum*. Biofilm formation has contributed in the emergence of *M. fortuitum* as an opportunistic pathogen, gaining importance in nosocomial infections [11].

It has been reported that biofilm development affinity of *Mycobacterium fortuitum* is higher in stainless steel, polycarbonate, and polyvinyl chloride rather than glass and copper. *M. fortuitum* exhibits heterogeneous morphology with a mycelia like texture when morphology and ultrastructure of its biofilm is resolved using SEM [12].

2.4.9 Techniques used for characterization of biofilms

Studies are mainly focused to identify factors involved in the initial stage of biofilm assembly, i.e., attachment. The diverse techniques are followed which are goal oriented.

a. **Microtitre Plate Test**: The most common method used for characterization of biofilm assembly *in vitro* is the microtiter plate test. It allows observation of bacterial adherence on abiotic surfaces. Staining techniques like crystal violet assay allows both the visualization and quantification of biofilm through microscopy and spectrophotometry measurement respectively. The advantage of microtiter plate test over other methods is that it is the cheapest and less labour-intensive method. The Congo red agar assay, resazurin assay and ring test are other techniques which are based on staining procedures coupled with spectrophotometric methods used for biofilm study.
b. **Microfermentor test**: This technique allows generation of abundant biomass, thus, having the advantage of permitting extraction of proteins and nucleic acids. Hence, it provides more information on biofilm assembly.
c. **Atomic force microscopy (AFM)**: It is a highly sensitive tool which can be used to examine the bacterial adhesion to surface. Moreover, bacterial morphology can be studied with high resolution using AFM. Minimum sample preparation is required in this method and it allows the acquisition of 3D images of the surface ultrastructure in physiological conditions. However, AFM imaging has several problems like small scanning area, slow scanning speed, possibility of image artifacts etc.
d. **Fluorescence microscopy**: It is a non invasive technique to assess biofilms, for example, the reactivity of an antimicrobial drug in a biofilm.

e. **Confocal laser scanning microscopy**: It is an optical microscopy technique in which more thick samples are studied. It is also used to analyse antimicrobials action; however, it has restricted magnification.

Other possible methods are environmental SEM (ESEM) and cryo-SEM, in which samples dehydration is not required. In cryo-SEM, liquid nitrogen is used to freeze the sample during imaging; however, resolution of such micrographs is less than the resolution of TEM or SEM [33].

2.5 Methods used to identify genes involved in biofilm formation

2.5.1 Transposon mutagenesis

Pathogenesis of a microorganism is not of much concern till it can be treated properly with available therapeutic approaches. But, mechanisms like biofilm formation and rapid mutation in the prokaryotic species have the ability to provide resistance against present therapeutic approaches. For example, *M. fortuitum* has already started to develop resistance against some of the drugs used for its treatment. Resistance of pathogen can be overcome by increasing the dosage of available antibiotics but this may not be a solution as the increased dose may be lethal for patients. Thus, study carried to identify genes involved in virulence or biofilm formation might be a helpful tool in tackling problem due to drug resistance. A method which can be utilized to identify such genes is random mutagenesis of *M. fortuitum* genes by inserting a transposon (Tn5) which would block the expression of genes. Since the genomic sequence of *M. fortuitum* is not known till now, thus, random mutagenesis seems to be most appropriate method to study gene functions.

The principle of transposon mutagenesis involves insertion of foreign DNA, a transposable element, into many sites in the bacterial genome, ideally on a completely random basis. A selectable phenotype within the transposon, such as an antibiotic resistance marker is required to select mutants. The Tn5 based transposon inserts randomly and can create gene knock-outs. Transposons can be inserted into the bacterial chromosomes with the electroporation of ‘suicide vectors’, or bacterial phages, carrying transposon into the bacterial cells.

In order to confirm the insertion of transposon Tn5 into genes of *M. fortuitum*, the electroporated cells were plated on NAT plates containing Kanamycin, Cycloheximide,

X-gal, IPTG and 5% glucose. Blue colonies of mutants were selected and screened further for biofilm formation so as to identify genes responsible for forming biofilm in *M. fortuitum*.

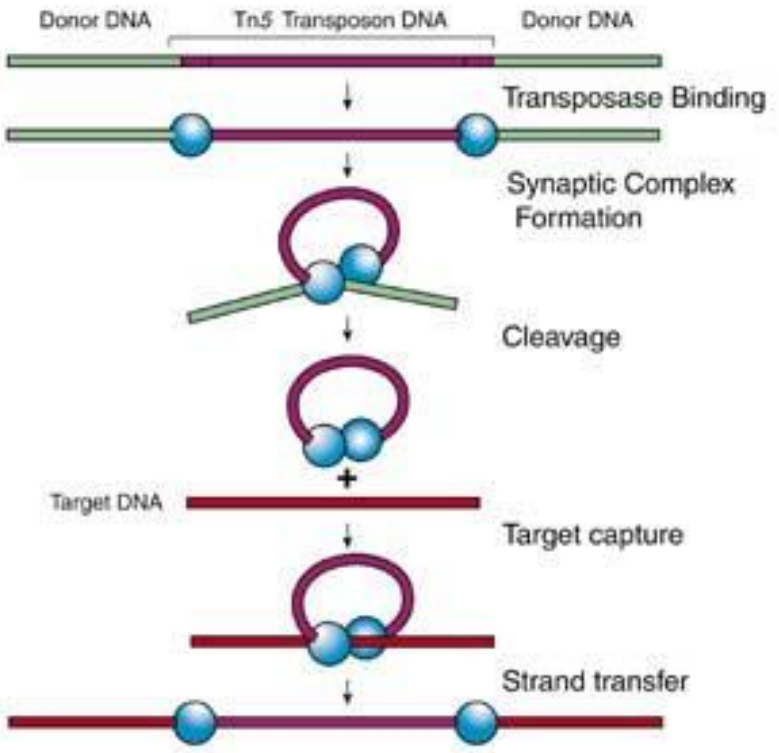


Fig.7: *Schematic representation of transfer of Transposon DNA from vector to target DNA, resulting in transposon mutagenesis* [42].

2.5.2 Gene knockout

Gene knockout: A gene knockout is a genetically engineered organism that carries one or more genes in its chromosomes that have been inactivated (have been "knocked out" of the organism). This method is also known as knockout organisms or simply knockouts, and mainly used to decipher the function of a gene whose sequence is already known but function is unknown. Function of knockout gene is usually interpreted from inferences made by differences between the knockout organism and normal individuals. Knockout is accomplished through a combination of techniques, beginning with the culturing of vector, transformation of this vector into the stem cells of the embryo. The vector is engineered in such a way that it recombines with the target gene, which is usually accomplished by incorporating sequences from the gene itself into the vector. This results

in the recombination in the region of that sequence within the gene, which ultimately leads to the insertion of a foreign sequence in the gene, disrupting its function. Since, the sequence of the gene is altered; it is translated into a nonfunctional protein, if it is translated at all. A conditional knockout allows gene deletion in a tissue specific manner. Since, recombination is a rare event, it is necessary that foreign sequence should be a reporter sequence to allow easy selection of cells in which knockout was successful [34].

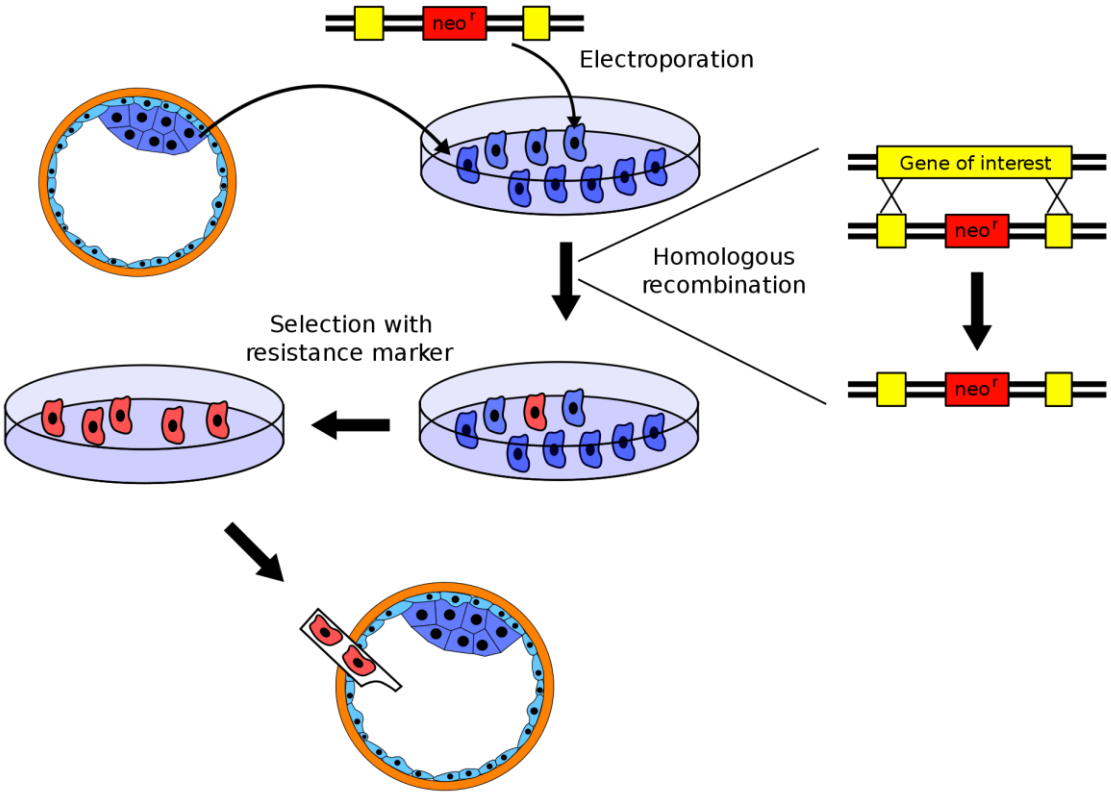


Fig.8: *A schematic diagram showing the steps to create gene knockout in an organism* [41].

2.5.3 Gene Silencing by Antisense Technology

Gene silencing is a method in which expression of a gene is suppressed at transcriptional or translational levels. Usually, an antisense RNA is introduced to block the translation of messenger RNA. mRNA is the nucleic acid molecule that carries genetic information from the DNA to the other cellular machinery involved in the protein production. "Sense" refers to the original sequence of the DNA or RNA molecule. A Sense strand is a 5' to 3' mRNA molecule or DNA molecule. The complementary strands or mirror strand to the

sense is called an antisense. The basic idea is to introduce an oligonucleotide molecule which is complementary to the mRNA produced by a gene, leading to its specific binding to target mRNA. This leads to the formation of an RNA dimer in the cytoplasm, which halts the protein synthesis. This occurs because the mRNA no longer has access to the ribosome and cytoplasm by ribonucleotide. Moreover, double stranded RNA will be recognized as foreign element and will be degraded by the cell, thus, preventing the production of undesired protein. In effect, the gene will be turned off [35]. Although DNA is already a double stranded molecule, antisense technology can be applied to it building a triplex formation. However, the exact mechanism of an antisense strand has not been determined but it has been hypothesized that it blocks RNA splicing, accelerates degradation of the RNA molecule, and prevents introns from being spliced out of the mRNA, impedes the exportation of mRNA into the cytoplasm, hinders translation, and results in the triplex formation in DNA" [36].

Antisense technology is particularly useful in knocking down essential genes as their knockout mutants will be unable to survive. It is also unseful in systems where gene inactivation is difficult. Recently, the antisense approach was used to decrease the level of sigA, and Rv3303c in *M. tuberculosis* [37].

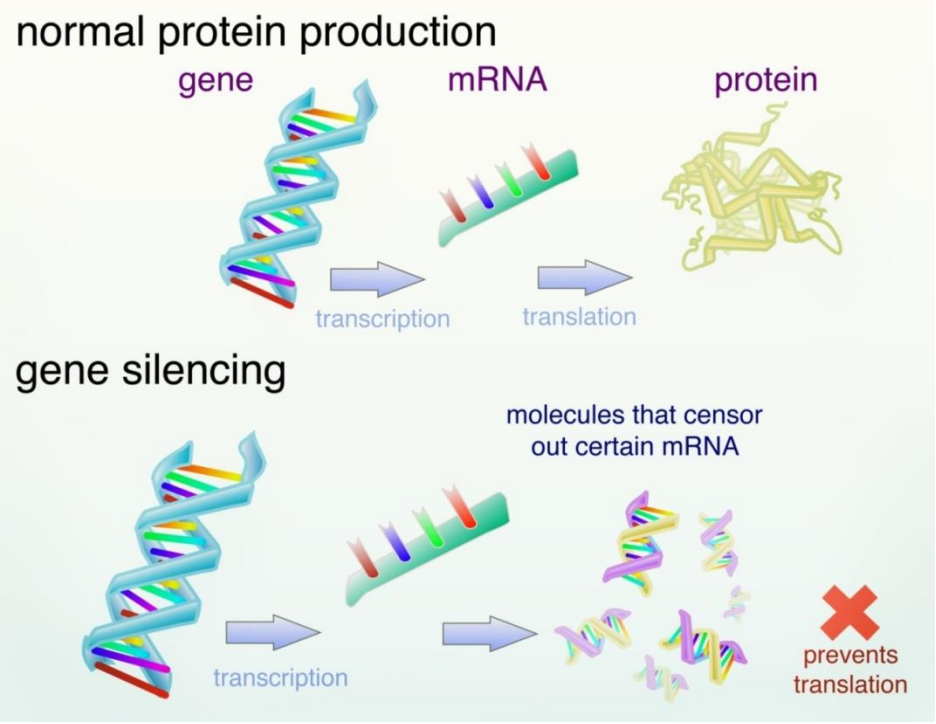


***Fig.9**: Mechanism to silence the gene using antisense technology* [40].

Chapter 3

Materials and Methods

3. MATERIALS AND METHODS

3.1 Construction of *M. fortuitum* library by random mutagenesis

3.1.1 Isolation of plasmid pRT291:

Chemicals required: 300µL of GTE (Glucose tris EDTA- ALS 1 (Alkaline lysis buffer 1)), 600µL of SDS-NaOH solution, 450µl of acetate solution, 2700µL of absolute ethanol, 96% ethanol, 70% ethanol, 40µL of autoclaved distilled water.

Equipments used: Centrifuge, Centrifuge tubes, Mini centrifuge tubes, Pipette and Tips.

Procedure:

1. The culture was centrifuged at 7000 rpm for 10 minutes.
2. Then pellet was dried properly.
3. 300µL of GTE (Glucose tris EDTA- ALS 1) was added and vortexed.
4. 600µL of SDS-NaOH solution was added and mixed gently.
5. It was then incubated at 37 ^{0}C for 5 minutes.
6. 450µL of acetate solution was added.
7. It was gently mixed for 5-6 times.
8. It was then incubated in ice for 30 minutes and centrifuged at 7000 rpm for 25 minutes.
9. Supernatant was transferred to fresh centrifuge tube and 2700µl of absolute ethanol was added.
10. The mixture was then incubated overnight at -20 ^{0}C.
11. The plasmid was then transferred into mini centrifuge tubes and centrifuged at 12000 rpm for 15 minutes and supernatant was removed.
12. 96% ethanol was added to mini centrifuge tubes and centrifuged at 10,000 rpm for 12 minutes.
13. Supernatant was removed and 70% ethanol was added and centrifuged at 10,000 rpm for 3 minutes.
14. Pellet was dried properly until ethanol evaporated.
15. 40µL of autoclaved distilled water was added and tube was placed at -20 ^{0}C.

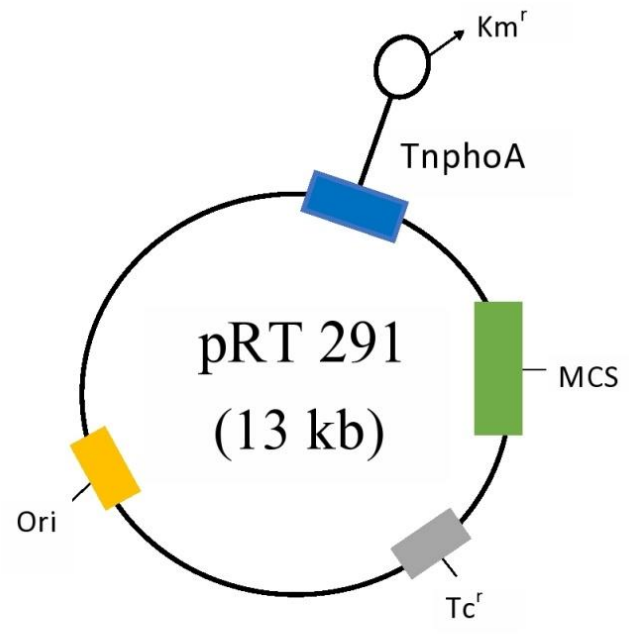


Fig.10: *Vector map of pRT291 having TnphoA, MCS, Tc^r, Ori (Origin of replication)*

3.1.2 Preparation of electrocompetent cells:

Chemicals required: 200 mL LBGT, 10% glycerol, seed culture.

Equipments used: Centrifuge, Ice box, Spectrophotometer, Flask, Pipette and Tips.

Procedure:

1. Seed culture was inoculated into 200 mL LBGT and incubated with shaking at 37^0C overnight.
2. OD_{600} was taken till it reached to 0.6-0.8.
3. The cells were kept on ice before harvesting for about 40-60 minutes.
4. Then, cells were centrifuged at 4000 rpm for 10 minutes at 4 ^{0}C.
5. Supernatant was discarded.
6. The pellet was washed with water or 10% glycerol. Again it was washed with reduction in the volume of agent each time.
7. Then, the pellet was suspended in 1-2mL 10% glycerol.
8. Cells were frozen at -80 ^{0}C.

3.1.3 Protocol for electroporation:

Chemicals required: 400 µL of electrocompetent cells, plasmid (pRT291), 2mL of LBGT, X-gal, IPTG, Cycloheximide, Kanamycin and 5% Glucose.

Equipments used: Cuvettes, Electroporation machine, Ice box, Pipettes and Tips.

Procedure:

1. 400 µL of electrocompetent cells with 25µg/mL of plasmid (pRT291) were taken in a mini centrifuge tube.
2. Mini centrifuge tube was incubated at room temperature for 10 minutes.
3. Sample from mini centrifuge tube was transferred into electroporation cuvette.
4. Cuvette was kept in ice for 5-10 minutes.
5. Electroporation conditions were set to capacitance-25 µF, resistance-1000Ω, voltage-2500 volts, and cuvette-2 mm.
6. Pulse was given twice and cuvette was again kept in ice.
7. 2mL of LBGT was added and incubated at 37 ^{0}C for 6 hours with shaking.
8. Then, sample was spread on selection plate containing X-gal + IPTG + Cycloheximide + Kanamycin + 5% Glucose.
9. After incubation, plates were observed for blue colonies.

3.1.4 Acid fast staining to check purity of culture:

Chemicals required: 70% ethanol, 50µL of culture (*M. fortuitum* wild type and 50 mutants), Carbol fuchsin, Acid alcohol, Malachite green, Distilled water.

Equipments used: Slides, Loop, Hot plate, Dropper and Microscope.

Procedure:

1. The slides were made grease free by cleaning with 70% ethanol.
2. Smear was prepared by adding 50µL of culture (*M. fortuitum* wild type and 50 mutants) on the slide and heat fixing it.
3. Slides were flooded with carbol fuchsin and kept on hot plate at 80 ^{0}C for 5-7 minutes.
4. After 5 minutes, the slides were left to cool down and then washed with distilled H_2O.
5. Then, few drops of acid alcohol were added to decolorize the slides and they were washed after 20-30 seconds.

6. Few drops of malachite green were added as a counter stain, kept for 45 seconds and then washed off with distilled H_2O.
7. The slides were air dried and then observed under the microscope at various magnifications of 10X, 40X and 100X.

3.2 Culture Preparation for Biofilm assay

3.2.1 Preparation of Seed Culture of Wild-type and Mutants

Chemicals Required: MB7H9 medium, Cultures (Wild-type, MTK1, MTK2, MTK3, MTK4 and MTK5), Kanamycin [30µg/mL] and Cycloheximide [50µg/mL].

Equipments Used: Pipette, Tips and Shaker.

Procedure:

1. 30 µg/mL of Kanamycin and 50 µg/mL of Cycloheximide were added to 10mL of MB7H9 medium.
2. A colony of mutant MT664 was picked from the plate and inoculated into the test tube containing medium.
3. Same procedure was repeated for other 5 mutants (MTK1, MTK2, MTK3, MTK4 and MTK5).
4. 50 µg/mL of Cycloheximide was added to 10 mL of MB7H9 medium.
5. A colony of wild-type was picked from plate and inoculated into the test tube containing medium.
6. All the test tubes were incubated in shaker at 37 ^{0}C for 3 days.

3.2.2 Acid fast staining to check purity of culture:

Chemicals required: 70% ethanol, 50µL of culture (*M. fortuitum* wild type, MTK1, MTK2, MTK3, MTK4 and MTK5), Carbol fuchsin, Acid alcohol, Malachite green, Distilled water.

Equipments used: Slides, Loop, Hot plate, Dropper and Microscope.

Procedure:

1. The slides were made grease free by cleaning with 70% ethanol.
2. Smear was prepared by adding 50µL of culture (*M. fortuitum* wild type, MTK1, MTK2, MTK3, MTK4 and MTK5) on the slide and heat fixing it.

3. Slides were flooded with carbol fuchsin and kept on hot plate at 80 ^{0}C for 5-7 minutes.
4. After 5 minutes, the slides were left to cool down and then washed with distilled H_2O.
5. Then, few drops of acid alcohol were added to decolorize the slides and they were washed after 20-30 seconds.
6. Few drops of malachite green were added as a counter stain, kept for 45 seconds and then washed off with distilled H_2O.
7. The slides were air dried and then observed under the microscope at various magnifications of 10X, 40X and 100X.

3.2.3 Preparation of Secondary Culture

Chemicals Required: MB7H9 medium, Cultures (Wild-type, MTK1, MTK2, MTK3, MTK4 and MTK5), Kanamycin [30µg/mL] and Cycloheximide [50µg/mL].

Equipments Used: Pipette, Tips and Shaker.

Procedure:

1. 500 µL of seed culture of mutants were inoculated in different medium flasks containing 30 µg/mL of Kanamycin and 50 µg/mL of Cycloheximide in 100 mL of MB7H9 medium.
2. 500 µL of seed culture of wild type was inoculated in medium flask containing 50 µg/mL of Cycloheximide in 100 mL of MB7H9 medium.
3. All flasks were incubated in shaker at 37 ^{0}C for 1 day.

3.3 Biofilm Assay

3.3.1 Plating of Wild-type and mutants cultures in 96-well plate

Chemicals required: MB7H9 medium, Cultures (Wild-type, MTK1, MTK2, MTK3, MTK4 and MTK5), Kanamycin [30µg/mL], Cycloheximide [50µg/mL] and Tween normal saline.

Equipments used: Centrifuge tubes, Centrifuge, Vortex, Spectrophotometer, 96-well plate, Pipette, Tips and Incubator with shaker.

Procedure:

1. Cultures were transferred to 50 mL centrifuge tubes.
2. Cultures were centrifuged at 5000 rpm for 5 minutes.

3. Supernatant was discarded and pellet was dissolved in 5 mL of tween normal saline.
4. Culture was vortexed to dissolve the pellet.
5. OD of the culture was set to 0.6 at 600 nm using tween normal saline.
6. Culture was diluted 10-fold in MB7H9 medium.
7. 200 µL of culture was added in the wells of microtiter plate in triplicates.
8. 6 such plates were prepared.
9. Plates were incubated in orbital shaker at 37 ^{0}C for 28 days and processed at different time intervals for biofilm formation.

3.3.2 Crystal Violet Assay

Chemicals required: Autoclaved distilled water, Methanol, Crystal violet and 33% Glacial acetic acid.

Equipments used: Pipette, Tips and Spectrophotometer.

Procedure:

1. Plate was taken out of the shaker.
2. Media of each well was discarded.
3. Each well was washed thrice with autoclaved water vigorously.
4. Methanol was added to each well to fix the cells and plate was left undisturbed for 10 minutes.
5. Methanol was discarded and 200 µL of crystal violet was added to each well.
6. Plate was left undisturbed for 15 minutes.
7. Then crystal violet was discarded and extra stain was washed off.
8. Plate was air dried and biofilm was dissolved in 200 µL of 33% glacial acetic acid.
9. After 10 minutes, absorbance was taken at 570 nm in spectrophotometer.

3.3.3 Carbol Fuschin Staining

Chemicals required: Autoclaved distilled water, Carbol fuschin stain and Absolute ethanol.

Equipments Used: Microscope, Pipette and Tips.

Procedure:

1. Plate was taken out of the shaker.

2. Media of each well was discarded.
3. Each well was washed thrice with autoclaved water vigorously.
4. 200 µL of Carbol fuschin stain was added to each well and plate was left undisturbed for 30 minutes.
5. Stain was discarded and extra stain was washed off.
6. Wells were decoloured with 200 µL of absolute ethanol was for 10 seconds.
7. Wells were washed off with water.
8. Plate was air dried.
9. Wells were observed under inverted microscope for biofilm formation.

3.3.4 CFU Count

Chemicals required: Autoclaved water, Tween normal saline and Nutrient agar tween.

Equipments used: Pipette, Tips, Incubator, Petriplates and Autoclaved Mini centrifuge tubes.

Procedure:

1. Plate was taken out of the shaker.
2. Media of each well was discarded.
3. Each well was washed thrice with autoclaved water vigorously to remove planktonic cells.
4. Adherent cells were scrapped from well using tip.
5. 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8} dilutions of cells were prepared by double dilution method in tween normal saline.
6. 10 µL of 10^{-2} diluted sample was spread on Nutrient Agar Tween plate.
7. Similarly, 10^{-4}, 10^{-6} and 10^{-8} diluted samples were spreaded on NAT plates.
8. Plates were incubated at 37 ^{0}C for 2-3 days in an incubator.
9. Plates were checked for growth and colonies were counted at different dilutions.

3.4 Genomic DNA isolation of *M. fortuitum* and mutants strains containing the transposon

Chemicals required: TES buffer, 2mg/mL of lysozyme, Buffer phenol, Chloroform: Isoamylalcohol, SDS, Proteinase K, Isopropanol (chilled), Ethanol, Autoclaved distilled water, Culture of *M. fortuitum* and mutants.

Equipments used: Centrifuge, Centrifuge tubes, Mini centrifuge tubes, Incubator, Water bath, Electrophoresis apparatus, Gel doc, Pipettes and Tips.

Procedure:

1. The culture was taken and pelleted in 50 mL centrifuge tube at 7000 rpm for 15 minutes.
2. The supernatant was discarded and 2 mL of TES buffer was added to pellet.
3. Then it was incubated at 80 ^{0}C for about 1 hour. After that 2mg/mL of lysozyme i.e. 80 µL was added to each tube and incubated at 37 ^{0}C for 1 hour.
4. 1.5% SDS and 100µg/mL Proteinase K were added in each tube.
5. Centrifuge tube was incubated at 50 ^{0}C for 1 hour.
6. Then buffer phenol was added in each tube and tubes were centrifuged at 12000g for 15 minutes.
7. Then chloroform: isoamylalcohol was added in 24:1 ratio in each tube and again tubes were centrifuged at 12000g for 15 minutes.
8. Then the aqueous layer was transferred to fresh tubes. Again chloroform: isoamylalcohol was added to tubes and centrifuged at 12000g for 10 minutes.
9. The aqueous layer was transferred to fresh mini centrifuge tubes and equal volume of isopropanol (chilled) was added.
10. Mini centrifuge tubes were incubated at 4 ^{0}C for overnight.
11. Next day, mini centrifuge tubes were centrifuged at 12000g for 15 minutes.
12. Supernatant was removed and 70% ethanol was added (1mL) and then mini centrifuge tubes were centrifuged at 12000g for 15 minutes.
13. Ethanol was evaporated and 100µL of autoclaved distilled water was added.
14. Mini centrifuge tubes were then placed in ice for about 2 hours for proper suspension of DNA.
15. Then electrophoresis was done to check the DNA.

3.5 Restriction digestion of gDNA by *EcoRV*

Chemicals required: Nuclease free water, *EcoRV*, Cut smart buffer, Agarose gel and TAE buffer.

Equipments used: Thermo-cycler, PCR vials, Mini spin, Electrophoresis apparatus and Gel doc.

Procedure:

1. Reaction for restriction digestion was prepared in PCR vials with following constituents:

gDNA	-	5.0µL
EcoRV	-	1.5µL
Cut Smart Buffer	-	3.0µL
Nuclease free water	-	20.5µL
Total	-	30.0µL

2. Reaction mixture was incubated in thermo cycler at 37 ^{0}C for 4 hours.
3. Agarose gel electrophoresis was done to visualize the digested DNA on agarose gel (0.8%).
4. After that pUC19 plasmid was isolated.

3.6 Restriction Digestion of pUC19 with *SmaI*

Chemicals required: Nuclease free water, *SmaI*, Cut smart buffer, Agarose gel and TAE buffer.

Equipments used: Thermo-cycler, PCR vials, Mini spin, Electrophoresis apparatus and Gel doc.

Procedure:

1. Reaction for restriction digestion was prepared in PCR vials with following constituents:

Vector	-	35.00µL
SmaI	-	2.25µL
Cut Smart Buffer	-	4.50µL
Nuclease free water	-	3.25µL
Total	-	45.00µL

2. Reaction mixture was incubated in thermo cycler at 37 ^{0}C for 4 hours.
3. Agarose gel electrophoresis was done to visualize the digested DNA on agarose gel (0.8%).

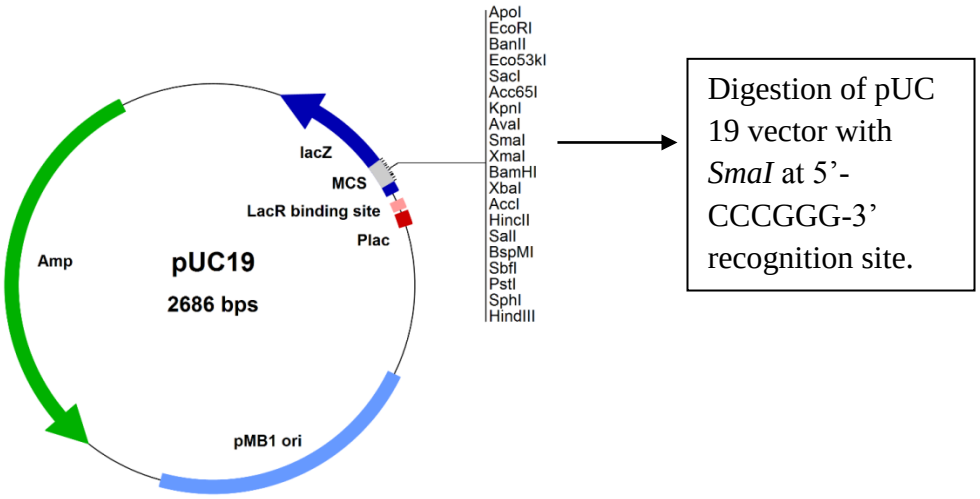


Fig.11. *Circular map representation of pUC 19 vector* [38]

3.7 Gel extraction of digested pUC19 vector

Chemicals required: Binding buffer NTI, Wash buffer NT3, Elution buffer NE and Ethanol.

Equipments Used: Centrifuge, Mini Spin, Scalpel, Gel Doc, Mini Centrifuge tubes, Vortex, Water bath, Clean up columns, Pipette and Tips.

Procedure:

1. A clean scalpel was taken and DNA fragment was excised from the agarose gel using the scalpel.
2. 200µL of NTI buffer was added in a mini centrifuge tube containing excised DNA fragment.
3. Sample was incubated at 50 ^{0}C for 5-10 minutes. Sample was vortexed briefly every 2-3 minutes until the gel slice was completely dissolved.
4. Then, clean up column tube was kept in collection tube and 500µL of sample was loaded in the tube.
5. Tube was centrifuged at 11,000g for 30 seconds.
6. Flow through was discarded and column tube was kept back in collection tube.
7. Above steps were repeated for remaining sample.
8. 700µL of wash buffer NT3 was added to the clean up column tube.
9. Tube was centrifuged at 11,000g for 30 seconds.
10. Flow through was discarded and column tube was placed back in collection tube.
11. Again, tube was centrifuged at 11,000g for 1 minute to remove the wash buffer completely.

12. Then, clean up column tube was placed in a mini centrifuge tube.
13. 30µL of elution buffer NE was added to the tube and it was incubated at room temperature for 5 minutes.
14. Tube was centrifuged at 11,000g for 1 min.
15. Sample was stored at 4 ^{0}C.

3.8 Ligation of *EcoRV* digested gDNA fragment with *SmaI* digested with pUC19

Chemicals required: Nuclease free water, *EcoRV* digested gDNA, T4 ligase enzyme, T4 ligation buffer, *SmaI* digested with pUC19.

Equipments used: Thermo-cycler, PCR vials and Mini spin.

Procedure:

1. Reaction for ligation was prepared in PCR vials with following constituents:

Digested gDNA	-	1µL
Digested pUC 19 vector	-	3µL
T4 ligase	-	1µL
T4 ligation buffer	-	1µL
Nuclease free water	-	4µL
Total	-	10µL

2. Reaction mixture was incubated at 16 ^{0}C for overnight.

3.9 Transformation of ligated product into competent *E.coli* cells

3.9.1 Preparation of competent cells

Chemicals required: Transformation buffer-1, Transformation buffer-2, LB medium.

Equipments used: Centrifuge, Vials, Micro centrifuge tubes, Centrifuge tubes, Incubator, Pipettes and Tips.

Procedure:

1. Single colony was picked and inoculated in 10 mL LB medium. It was incubated at 37 ^{0}C for overnight.
2. 1 mL of overnight culture was added to 100 mL pre-warmed LB medium and incubated with constant shaking at 37 ^{0}C until O.D$_{600}$ reaches about 0.5.

3. The culture was cooled in ice for 5 minutes and transferred to sterile round bottom centrifuge tube.
4. The cells were pelleted by centrifugation at low speed 4000g for 5 minutes at 4^0C.
5. Supernatant was carefully discarded and cells were kept in ice.
6. The cells were gently resuspended in 30 mL of transformation buffer-1 and suspension was kept on ice for 90 minutes.
7. Cells were pelleted down by centrifugation at 4000 rpm for 5 minutes at 4 ^{0}C. The supernatant was discarded.
8. The cells were then carefully resuspended in 4 mL ice cold transformation buffer–2.
9. Aliquots of 100-200 µL were prepared in sterile centrifuge tubes.
10. Cells were stored at -80 ^{0}C.

3.9.2 Transformation of Ligated product in *E. coli DH5α*

Chemicals required: 15 µL ligated product, 200µL competent cells, 800µL of LB.

Equipments used: Vials, Mini centrifuge tubes, Incubator, Water bath, Petri plates.

Procedure:

1. Competent cells were thawed.
2. 15 µL of ligated product was added in 200 µL of competent cells.
3. It was mixed by swirling and incubated in ice for 30 minutes.
4. After that, it was incubated in water bath at 42 ^{0}C for 90 minutes.
5. Then, it was chilled on ice for about 5-15 minutes.
6. 800 µL of LB medium was added to the vial.
7. Vial was incubated at 37 ^{0}C for 45 minutes.
8. After 45 minutes of incubation, the cells were spread on selection plate with appropriate antibiotics.
9. White mutant colonies were selected from the selection plate and inoculated in LB- broth for plasmid isolation.
10. After isolation of plasmid, the plasmid was sent for Sanger sequencing in Xcelris Lab Ltd.
11. Then, Comparative genomic analysis was done for sequences obtained and was aligned with the sequence showing most identical homology.

Chapter 4

Results

4. RESULTS

4.1 Acid Fast Staining Results

Acid fast staining of mutants present in the borrowed library was done to check the purity of the cultures.

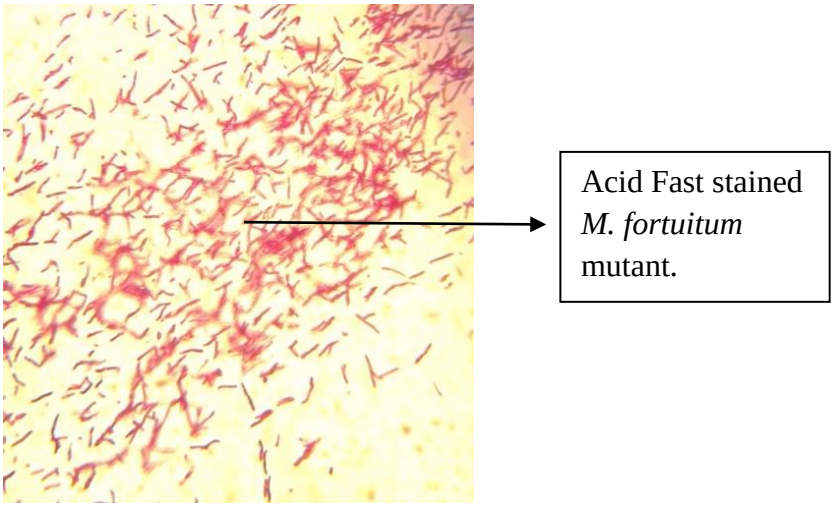


***Fig.12**: Acid fast staining of M. fortuitum mutant showing pure culture of red colored and rod shaped mycobacteria.*

4.2 Crystal Violet Assay Results

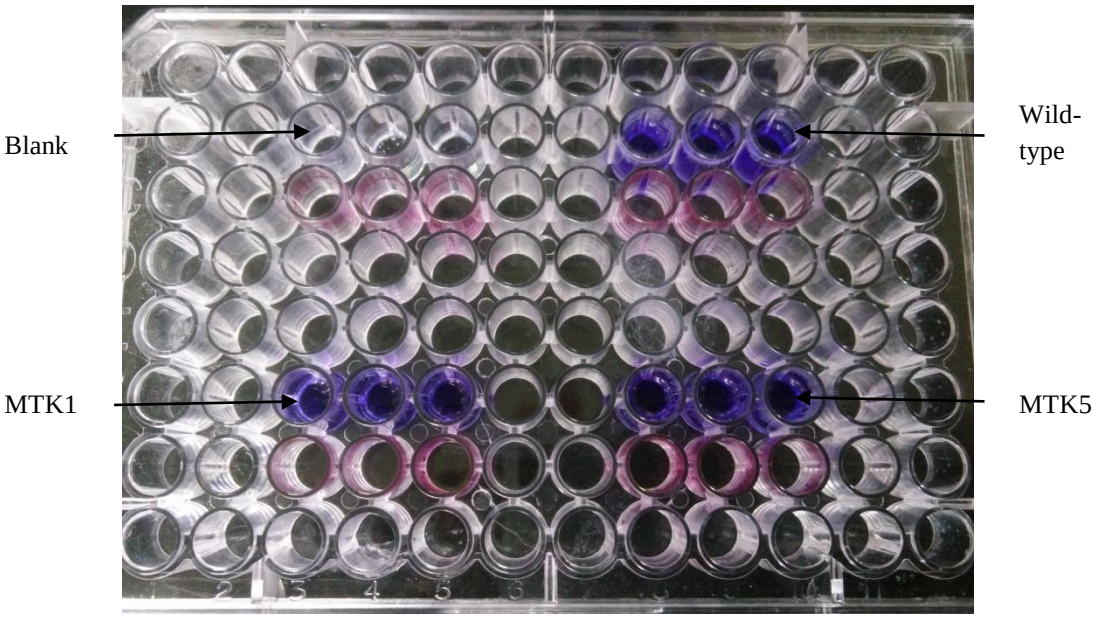


***Fig.13:** Crystal violet assay showing color variation in wild-type and mutants (MTK1 and MTK5,) indicating difference in amount of biofilm formation after 28 days of incubation.*

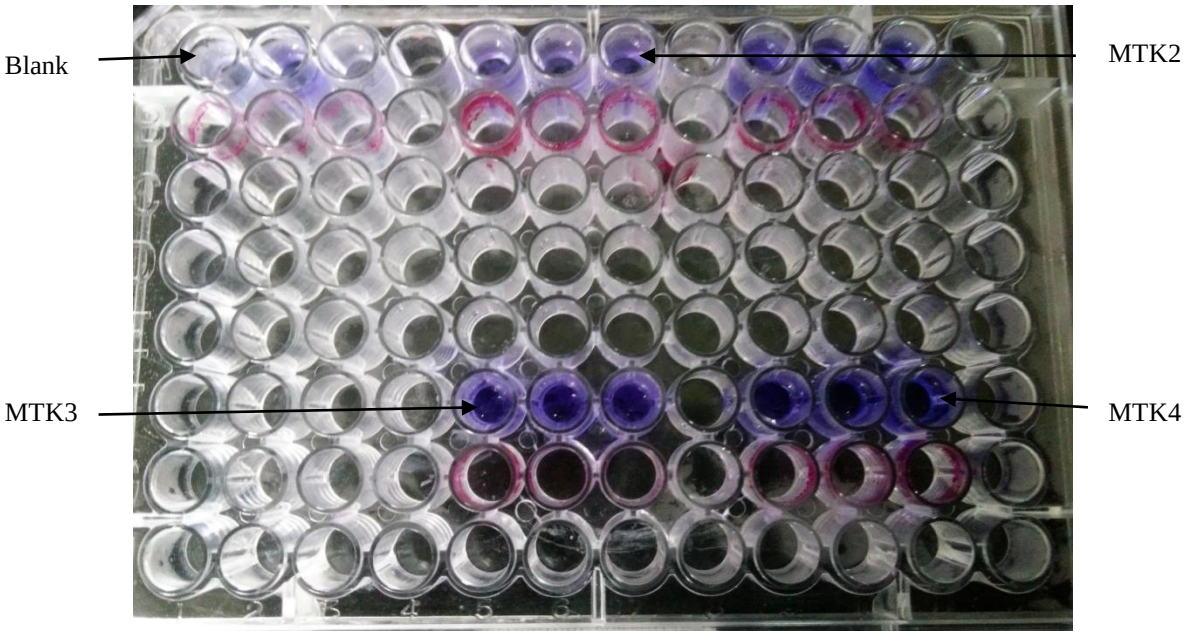


Fig.14: *Crystal violet assay showing color variation in mutants (MTK2, MTK3 and MTK4), indicating difference in amount of biofilm formation after 28 days of incubation.*

Crystal Violet Assay Graphs

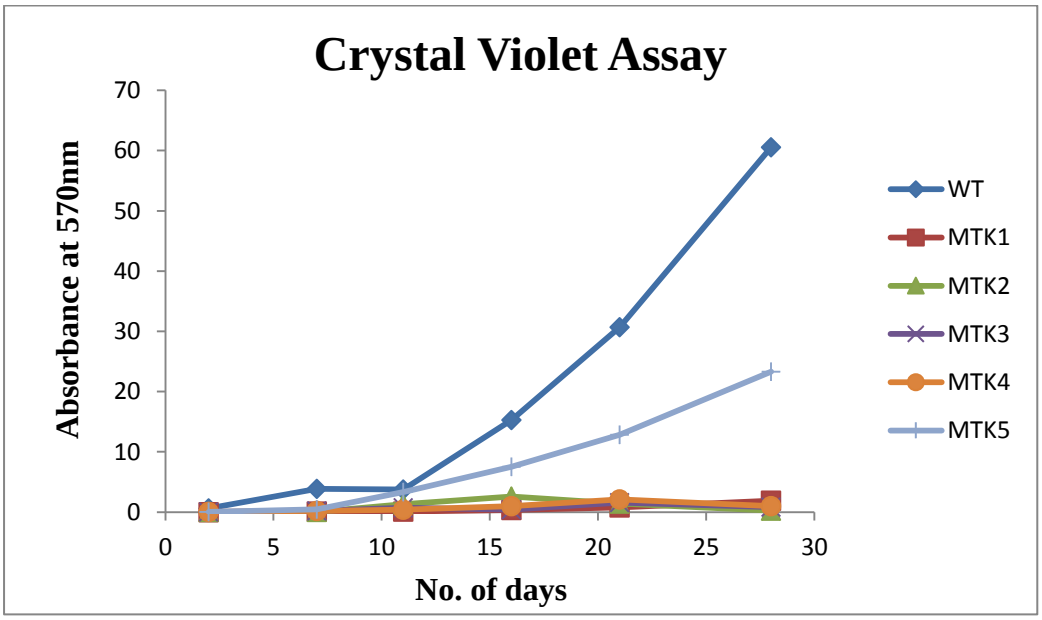


Fig. 15: *Biofilm formation curves of M. fortuitum wild- type and transposon mutants MTK1, MTK2, MTK3, MTK4 and MTK5. Graph depicts attenuation in biofilm formation of mutants MTK1, MTK2, MTK3 and MTK4 as compared to wild-type.*

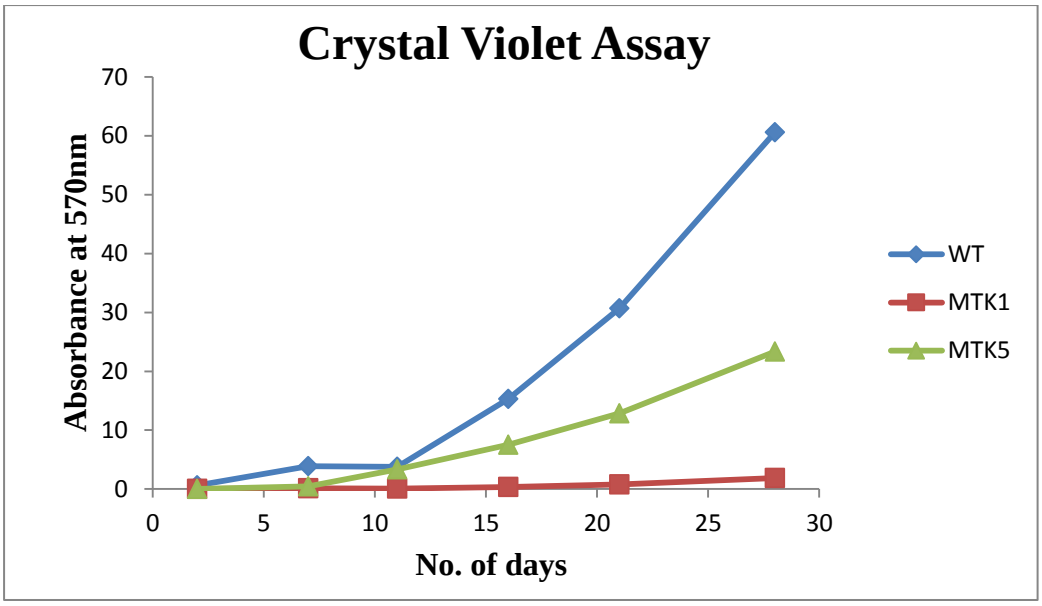


Fig.16: *Biofilm formation curves of M. fortuitum wild- type and transposon mutants MTK1 and MTK5. Graph depicts increase in biofilm formation in wild-type and mutants till 28 days of incubation.*

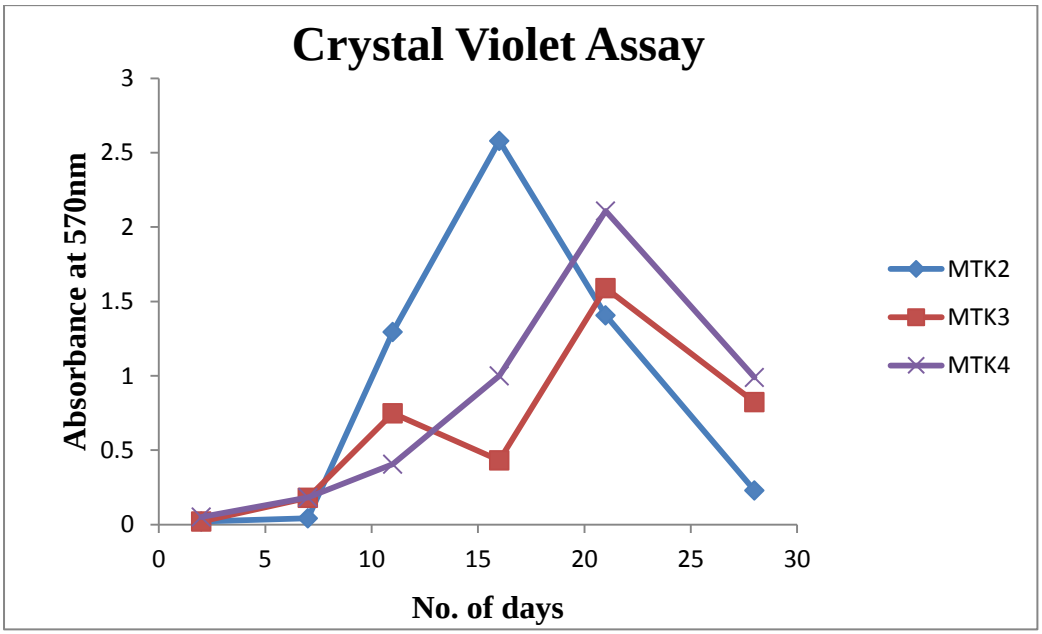
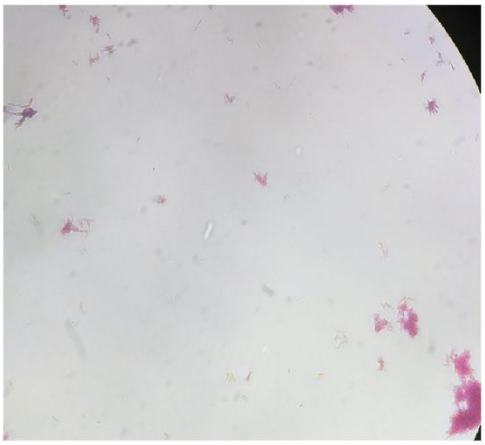
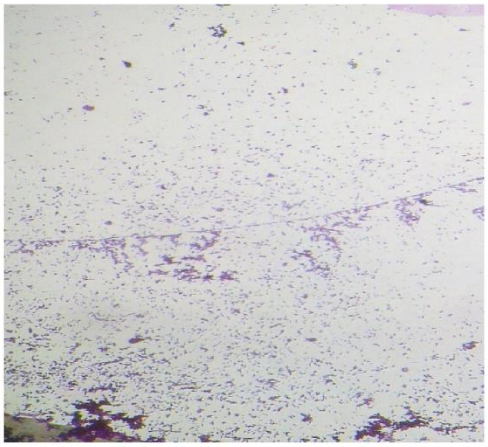


Fig.17: *Biofilm formation curves of M. fortuitum transposon mutants MTK2, MTK3 and MTK4. Graph depicts decrease in the biofilm formation after 16 days of incubation in mutants MTK2 and MTK3 and after 21 days in mutant MTK4.*

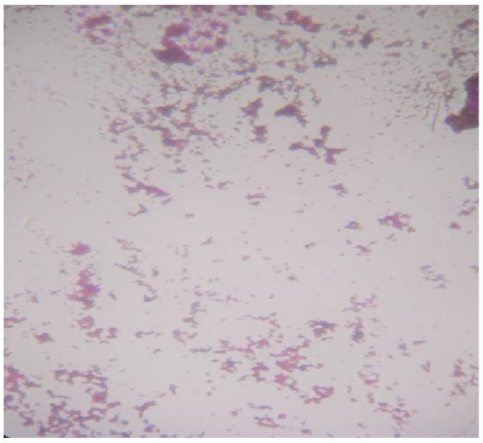
4.3 Carbol Fuschin Staining Results



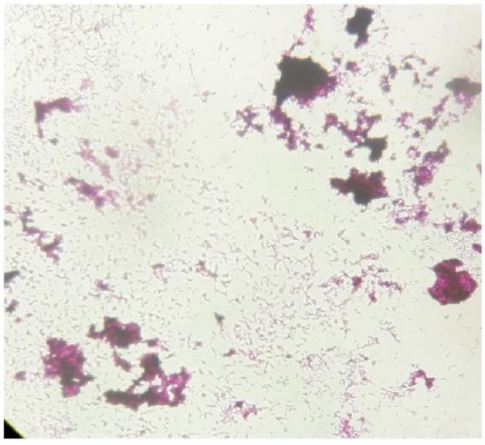
Day 2



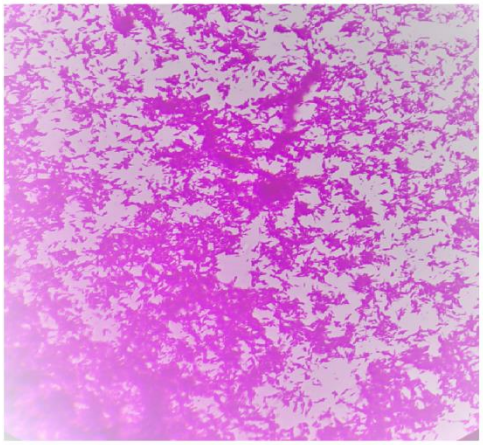
Day 7



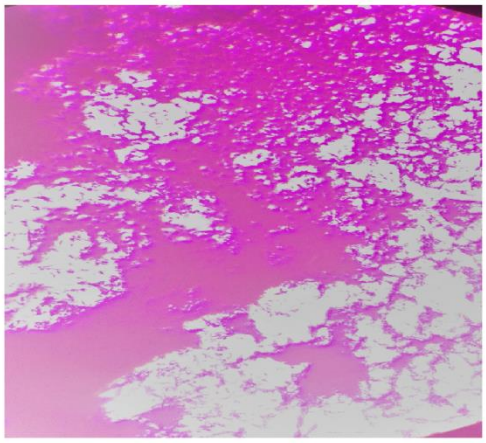
Day 11



Day 16

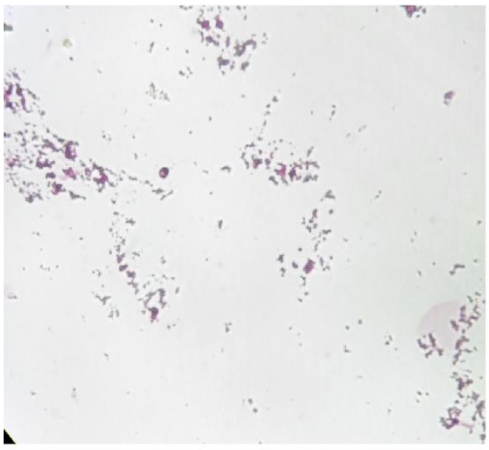


Day 21

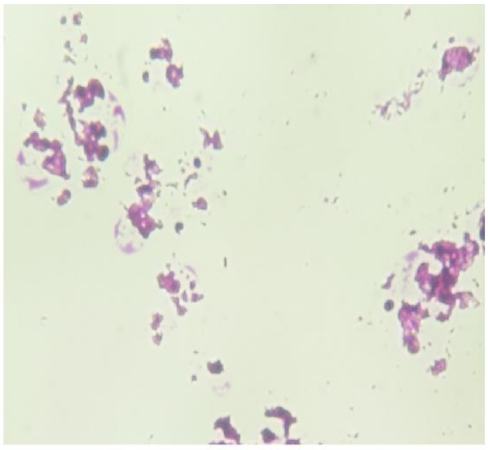


Day 28

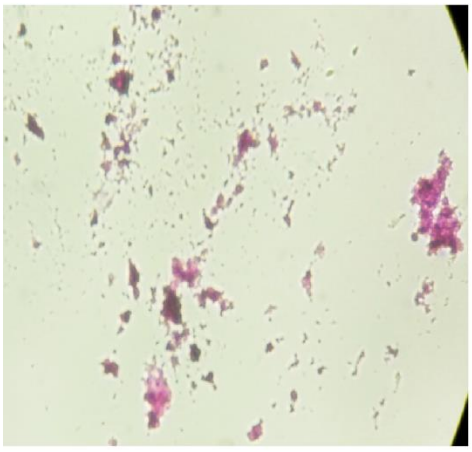
***Fig.18**: Figure depicting increase in the amount of Biofilm Formation in M. fortuitum wild-type from Day 2 to Day 28.*



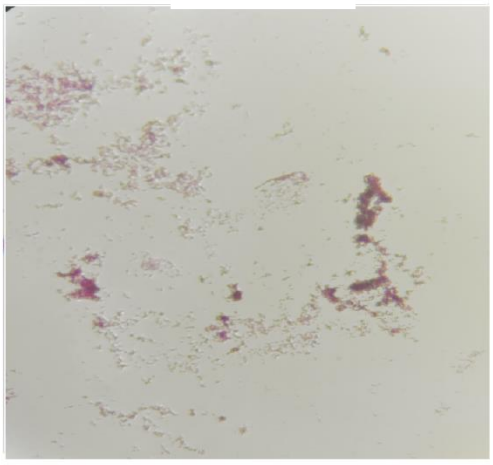
Day 2



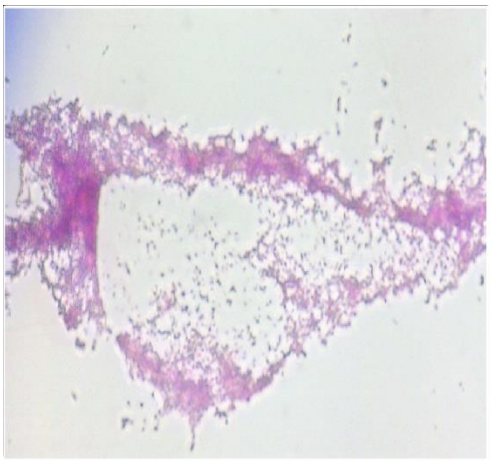
Day 7



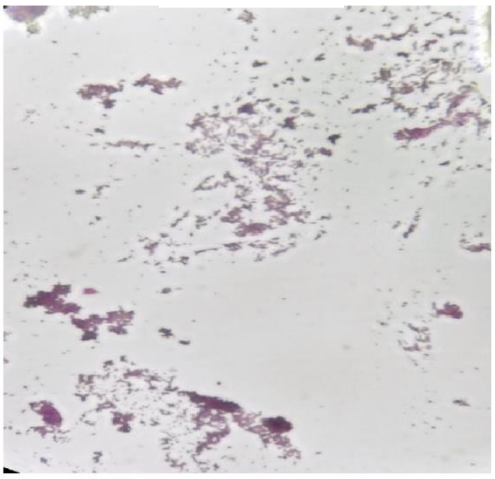
Day 11



Day 16

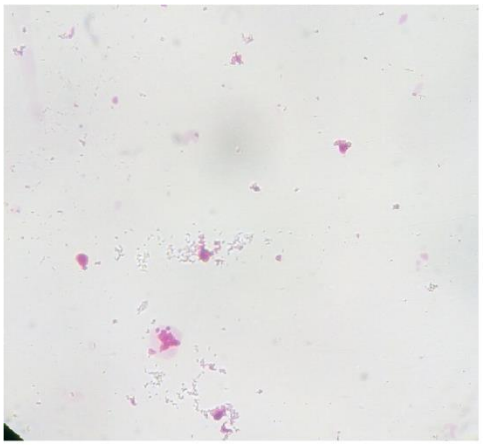


Day 21

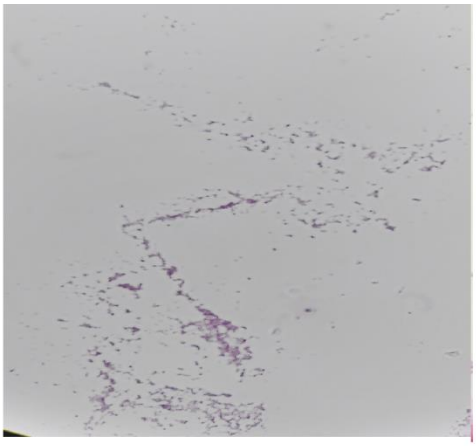


Day 28

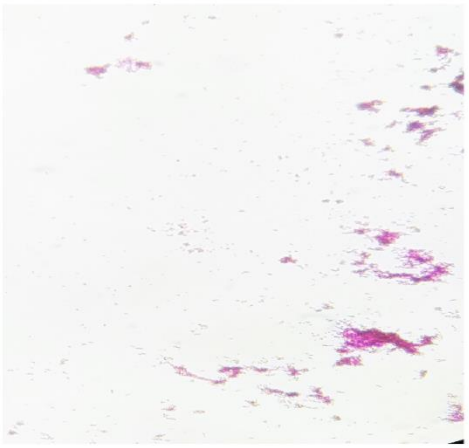
Fig.19: *Figure depicting increase in the amount of Biofilm Formation in MTK1 from Day 2 to Day 28.*



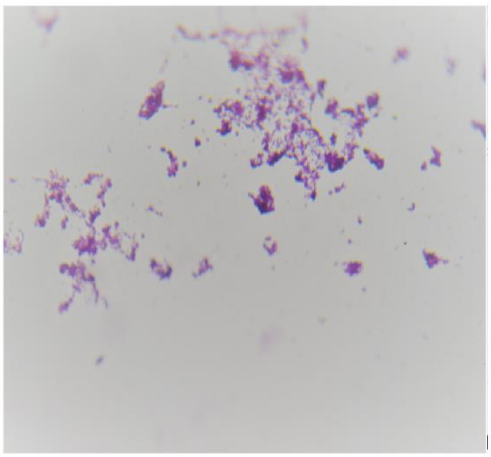
Day 2



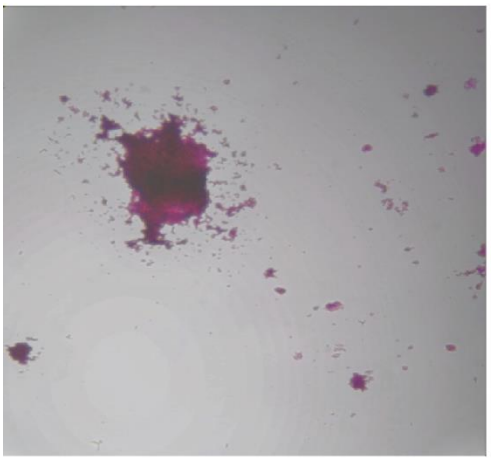
Day 7



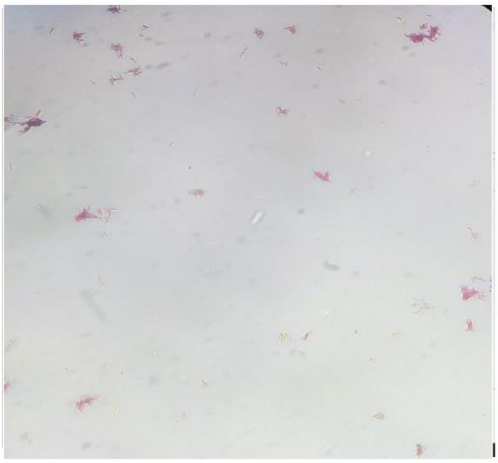
Day 11



Day 16

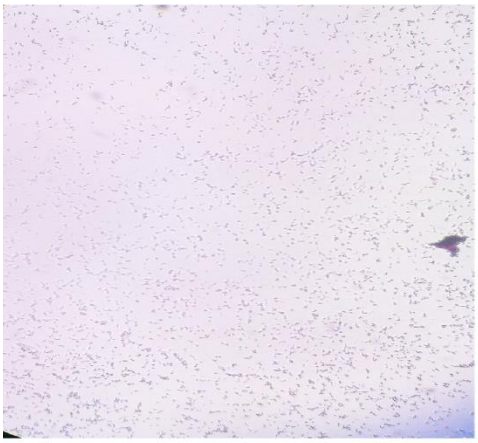


Day 21

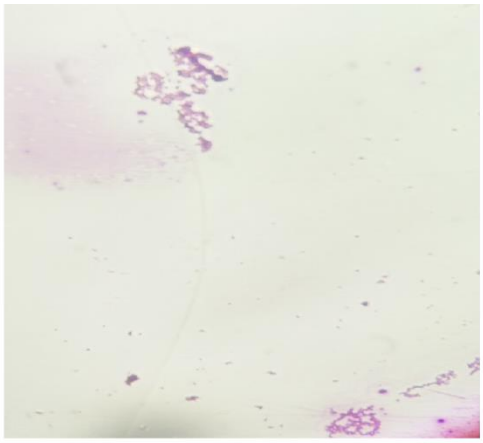


Day 28

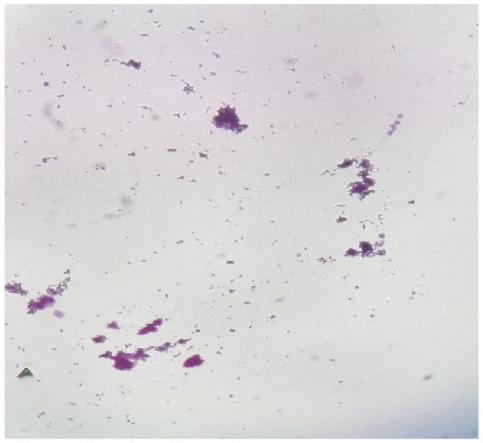
Fig.20: *Figure depicting attenuation in Biofilm Formation in MTK2 as compared to wild-type from Day 2 to Day 28.*



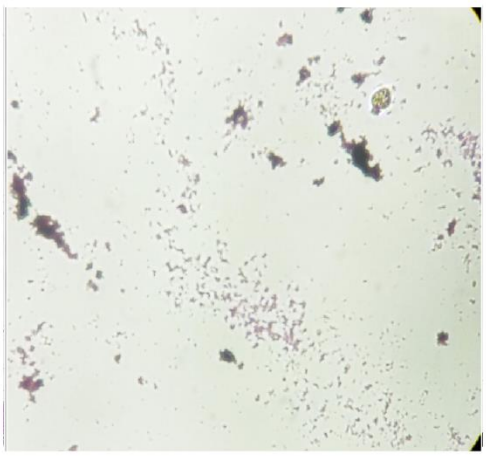
Day 2



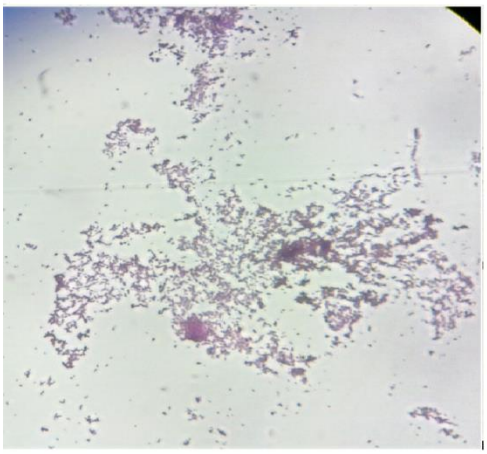
Day 7



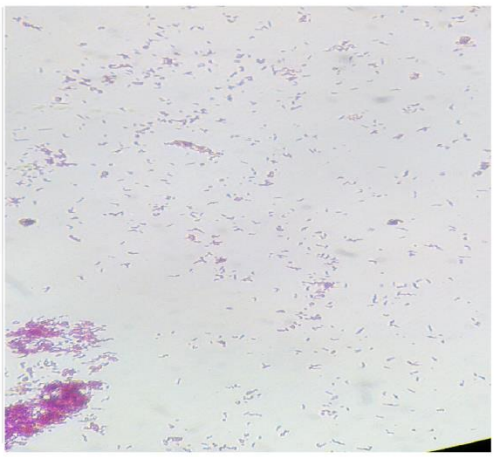
Day 11



Day 16

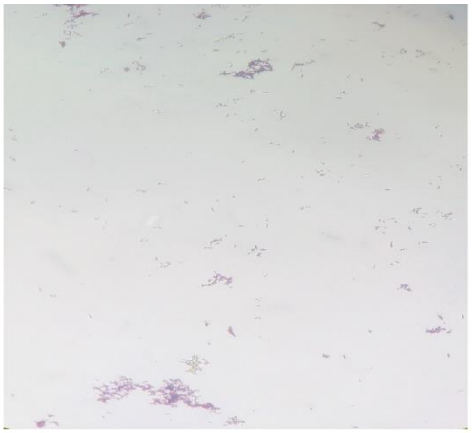


Day 21

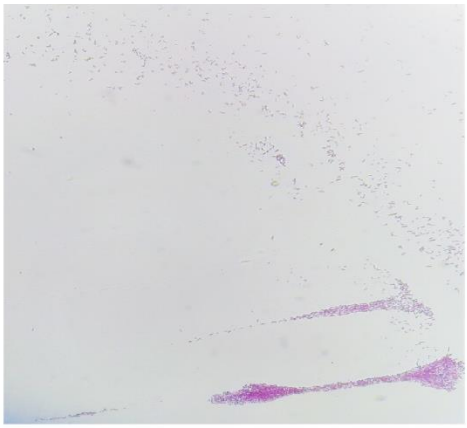


Day 28

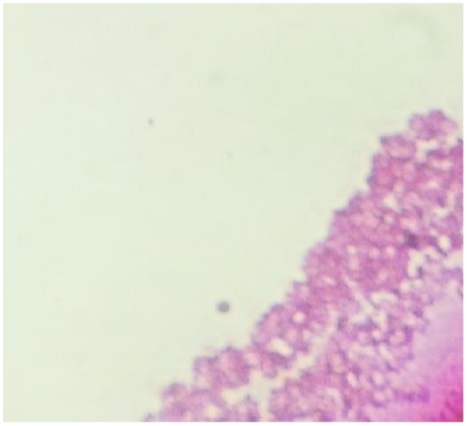
Fig.21: *Figure depicting attenuation in Biofilm Formation in MTK3 as compared to wild-type from Day 2 to Day 28.*



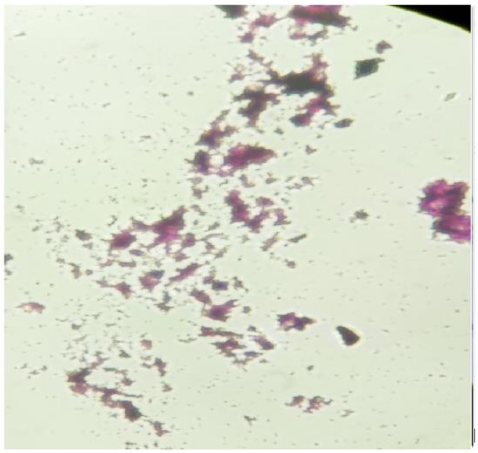
Day 2



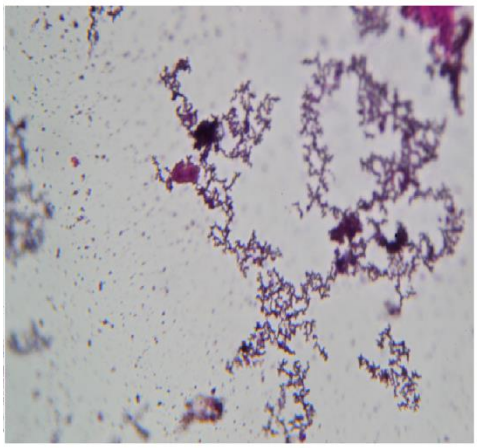
Day 7



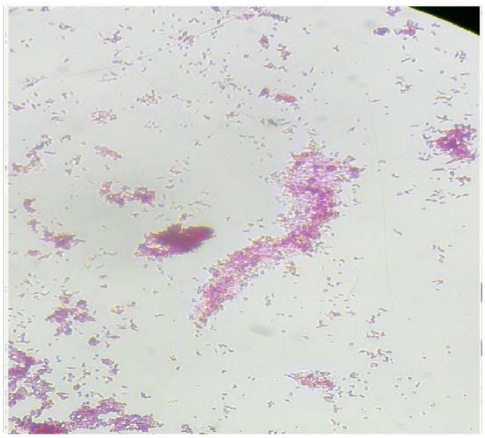
Day 11



Day 16

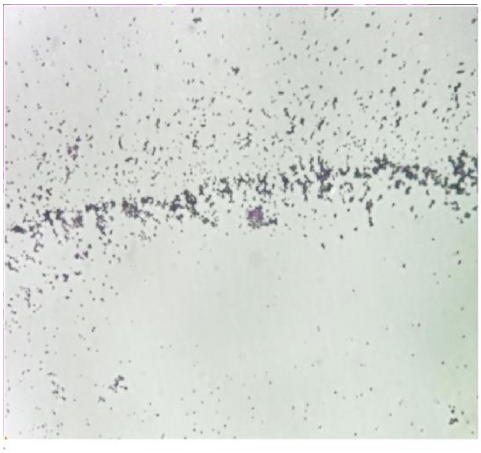


Day 21

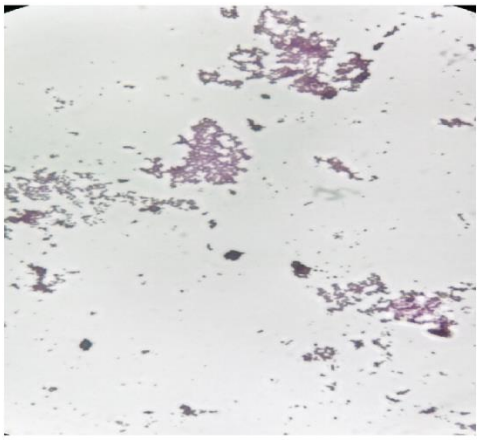


Day 28

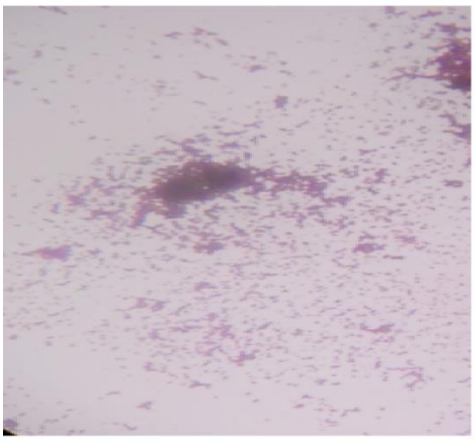
Fig.22: *Figure depicting attenuation in Biofilm Formation in MTK4 as compared to wild-type from Day 2 to Day 28.*



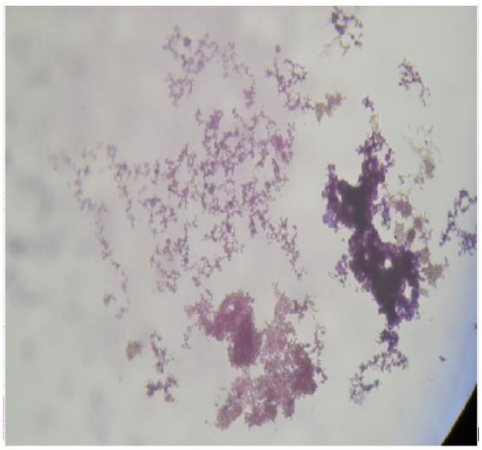
Day 2



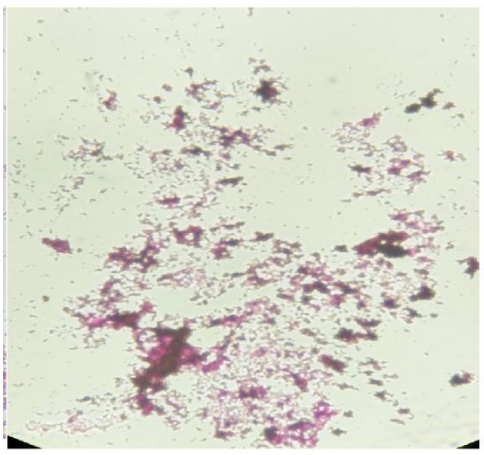
Day 7



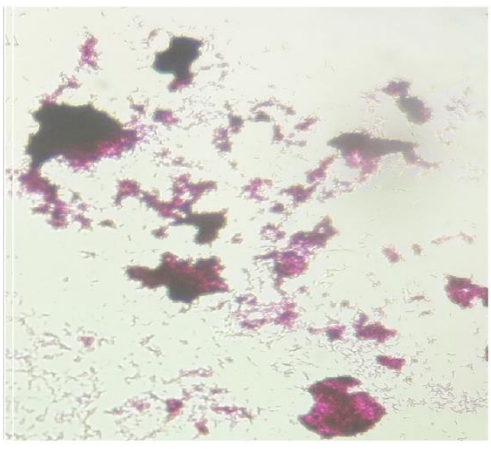
Day 11



Day 16

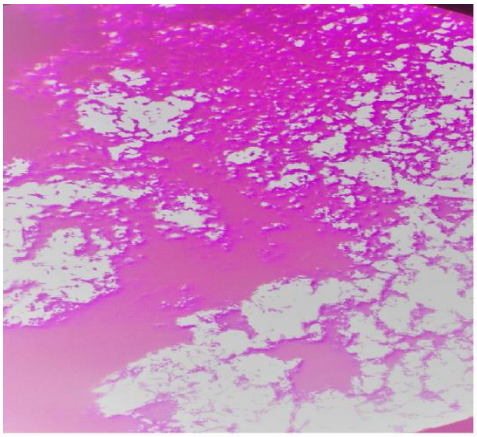


Day 21

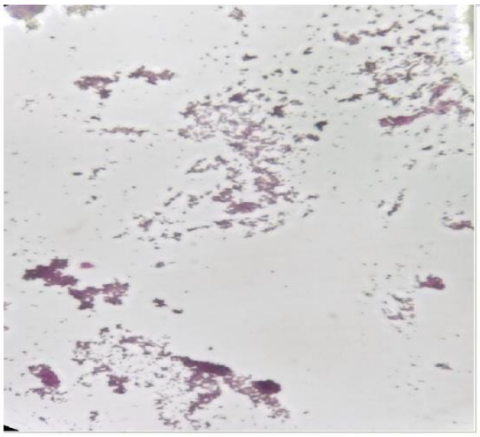


Day 28

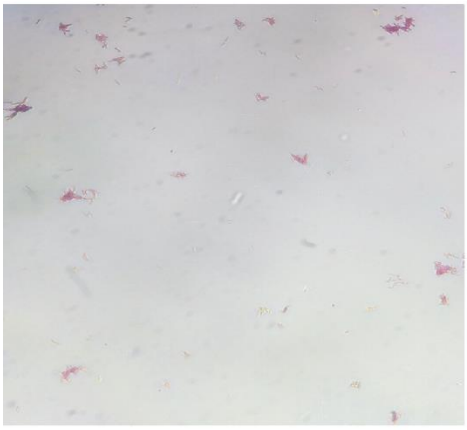
Fig.23: *Figure depicting increase in the amount of Biofilm Formation in MTK5 from Day 2 to Day 28.*



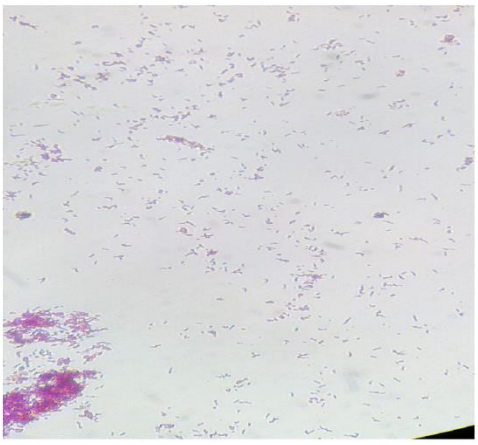
Wild-type



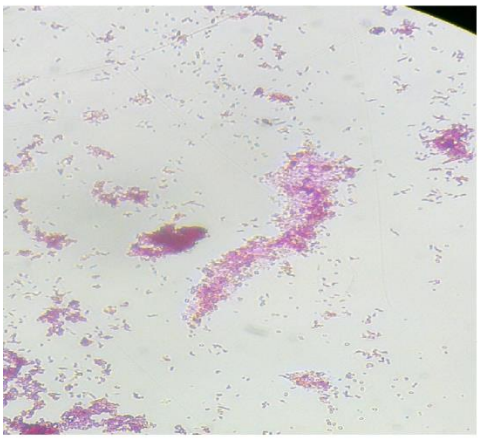
MTK1



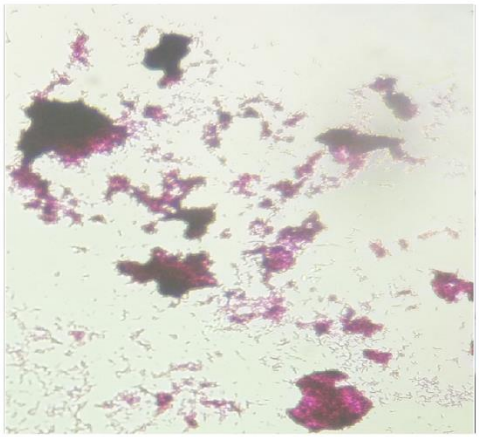
MTK2



MTK3



MTK4



MTK5

Fig.24: *Figure depicting variation in the amount of Biofilm Formation formed by M. fortuitum Wild-type and mutants after 28 days of incubation.*

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4.4 CFU Count Results

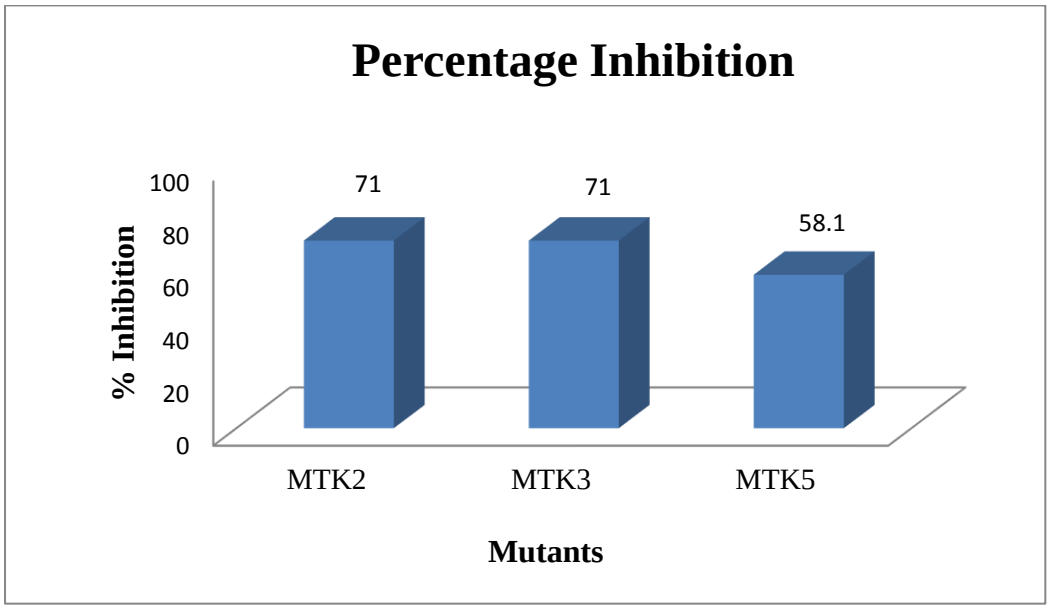

Fig.25: *Graph depicting percentage inhibition in the M. fortuitum mutants with respect to wild-type (taken as 100%) calculated through CFU count data.*

4.5 Genomic DNA Isolation Results

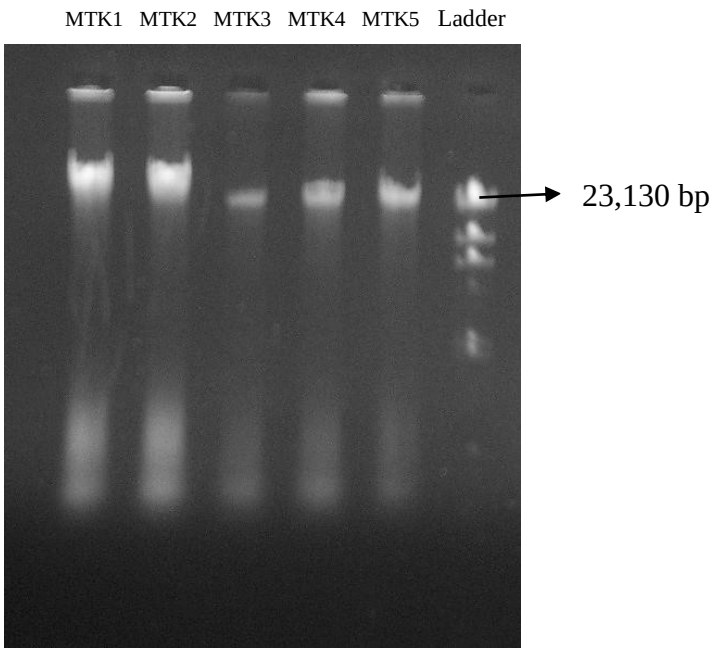

Fig.26: *Gel image of isolated genomic DNA of M. fortuitum mutants on 0.8% Agarose gel.*

4.6 Gel Extracted pUC19 vector Results

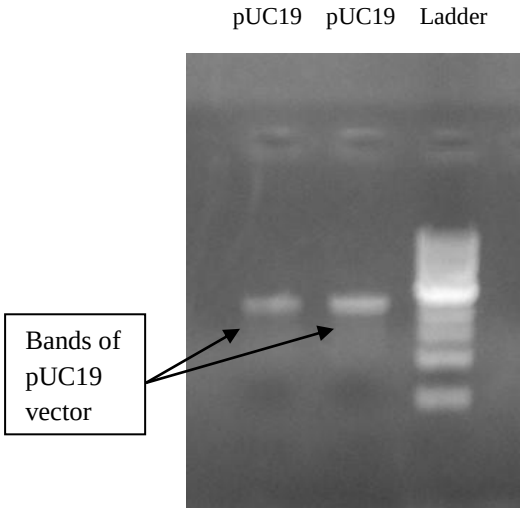

Fig.27: *Gel image of restriction digested pUC19 vector which was resolved on 0.8% Agarose gel.*

4.7 Restriction digested genomic DNA of mutants

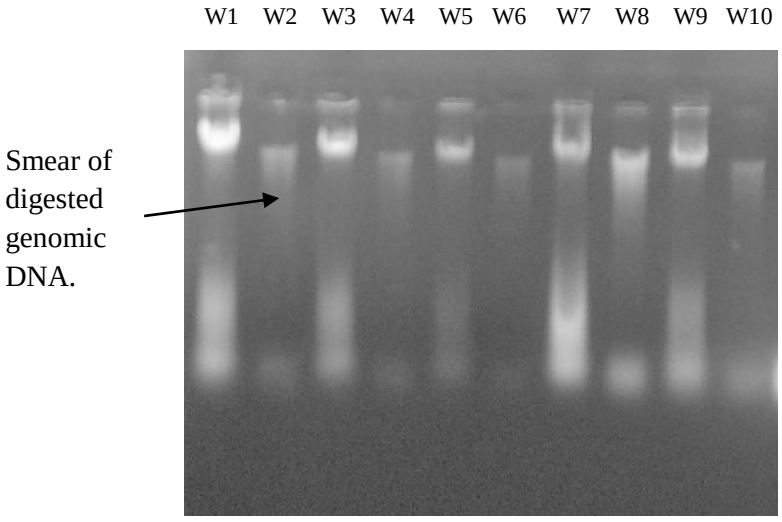

Fig.28: *Gel image of digested and undigested genomic DNA of M. fortuitum mutants resolved on 0.8% agarose gel.*

W1- MTK1 Undigested gDNA
W2- MTK1 Digested gDNA
W3- MTK2 Undigested gDNA
W4- MTK2 Digested gDNA
W5- MTK3 Undigested gDNA
W6- MTK3 Digested gDNA
W7- MTK4 Undigested gDNA
W8- MTK4 Digested gDNA
W9- MTK5 Undigested gDNA
W10- MTK5 Digested gDNA

4.8 Homology Study Results

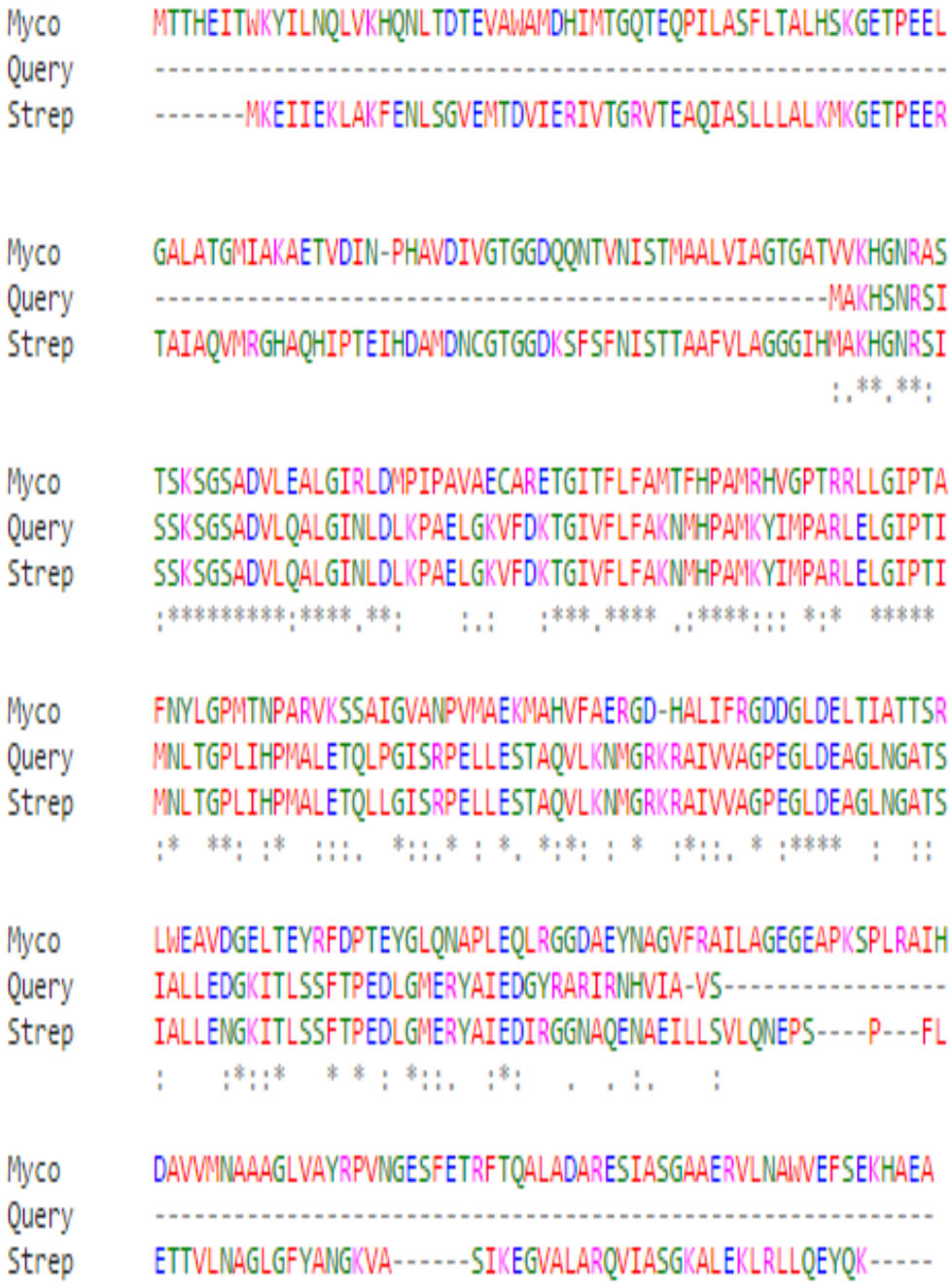

Fig. 30: *Multiple sequence alignment of protein sequence of MTK2 mutant altered gene showing 41% and 98% homology with anthranilate phosphoribosyltransferase protein of Mycobacterium tuberculosis (Myco) and Streptococcus oralis (Strep), respectively.*

Chapter 5

Discussion & Conclusion

5. DISCUSSION & CONCLUSION

In present scenario, *M. fortuitum* is one of the most studied microorganisms due to its emergence as an etiological agent and its role in nosocomial infections. It has been reported that biofilm formation has a significant role in emergence of *M. fortuitum* as a pathogen. Moreover, evidence suggests that biofilm formation also helps in the persistence of mycobacteria against various environmental challenges and immune system of humans. Thus, there is a need to find a solution to attenuate biofilm formation in this organism in order to reduce the incidences of infection.

Present study was conducted in order to find the solution to above problem by identifying genes responsible for biofilm formation. In this study, transposon mutagenesis technique was used to create the mutants of *M. fortuitum* as its genomic sequence is not completely known till now. Gene silencing and gene knockout methods can be used only when the sequence of the gene to be altered is known. Thus, these methods were ruled out in this study.

In this study, microtitre plate test was used to allow assembly of biofilm as it is cost effective method with more feasibility and less chances of contamination as compared to other methods like microfermentor test. Crystal violet assay was used to characterize biofilm formation as this method allows quantification as well as microscopic examination of biofilm.

It has been reported that *M. fortuitum* takes 28 days to completely assemble biofilm on microtitre plate. This observation was confirmed in my study as wild-type took 28 days for complete formation of biofilm when incubated in microtitre plate. Additionally, through this study it was observed that *M. fortuitum* mutant MTK2 showed attenuation in the biofilm formation as compared to the wild-type. Crystal violet assay and carbol fuschin results showed that the amount of biofilm formation on microtitre plate was less as compared to wild-type and decreased drastically after 16 days of incubation.

Genomic analysis of this mutant showed 41% and 98% homology with “Anthranilate phosphoribosyl transferase” gene of *Mycobacterium tuberculosis* and *Streptococcus oralis*, respectively. Anthranilate phosphoribosyl transferase is involved in the tryptophan biosynthetic pathway. Thus, this gene has an essential role in the synthesis of aromatic amino acids.

The present results indicate the probable role of this gene in the biofilm formation of *M. fortuitum* and thus, can be used as potential drug target against *M. fortuitum* and other pathogenic *Mycobacterium* species.

However, there exists a significant knowledge gap in the molecular mechanisms underlying phenotypic and structural developments of mycobacterial biofilms. With new high-throughput genetic and biochemical tools as well as advanced microscopic techniques like AFM, SEM, etc., there is an enormous opportunity to explore the biological complexities and intercellular dynamics of multicellular structures and their likely involvement in the extraordinary recalcitrance of mycobacterial infections.

Chapter 6

Appendix

6. APPENDIX

6.1 Bacteriological media

All the media were prepared in distilled water and autoclaved at 15 pounds per square inch for 15 min., unless otherwise indicated.

LB Broth (Luria Bertani Broth)

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g

The components were dissolved in 950mL distilled water and the pH was adjusted to 7.5 with 5N NaOH and finally, the volume was made to 1000 mL with distilled water. Agar at a concentration of 1.5 % was added whenever solid medium was required.

Nutrient Broth

Peptone	5 g
Yeast extract	1.5 g
Beef extract	1.5 g
NaCl	5 g

The components were dissolved in 950mL distilled water and the pH was adjusted to 7.5 with 5N NaOH and finally, the volume was made to 1000 mL with distilled water.

Nutrient Agar Tween80 (NAT)

Nutrient Broth	13 g
Tween 80	500 µL (0.05%)

The components were dissolved in 950mL distilled water and the pH was adjusted to 7.5 with 5N NaOH and finally, the volume was made to 1000 mL with distilled water. Agar at a concentration of 1.5 % was added whenever solid medium was required.

Middle brook (MB)7H9 broth

MB7H9 broth base	4.7 g
Tween 80	1.5 mL (0.15%)
Glycerol	5 mL (0.5%)

The components were dissolved in 950mL distilled water and the pH was adjusted to 7.2 with 5N NaOH and finally, the volume was made to 1000 mL with distilled water.

6.2 Reagents for Acid Fast Staining

i. Carbol fuchsin (Primary stain)

Basic fuchsin	3 g
Phenol	5%
Ethanol (96%)	10 ml

10 mL of Basic fuchsin prepared in 96% ethanol was mixed with 90 mL of phenol and the solution was filtered through Whatman filter paper no. 1.

ii. Acid alcohol (Decolorizer)

HCL (conc.)	3 mL
Ethanol (96%)	97 mL

iii. Malachite green solution (Counter stain)

Malachite green	0.5 g
Distilled water	100 mL

6.3 Antibiotics and Substrates

All antibiotic solutions were filter sterilized by a 0.22 µm filter (Millipore) and stock solutions were stored at -20 ^{0}C for long-term use.

Reagent	Stock Solution	Final Concentration (in *E. coli*)	Final Concentration (in *Mycobacterium*)
Ampicilin	5mg/mL in H_2O	100µg/mL	-
Kanamycin	15mg/mL in H_2O	50µg/mL	30µg/mL
Cycloheximide	5mg/mL in H_2O	100µg/mL	50µg/mL
X-gal	20mg/mL in DMF	40µg/mL	40µg/mL

6.4 Reagents and Buffers

All the reagents and buffers for DNA and protein protocols were prepared in Milli Q grade water and sterilized by autoclaving for at 15-psi pressure 15 minutes unless otherwise indicated.

6.4.1 Commonly used Buffers

i. Tris HCL buffer

Tris-HCL buffer of desired strength was prepared by dissolving appropriate amount of Tris in distilled water and adjusting the pH with concentrated HCl. For bacteriological work 10 mM Tris-HCl (pH 8.0) was prepared.

ii. Ethylene diamine tetra acetic acid (EDTA)

0.5 M solution of disodium salt of EDTA was prepared in distilled water, pH was adjusted to 8.0 with NaOH pellets and stored at 4 ^{0}C.

iii. Normal Saline

NaCl	8.9 g
Distilled water	1000 mL (final volume)

iv. Tween Normal Saline

NaCl	0.9%
Tween 80	0.1%
Distilled water	100 mL

6.4.2 Reagents for Genomic DNA isolation

i. TE Buffer

Tris-HCl (pH 8.0)	10 mM
EDTA	1 mM

ii. Tris EDTA Saline (TES) Buffer

Tris-HCL (pH 8.0)	10 mM
EDTA	1 mM
NaCl	150 mM

iii. **Lysozyme**

Lysozyme	50 mg/mL in distilled H_2O

iv. Proteinase K

Proteinase K	20 mg/mL in distilled H_2O

v. Buffer Phenol

Phenol was melted at 60 ^{0}C in water bath for 2 hours. 1M Tris Cl (pH 8.0) was added to the molten phenol and it was incubated at room temperature for half an hour. Tris Cl was removed with the help of glass pipette and pH of the phenol was checked. If pH of phenol was <7 then again Tris Cl was added and above process was repeated, until the pH of phenol reached 7.4. Then, it is stored submerged in 10 mM Tris-HCl (pH 8.0) in dark bottle at 4 ^{0}C.

vi. Chloroform: Isoamyl alcohol

Solution contains 24 parts of chloroform and 1 part of Isoamylalcohol. The solution is stored in dark bottle at 4 ^{0}C.

6.4.3 Buffers for Plasmid Isolation from *E. coli*

i. Alkaline Lysis Solution I

Tris-HCL (pH 8.0)	25 mM
EDTA (pH 8.0)	10 mM
Glucose	50mM

ii. Alkaline Lysis Solution II

NaOH	0.2N
SDS	1.0%

iii. Alkaline Lysis Solution III

5M Potassium acetate	60 mL
Glacial Acetic acid	11.5 mL
Distilled water	28.5 mL

6.4.4 Buffers for Electrophoresis

i. TAE Buffer (50 X)

Tris Base	242 g
Glacial Acetic Acid	57.1 mL
0.5 M EDTA (pH 8.0)	100 mL
Final Volume	1000 mL

6.4.5 Buffer for Transformation

i. Transformation Buffer 1 (TFB I)

RbCl	100 mM
$MnCl.4H_2O$	50 mM
Potassium Acetate	30 mM

$CaCl_2.2H_2O$	10 mM
Glycerol	15%
Distilled water	100 mL

pH of the buffer was adjusted to 5.8 with diluted acetic acid.

ii. Transformation Buffer II (TFB II)

RbCl	10 mM
MOPS buffer	10 mM
$CaCl_2.2H_2O$	75 mM
Glycerol	15%

pH of the buffer was adjusted to 6.8 with KOH.

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PUBLICATIONS

1. **Analysis of transposon mutants' library in search of genes responsible for biofilm formation in *Mycobacterium fortuitum***

   Kinam Gupta, Poonam, Jitendraa Vashistt, Rahul Shrivastava

   Poster presentation at - 57th Annual Conference of Association of Microbiologists of India held at Gauhati University, Guwahati from November 24-27, 2016.

Poster

Analysis of transposon mutants library in search of genes responsible for biofilm formation in *Mycobacterium fortuitum*

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ABSTRACT

Mycobacterium fortuitum is a non tuberculous, rapidly growing mycobacterium which is present ubiquitously in nature. *M. fortuitum* is an emergent pathogen attaining relevance in human health as it is one of the leading causes of opportunistic infections such as local cutaneous diseases, osteomyelitis, joint infections, ocular disease, post surgical infections etc. An obstruction is imposed on the way of its treatment as it is evolving resistance to the currently prescribed drugs. Growing incidence of nosocomial infections caused by *M. fortuitum* may be correlated with its ability to assemble biofilm on implantable devices and other surgical instruments. Membrane genes plays an important role in the biofilm formation thus, the present study aims to identify membrane genes responsible for biofilm formation in *M. fortuitum* as virulence factors by random mutagenesis and subsequent use of these findings in establishing effective drug targets. As the genomic sequence of *M. fortuitum* is not known, random mutagenesis of wild type *M. fortuitum* (ATCC 6871) was done by electroporation of vector pRT291 containing TnphoA having transposon Tn5 with kanamycin resistance and structural alkaline phosphatase gene lacking signal sequence. Inactivation of membrane spanning genes in *M. fortuitum* mutants was confirmed by blue colour resulting due the fusion of alkaline phosphatase gene of TnphoA with membrane gene. A library of about 5000 mutants was prepared, out of which 227 blue mutant colonies showing mutation in membrane gene were selected. These mutants were further assayed for alkaline phosphatase activity to shortlist 50 mutants with highest alkaline phosphatase activity for further experiments. The mutants were individually subjected to the standardised biofilm forming protocol, to check for any deviation in the amount of biofilm formed. Two mutants were observed to shown attenuation in the amount of biofilm formation. Genomic analysis followed by homology study would be done for identification of genes for biofilm formation in *M. fortuitum*. Genes identified by the study would serve as potential drug targets for development of novel drugs or other intervention strategies.

INTRODUCTION

- *Mycobacterium fortuitum* is an emergent pathogenic species of Mycobacterium genus.
- Major cause of nosocomial infections
- Presence of a mycolic acids in cell wall
- Long generation time
- Ability to form biofilm which enhances its virulence
- Biofilm makes it resistant to high concentrations of antimicrobial drugs
- Difficult to eradicate as compared to their planktonic counterparts
- Membrane genes play important role in the biofilm formation
- Identification of genes responsible for biofilm formation can help in development of novel drugs against Mycobacterium

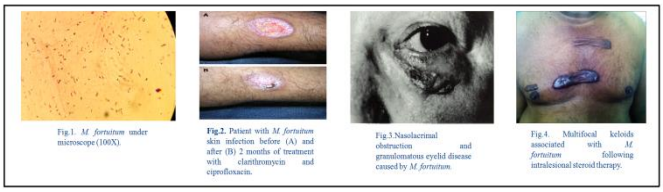


Fig.1. *M. fortuitum* under microscope (100X).

Fig.2. Patient with *M. fortuitum* skin infection before (A) and after (B) 2 months of treatment with clarithromycin and ciprofloxacin.

Fig.3.Nasolacrimal obstruction and granulomatous eyelid disease caused by *M. fortuitum*.

Fig.4. Multifocal keloids associated with *M. fortuitum* following intralesional steroid therapy.

MATERIALS AND METHOD

Construction of transposon mutant library of M. fortuitum using pRT291

↓

Selection of transposon mutants with X-gal, IPTG (Isopropyl ß-D-1-thiogalactopyranoside) and glucose

↓

Determination of alkaline phosphatase activity by pNPP assay

↓

Attenuated mutants proceeded for cloning in pUC19 vector

↓

Selected mutants were subjected to standardized biofilm formation protocol

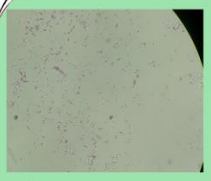
↓

Mutants ability to form biofilm was checked by staining with crystal violet and measuring optical density at 570 nm

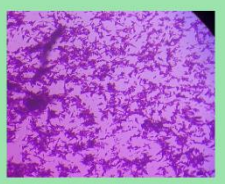
↓

Mutants were stained with carbol fuschin to check biofilm formation

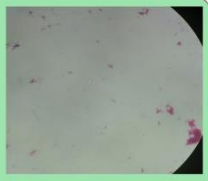
RESULTS



MT721 mutant showing no biofilm formation at 11th day



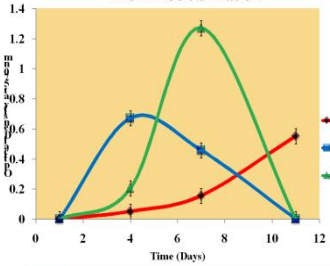
Wild type M. fortuitum showing biofilm formation at 11th day

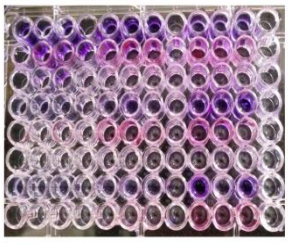


MT725 mutant showing no biofilm formation at 11th day

MT721 and MT725 TnphoA mutants of *M. fortuitum* showed attenuation in biofilm formation as compared to wild type *M. fortuitum*

Biofilm Solubilization





SIGNIFICANCE

MT 721 and MT 725 mutants showed attenuation in the biofilm formation as compared to wildtype. Genomic analysis of these mutants showed 50% homology with replication inactivation and membrane attachment genes and 54% homology with anthranilate phosphoribosyl transferase gene of *Mycobacterium* species. The present results indicate the probable role of These two genes in the biofilm formation of *M. fortuitum* and thus, can be used as potential drug target against pathogenic *Mycobacterium* species.

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