STUDY OF *MYCOBACTERIUM FORTUITUM FABG4* GENE MUTANT UNDER VARIOUS STRESS CONDITIONS

Dissertation submitted in partial fulfillment of the requirement for the degree of

Master of Science

In

Microbiology

By

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Under the supervision

of

Dr. Rahul Shrivastava

To

Department of Biotechnology & Bioinformatics

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May, 2024

DECLARATION

I hereby declare that the work presented in this report entitled "**Study of *Mycobacterium fortuitum fabG4* gene mutant under various stress conditions**" in partial fulfillment of the requirements for the award of the degree of **Master of Science in Microbiology** submitted in the **Department of Biotechnology & Bioinformatics**, Jaypee University of Information Technology, Waknaghat is an authentic record of my own work carried out over a period from August 2023 to May 2024 under the supervision of **Dr. Rahul Shrivastava** Associate Professor, Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Solan, Himachal Pradesh.

I also authenticate that I have carried out the above mentioned project work under the proeficiency stream

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

DIKSHA SUMAN, (Enrollment no.- 225112001)

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

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CERTIFICATE

This is to certify that the work reported in the M.Sc. Microbiology thesis entitled **"Study of *Mycobacterium fortuitum fabG4* gene mutant under various stress conditions"** which is being submitted by **Ms Diksha Suman (225112001)** in fulfillment for the award of Masters of Science in Microbiology by the Jaypee University of Information Technology, is the record of candidate's own work carried out by her under my supervision. This work is original and has not been submitted partially or fully anywhere else for any other degree or diploma.

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ACKNOWLEDGEMENT

I would like to express my sincere gratitude to the Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology (JUIT), Solan, Himachal Pradesh, for their unwavering support and guidance throughout my thesis project. The department's resources and facilities played a crucial role in enabling me to conduct my research effectively.

My deepest gratitude goes to my esteemed supervisor, **Dr. Rahul Shrivastava**, Associate Professor in the Department of Biotechnology and Bioinformatics at JUIT. His invaluable guidance, insightful feedback, and unwavering support were instrumental in shaping this project. I am particularly grateful for the liberty he granted me to explore my potential and pursue independent research avenues.

I would like to extend my thanks to **Dr. Ayushi Sharma** for her significant contributions and support during the course of this project.

I am also thankful to my fellow mate, **Sourav Kumar**, for his camaraderie and assistance in the laboratory. His help during experiments significantly smoothened the research process.

Finally, I would like to express my appreciation to the lab technicians, **Mr. Baleshwar Parshad** and **Mrs. Mamta Mishra**, for their dedication and promptness in providing the necessary materials and equipment for my experiments. Their contributions ensured the smooth running of the research process.

Last but not least I would like to thanks my **Parents** whose unwavering love and support provided a constant source of strength throughout this journey.

Thank you all

Diksha Suman

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List of Abbreviations

NTM	**Non tuberculous mycobacteria**
FAS-II	**Fatty acid synthesis- type II**
WT	**Wild type**
MT	**Mutant type**
ACP	**Acyl Carrier Protein**
PCR	**Polymerase Chain Reaction**
MTB	***Mycobacterium tuberculosis***
IDSA	**Infectious Disease Society of America**
ATS	**American thoracic Society**
M. fortuitum	***Mycobacterium fortuitum***
M. smegmatis	***Mycobacterium smegmatis***
TB	**Tuberculosis**
SGM	**Slow Growing mycobacteria**
RGM	**Rapidly Growing mycobacteria**
E. coli	***Escherichia coli***
pUC-19	**Plasmid of University of California-19**
pH	**Potential of Hydrogen**
mAG	**mycolylarabinogalactan**
HIV	**Human Immunodeficiency Virus**
WHO	**World Health Organization**
XDR	**Extensively Drug resistant**
MDR	**Multi Drug resistant**
BCG	**Bacillus Calmette Guerin**
M. bovis	***Mycobacterium bovis***
M. microti	***Mycobacterium microti***
M. africanum	***Mycobacterium africanum***
M. avium	***Mycobacterium avium***
M. gordonae	***Mycobacterium gordonae***
M. xenopi	***Mycobacterium xenopi***
M. intracellulare	***Mycobacterium ntracellulare***
M. marinum	***Mycobacterium marinum***

M. kansasii	***Mycobacterium kansasii***
M. abscessus	***Mycobacterium abscessus***
M. chelonae	***Mycobacterium chelonae***
M. saopaulense	***Mycobacterium saopaulense***
M. franklinii	***Mycobacterium franklinii***
M. brisbanense	***Mycobacterium brisbanense***
M. neworleansense	***Mycobacterium neworleansense***
CDC	**Centre of Disease Control**
IDSA	**Infectious Disease Society of America**
COPD	**Chronic obstructive pulmonary disease**
DNA	**Deoxyribonucleic acid**
NADPH	**Nicotinamide Adenine Dinucleotide Phosphate Hydrogen**
ATCC	**American Type Culture Collection**
SDS	**Sodium Dodecyl Sulphate**
CFU	**Colony Forming Unit**
NaOH	**Sodium hydroxide**
NAT	**Nutrient Agar with Tween-80**
EDTA	**Ethylenediaminetetraacetic acid**
NaCl	**Sodium Chloride**
TAE	**Tris – acetate- EDTA**
°C	**Degree Celcius**
ml	**Mililiter**
µl	**Microlitre**
g	**Gram**
mg	**Milligram**
V	**Voltage**

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Abstract

Mycobacterium fortuitum is a nontuberculous mycobacteria (NTM) which grows rapidly and is found in most environments. *M. fortuitum* is fast-growing and pathogenic bacteria which can infect bones, joints as well as the skin. It is an immobile, rod-shaped, gram-positive bacterium. *M. fortuitum* is highly adaptable to different environments, and it survives in a variety of often difficult conditions. The process of fatty acid production is essential for the mycobacterial cell membrane to develop.

The *fabG4* is an important genes involved in biosynthesis of fatty acid, mycolic acid synthesis and crucial for multiplication and survival of mycobacterial species. The FAS-II (fatty acid synthase type-II) enzyme 3-oxoacyl-ACP reductase (*fabG4*) is important for pathogenesis and virulence in mycobacterium species. The knockdown strain of *M. fortuitum fabG4* gene constructed in our lab was used for the present study. The knockdown strains along with wild type strain of *M. fortuitum* were studied under various *in vitro* stress conditions of acid stress, hypoxic stress, oxidative stress, detergent stress, and nutrient starvation to gain an insight into their role of *fabG4* under these stress conditions. This study showed that MF*fabG4* gene was essential for the survival under acidic, hypoxic, nutrient starvation, and oxidative stress conditions and not involved in survival under detergent stress condition. This study emphasizes the roles that *fabG4* play in stress tolerance of *M. fortuitum*, and it would contribute to understanding the pathophysiology of an important human pathogen *M. fortuitum*.

Keywords: *M. fortuitum*, stress conditions, *fabG4*, Knockdown, FAS-II.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Members of the genus *Mycobacterium* are included under the phylum Actinobacteria. The genus contains *Mycobacterium leprae* and the exclusively pathogenic *Mycobacterium tuberculosis* (MTB) complex. While *M. leprae* causes leprosy, members of the MTB complex cause both extrapulmonary and pulmonary tuberculosis. There are 200 additional species in the genus "*Mycobacterium*" that are known as atypical or NTM. While non tuberculous mycobacterium don't cause tuberculosis, they can produce illnesses that are comparable to those brought on by MTB in both immunocompromised people and immunocompetent people[1].

Inhalation of these microorganisms is the usual cause of NTM infection. NTM has the ability to penetrate the body's natural defenses and take up residence in the airways of patients with impaired lungs. The NTM infection causes lung tissue damage and inflammation over time. In addition to creating cavities and making it harder to remove mucus, this damage can leave scars and exacerbate respiratory issues[2].

Human pathogenic NTM *M. fortuitum* is a fast-growing organism that is distributed widely within the environment. It is one from the many, most common NTM infections in Asia, especially in Korea, Iran, and India. It is the cause of pulmonary, extrapulmonary, and infections specific to biofilms in medical implants, as well as hospital-associated illnesses. It appears that *M. fortuitum* has the capacity to endure in human alveolar macrophages. It demonstrates resistance to common disinfectants and antitubercular medications.

NTM have received a lot of attention recently because of their rising infection rates and broad spectrum of pathogenicity across a variety of hosts, including humans. NTM may be categorized into two groups: slow-growers and rapid-growers, depending on when in their generation cycle. It can be difficult to identify and diagnose NTM due to its wide range of geographic distribution, antibiotic susceptibility profile, species diversity, and clinical symptoms.

The American Thoracic Society (ATS) and the Infectious Disease Society of America (IDSA) have recommended adhering to adequate diagnostic criteria for the identification of NTM together with *M. tuberculosis* in sputum samples since infections from NTM may also mislead to medication against *M. tuberculosis*[6].

M. fortuitum quickly spreads, which increases its capacity to spread infections and makes it immune to biocides. Infections caused by *M. fortuitum* are becoming more and more drug-resistant, necessitating prolonged treatment with several medications. There is a knowledge gap in the molecular and phenotypic development of *M. fortuitum* infection, which calls for the discovery and investigation of therapeutic targets linked to associated pathogen processes.

A combination of genomic, and in silico methods may be used to identify gene targets against any mycobacterial pathogen. Genes involved in mycobacterial survival and development that are homologous to *M. fortuitum* were examined using a knockdown method and an antisense technique[4].Its adaptability to a variety of settings shows that it has built-in defenses against different types of stress. In order to get better understanding of these pathways, scientists have compared created mutants to the wild type strain of *M. fortuitum* in stress tests. The bacterium's response to a variety of stresses has been examined in this investigations, including nutrient deprivation (nutrient starvation), exposure to detergents (detergent stress), the presence of reactive oxygen species (oxidative stress), and acidic environments (acid stress).

Study of genes and pathways essential for *M. fortuitum*'s growth and viability by examining the survival rates and physiological changes of both wild type strain and mutant strains under these conditions would help in better understanding of pathophysiology of the pathogen and consequently in rationale discovery of drugs and vaccines for effective control of the pathogen.

1.2 Aims and Objectives

- To study the function of *M. fortuitum* *fabG4* gene in response to various stress conditions:
    - Acidic Stress.
    - Hypoxic stress.
    - Oxidative Stress
    - Nutrient deprivation.
    - Detergent Stress
- To establish the function of *fabG4* survival and growth of *M. fortuitum* under various stress conditions.

CHAPTER 2

REVIEW OF LITERATURE

2. Review of literature

2.1 Genus *Mycobacterium*

Mycobacterium is a genus with around 190 species that is one of the member of order Corynebacteriales, the phylum/class Actinobacteria, the family *Mycobacteriaceae* family, and the kingdom bacteria[3]. Mycobacteria are rod-shaped, aerobic, non-sporulating and nonmotile bacilli that are distinguished by the cell wall of mycobacteria which is very high in lipids and contains the mycolic acids. All mycobacterial species are "acid-fast" due to these long-chain (Carbon 60 to Carbon 90) fatty acids, which protect the germs from acid-alcohol decolorization after staining with phenicated dyes like fuchsine and allow microscopic identification.

In 1896, Lehmann and Neuman proposed the genus *Mycobacterium* based on phenotypic features[4]. There are two main types of mycobacteria relevant to human health: Non-tuberculous mycobacteria (NTM) and *M. tuberculosis* (MTB). The extremely contagious and sometimes fatal infectious illness tuberculosis (TB) is caused by MTB. NTM, on the other hand, are a varied collection of environmental bacteria that seldom make healthy people unwell[5].

The genus contains *Mycobacterium* about 200 species, including nontuberculous mycobacteria (NTM), and is broadly divided into two groups: the slowly-growing mycobacteria (SGM), which forms colonies in longer than 7 days and the rapidly growing mycobacteria (RGM), which forms colonies in fewer than 7 days. Despite having different genetic and biological makeups, NTM cause strikingly similar illness presentations in many groups that are susceptible to them. Both the impacted patients and the physicians should take note of this regularity and the lessons that may be drawn from it[6].

2.2 Cell wall of Mycobacteria

With a complex of three part organization, the lipid content of mycobacterial cell walls ranges from 30 percent to 40 percent[7]. A significant amount of these lipids are "loosely bound," which means that organic solvents may be used to extract them, but "firmly bound lipids" can only be recovered after the leftovers from earlier extractions have saponified[8]. The bulk of mycobacteria's physiologically active lipids are found in the "loosely bound" fraction, whereas the majority of the "firmly bound" fraction is made up of residues of the mycolic acid esterified to residues of arabinose within the cell wall skeleton.[9]. The outer most covering of the mycobacterial cell envelope serve as barriers to permeability.

Compounds that rupture the cell membrane can greatly increase the sensitivity of multiple drug-resistant atypical mycobacteria to medicines, and lipophilic carriers can promote drug diffusion across the cell envelope. Following saponification and delipidation, the following compounds were found in the cell wall residues: alanine, galactose, diaminopimelic acid, muramic acid, glucose, glutamic acid, and arabinose [10].

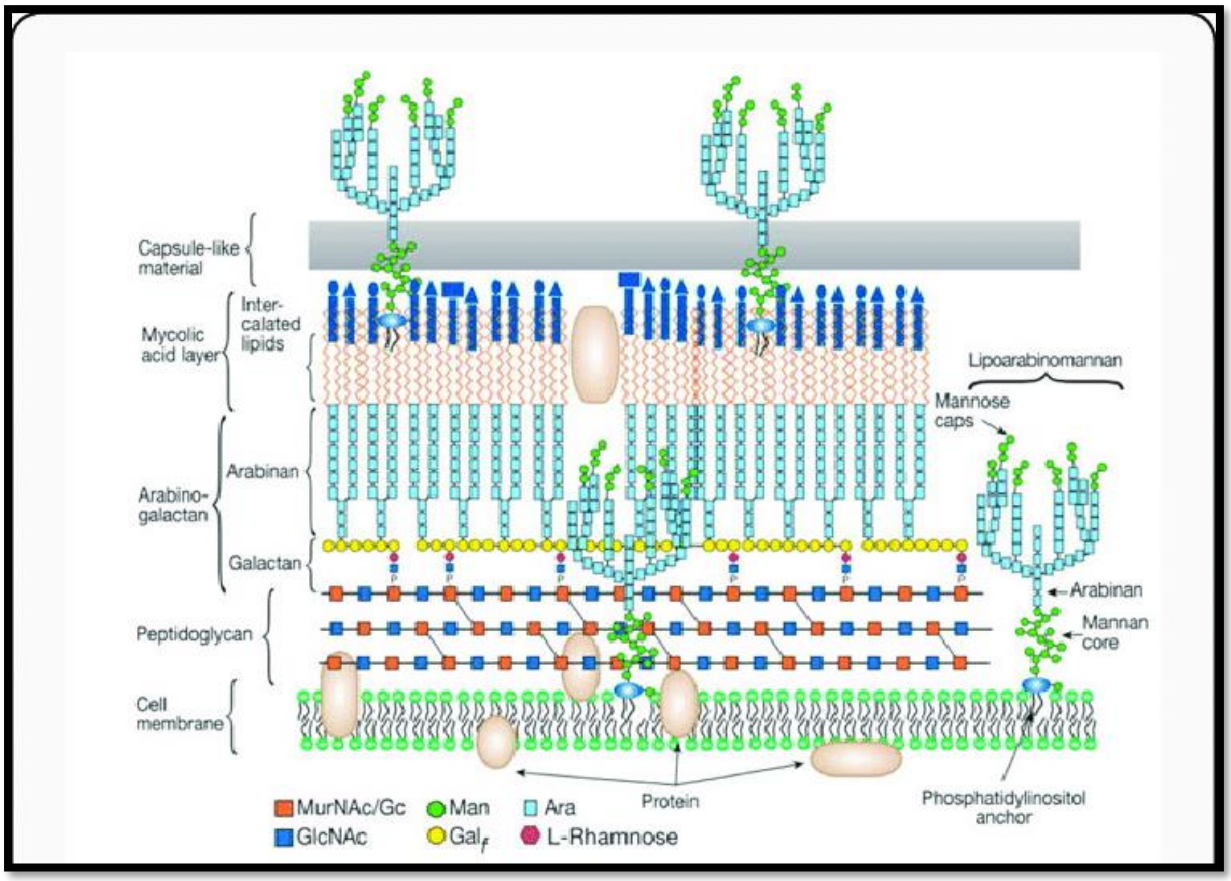


Fig 1: Cell wall of Mycobacterium [10]

A phosphodiester bond forms a covalent connection between the chemotype four peptidoglycan and mycolylarabinogalactan (mAG), which is very distinctive part of the mycobacterial cell wall. The fundamental structure or framework of the appropriate wall is made up of mAGP. It is built up of a large mycolic acids complex (X-90 branching fatty acids), which are found as tetramycolylpentaarabinofuranosyl clusters and are covalently bonded[11].

2.3 *Mycobacterium tuberculosis*

Among the infectious diseases, tuberculosis (TB) remains one of the deadliest and is one of the oldest illnesses ever documented in human history[12]. Koch's bacillus, or *M. tuberculosis* (MTB), that is a non-motile and it is rod in its shape bacterium that has a distinct waxy cell wall that is abundant in mycolic acids. Because of its waxy covering, MTB cannot be identified

using conventional Gram staining methods; instead, specialist acid-fast staining techniques such as Ziehl-Neelsen or fluorescence stains are used[13]. As an obligatory aerobe that needs a lot of oxygen, MTB hs ability to live in the low oxygen level of the lungs, which is where the infection primarily occurs. After inhaled droplets of infection, MTB settles inside macrophages, the immune system's first line of defense.

But MTB has a plethora of virulence factors up its sleeve that allow it to avoid immune destruction and survive for long stretches in the host. This typical slow-moving chronic infection can develop into active tuberculosis (TB) illness, which manifests as fever, cough, weight loss, and nocturnal sweats. Remarkably, more then quarter of the world's population is thought to carry a MTB infection, in which the germs are dormant but do not actively cause illness. These people are susceptible to acquiring active tuberculosis should their immune systems be compromised by HIV infection, malnourishment, or certain drugs[14].

The World Health Organization (WHO) 2022 Global Tuberculosis Report states that *M. tuberculosis* (MTB) continues to pose a serious danger to international health. 10.6 million cases of tuberculosis were reported in 2021; of those cases, 1.4 million people died from the illness. These figures demonstrate the continued prevalence of tuberculosis, especially in low- and middle-income nations. The research highlights the problem of drug-resistant tuberculosis strains, noting that in 2021 there will be an estimated 456,000 additional cases of resistance to the important first-line medication rifampicin[15].

TB may be prevented and treated. Pyrazinamide, Isoniazid, ethambutol, and rifampicin are the 4 first-line medications that were developed around 60 years ago and are used for six months as part of the current chemotherapy for drug-susceptible tuberculosis. Treatment for extensively drug-resistant (XDR) and multidrug-resistant (MDR) tuberculosis (TB) lasts around two years, although it has significant toxicity and only modest success rates. The release of the MTB genome in 1998 facilitated the advancement of TB medication research and development. The only vaccination approved to protect infants from developing severe TB illness is BCG, which was created over a century ago [30]. On the other hand, the M72/AS01E vaccine candidate's phase-II study has produced encouraging findings.

There are four species that make up the *M. tuberculosis* complex:, *M. bovis, M. tuberculosis, M. microti,* and *M. africanum*. Although *M. bovis* is present in the cattle, it may infect people and create a disease similar to TB[16]

2.3.1 Anti-Tubercular Drug Resistance

The quantity of *M. tuberculosis* strains which are resistant to the one or more then one anti-tubercular medications is also significant[17]. Patients who were previously untreated and newly infected are now presenting with resistant strains. Over one-third of these newly acquired infections in some parts of the world are caused by isolates that are resistant to at least one anti-tubercular medication[18]. Twenty percent of 17,600 isolates in a collaborative CDC-WHO research from 2002–2004 using a global network of TB labs were multi-drug resistance, and two percent were severely drug resistant (resistant to more than three classes of anti-tubercular medicines). This makes treating TB extremely difficult, if not impossible, particularly in an area with few resources[19].

2.4 Non-tuberculous mycobacteria

True pathogens, opportunistic pathogens, colonizers, and contaminants are all included in this category of species. Based on how quickly these creatures develop, two major types may be distinguished[20]. While the fast-growing mycobacteria grow on solid medium in 72 hours to 1 to 2 weeks, the slow-growing mycobacteria take anything from 1 to 12 weeks to show signs of growth[21].

NTM are found in all parts of the environment including natural source of water, soil, and domestic water. Four separate clinical syndromes are used to categorize human disease caused by NTM: disseminated disease, cutaneous disease, lymphadenitis, and chronic pulmonary disease[22]. Among these, chronic lung disease is the condition that is clinically encountered the most frequently. NTM can induce pulmonary illness in susceptible persons, characterized by persistent cough, sputum production, and lung infiltrates, even if they are widely distributed in the environment.

Globally, NTM pulmonary disease incidence and prevalence have been rising, which is explained by an aging population with more comorbid conditions and advancements in laboratory diagnostics[23]. The ATS (American Thoracic Society) and the BTS (British Thoracic Society) have recommendations for the diagnosis and treatment of NTM pulmonary illness. Depending on the system implicated, the severity of the illness, and the NTM species identified, there are differences in the clinical presentation of NTM pulmonary disease. NTM often affects the lungs, resulting in pulmonary infiltrates, sputum production, and persistent cough. But other organs can also be affected, including the skin, soft tissue, bones,

gastrointestinal system, lymph nodes, and bone marrow. Additionally, there might be widespread illness in several organ systems[24].

Because NTM pulmonary illness is not reported as a public health concern, positive specimens may indicate colonization rather than disease, and ATS/BTS criteria must be used for diagnosis, it is difficult to estimate the frequency of non tuberculous mycobacterium pulmonary disease. On the other side, data from Ontario, Canada, the United States, and Northern Australia have indicated rising trends in the isolated prevalence of NTM. Few research have been done to confirm that NTM occurs in South Africa, even if the country's workforce includes gold miners, despite this growing trend worldwide[25].

Despite the best prescribed regimen medicines, the treatment results for NTM pulmonary illness are dismal; after 12 months, non-cavitary disease patients achieve rates of 40–55% sputum conversion[26]. An aging population with more coexisting conditions and advancements in laboratory diagnostics have been interlinked to the growth in NTM pulmonary disease. However, it is challenging to accurately assess the prevalence of this illness within some parts of the world due to the lack of data, notably in sub-Saharan African nations[27].

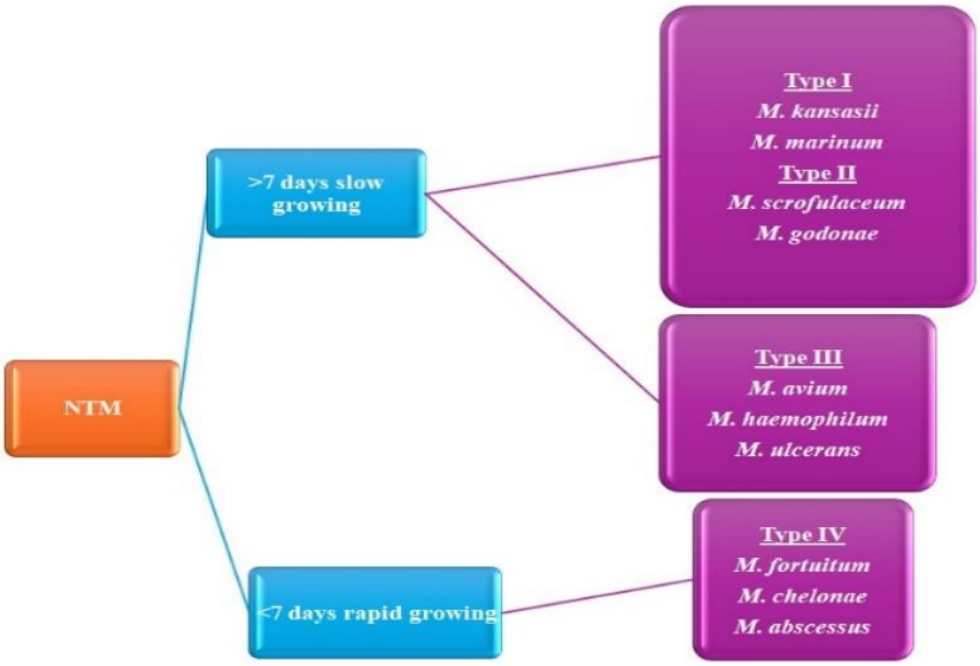


Fig 2: Classification of Non-tuberculous mycobacteria[28].

2.5 Epidemiology of NTM

In the global context, there has been a noticeable rise in NTM infection cases over the past few years. The number of vulnerable people, the advancement of laboratory detection methods, the rise in NTM infection awareness, and the growth in NTM source of infections can all be related to the increase. The epidemiology of mycobacterium which do not cause tuberculosis infections is difficult to describe because of various obstacles, includes the constant presence of non-tuberculous mycobacterium in the environment, This obscures the importance of a positive culture in patients, the appearance of unclear symptoms, and the indifference to NTM infections[29].

Given that the incidence of the non tuberculous infections are becoming more common annually, the situation regarding NTM infections in Europe is dire. Throughout European area, *M. gordonae, M. avium, M. xenopi, M. fortuitum,* and *M. intracellulare* have been recognized as the most common non tuberculous species. Additionally, Roax et al. in France found that individuals with cystic fibrosis had a greater frequency of NTM infections. An incidence rate of 18.9 and an outpatient and inpatient rate of 8.1 per 100,000, respectively, were discovered in a 68-month of Greek epidemiological data set.*M. marinum* infections were more common in extrapulmonary samples, although *M. avium* infections in pulmonary samples are quite widespread in Denmark.

Among the pathogenic NTM like *M. fortuitum, M. avium,* and *M. abscessus* were determined to be the most common species in Brazil. Comorbidities in positive HIV patients have been documented in nosocomial infections brought on by NTM. The isolation of NTM species from lung illnesses, such as *M. avium* complex, *M. kansasii,* and *M. fortuitum,* was evaluated by Costa et al.. According to further data from 1993 to 2011, *M. avium* complex, *M. kansasii, M. fortuitum* , and *M. abscessus* were the species that were isolated more frequently[30].

Numerous Indian papers highlight the significance of NTM infections. Given that TB is common in India, NTM infections have not received the attention they merit. According to the American Thoracic Society's criteria, NTM has garnered attention; several studies have evaluated the incidence of NTM in different Indian states.There is no information available on the epidemiology of NTM infections in any state. *M. fortuitum* was shown to be one of the most common NTM between extrapulmonary TB samples in a Mumbai research. There have also been reports of NTM-related eye infections in Southern and Eastern India.

Study examined the rising incidence of NTM in India and emphasized the issue of NTMs being more resistant to drugs. 13% of the isolates in a Himachal Pradesh research conducted between June 2013 and June 2014 on pulmonary and extrapulmonary samples were found to be NTM. The report recommended monitoring the presence of NTM in diagnostic samples.

The research cautioned against disregarding NTM as potential pollutants. Immunocompromised people are not the only ones who can have NTM infections. Immunocompetent people have also been documented to suffer musculoskeletal infections caused by NTM.

2.6 *Mycobacterium fortuitum*

One species of nontuberculous mycobacteria (NTM) that proliferates quickly and is found in all environments is *M. fortuitum*. Water, soil, and a variety of artificial settings including sewage, tap water, and medical equipment all contain it[29]. Human infections caused by *M. fortuitum* are well-known, especially in cases when the victim has had invasive surgical operations or has a weakened immune system. The study suggested that the presence of *M. fortuitum* infection for a strain that was isolated from a patient who had a cutaneous abscess following an injection[30]. When grown on solid media, this bacterium grows more quickly than other mycobacteria, usually producing colonies in 3–7 days.

Additionally, all mycobacteria have the trait of being acid-fast, which means that the stain is retained even after being exposed to acid. Skin infection, soft tissue infections, lung infections, disseminated infections, and postsurgical infections are just a few of the ways that *M. fortuitum* infections might appear[31], [32]. *M. fortuitum* mostly causes skin infection and soft tissue infections following severe injuries, surgeries, or aesthetic operations. These infections may show up as cellulitis, nodules, ulcers, or abscesses. They are often confined, but if they are not immediately and properly treated, they may spread. Although *M. fortuitum* pulmonary infections are uncommon, they can happen to those with weakened immune systems or pre-existing lung diseases. Chest discomfort, dyspnea, and coughing are some of the symptoms that these illnesses may cause[32].

Although they are uncommon, *M. fortuitum* infections that spread widely can be fatal, particularly in those with weakened immune systems[33]. These infections happen when the bacteria infects other organs and tissues after first spreading to the original location. Depending on the organs affected, disseminated *M. fortuitum* infections can cause a variety of symptoms, such as fever, weight loss, exhaustion, and symptoms unique to that organ[34].

M. fortuitum can cause postsurgical infections after invasive treatments including surgery, injections, or the implantation of medical devices. The bacteria can lead to localized or systemic illnesses by contaminating surgical sites or medical equipment. Sterile method rigorous adherence, appropriate cleaning of medical equipment, and post-procedure patient monitoring are necessary for the prevention of postsurgical *M. fortuitum* infections.

2.7 Taxonomy

M. fortuitum is a member of the Actinobacteria phylum's *Mycobacterium* genus, which is a member of the Mycobacteriaceae family[35]. The genus *Mycobacterium* has a broad variety of bacteria, including harmful and non-pathogenic species[36]. To differentiate itself from slower-growing mycobacteria like *M. tuberculosis*, *M. fortuitum* is categorized as a quickly growing nontuberculous mycobacterium (NTM) within this genus. Numerous factors, such as genetic sequencing, the makeup of the cell wall, metabolic parameters, and development characteristics, are taken into account in this categorization[37]. Based on genetic distinctions and phenotypic variances, *M. fortuitum* is further divided into subspecies. Within the species, subspecies like *M. fortuitum* subspecies and *M. fortuitum* subspecies peregrinum have been found[35], [38].

2.8 Epidemiology of *M. fortuitum*

M. fortuitum is very common environmental bacterium that may be found in water and soil all around the world. Since *M. fortuitum* is so widely distributed, it is challenging to identify particular endemic locations; infections have been observed in Europe, North America, Asia, Africa, and Australia.

Compared to *M. avium* complex (MAC) or *M. abscessus* complex (MAB), *M. fortuitum* is thought to be a less frequent cause of NTM (non-tuberculous mycobacteria) infections. But it seems to be becoming more common, especially in immunocompromised people and those with underlying medical issues. Research points to a connection between medical tourism and the development of postoperative wound infections in travelers to the fast spreading NTM cases, such as *M. fortuitum*.

Although the exact mode of M. fortuitum transmission is unknown, it is not often person-to-person. Inhaling polluted aerosols from soil or water sources is probably the main pathway. Tattoo ink that hasn't been adequately sanitized or manicure salon whirlpools are two examples of contaminated surroundings that have been connected to outbreaks. After surgery, M.

fortuitum can also result in opportunistic infections, especially when implanted devices like catheters or prosthesis are involved.

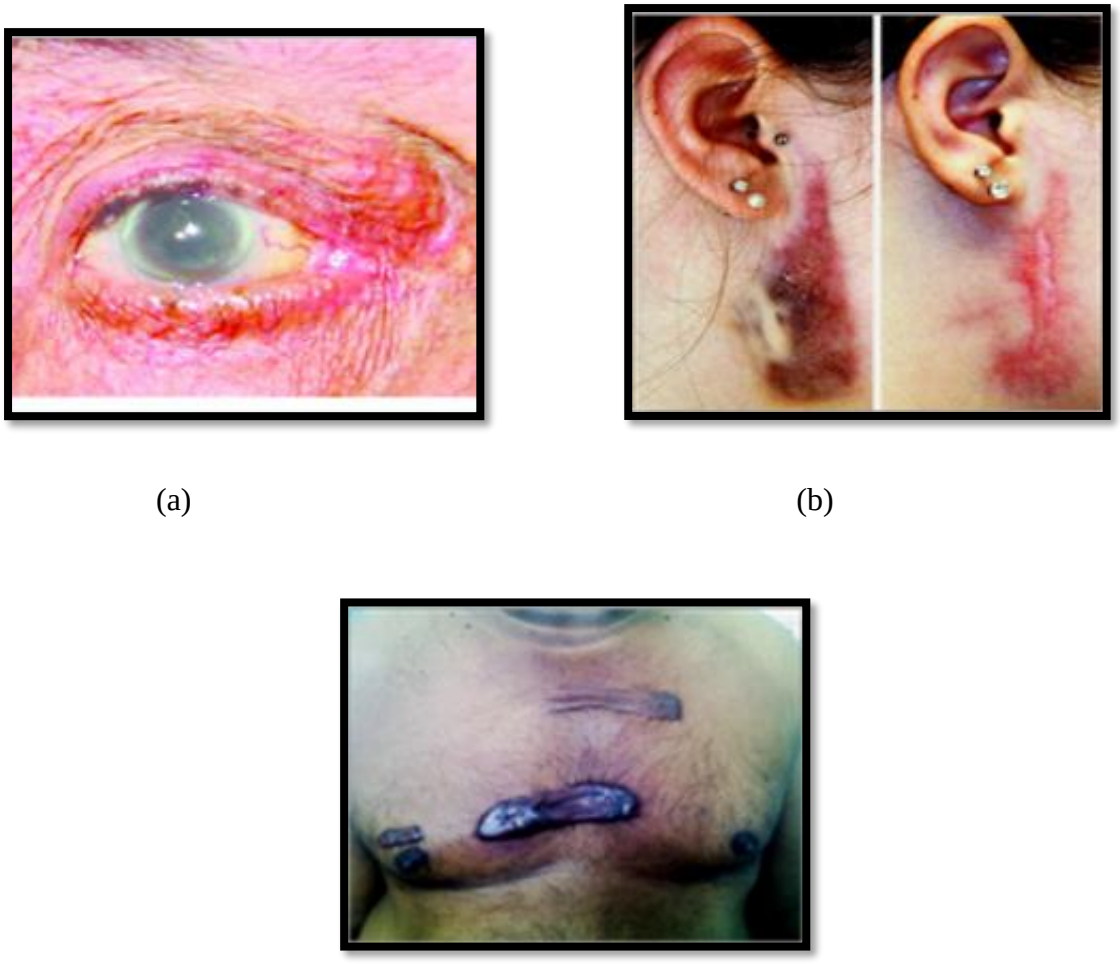
2.9 Pathogenesis of *M. fortuitum*

Relapses are prevalent, clinical diagnosis is difficult, and *M. fortuitum* is not well characterized. It also does not respond to common anti-tubercular therapy and it is developing antibiotic resistance. *M. fortuitum* is also responsible for 50–85% of surgical and post-surgical wound infections along with infections linked to RGM-associated catheters. It is the primary cause of skin disorders that come back. It can also causes broad extrapulmonary nosocomial infections, like post-thoracotomy sternal osteomyelitis, infections following cardiac bypass and augmentation mammaplasty surgeries, intermittent wound infections after the plastic surgery, and abscesses from electromyography needle injection in soft tissue and the skin.

The main source of *M. fortuitum* outbreaks linked to healthcare facilities is contaminated ice machines. Leg-furunculosis is caused by *M. fortuitum* in individuals who have used public footbaths. There have also been reports of *M. fortuitum*-related cervical lymphadenitis and keratitis, which are linked to corneal infections following laser surgery. The *Mycobacterium* is one of the main causative agents of pulmonary NTM illnesses and can also infect the central nervous system. Moreover, it results in widespread infections in those with impaired immune systems. Mycobacteriosis, bronchiectasis, and cystic fibrosis are risk factors for lung infections which is caused by the *M. fortuitum*.

2.9.1 Infection caused by *M. fortuitum*

It is a wide variety of illnesses that can be caused by *M. fortuitum*. Both pulmonary and extrapulmonary infections can result from *M. fortuitum*. It can also cause skin infection and soft tissue infection. Some representative pictures of *M. fortuitum* infections are as shown in Fig: 3 below:



(a) (b)

(c)

Fig 3: This figure shows (a) Eye infection caused by *M. fortuitum* and (b), (c) shows the skin infection caused by *M. fortuitum*[39].

2.9.2 Soft tissue and Skin infection

It is now recognized that *M. fortuitum* is one of the bacteria linked to infections of the skin and soft tissues. After surgery or trauma and skin infection, it can spread to the lungs, bones, lymph nodes, meninges, and joints. In those who has weak immune systems, this can often prove fatal. Breast infections that result from breast implants are often caused by *M. fortuitum*. They allow *M. fortuitum* to penetrate puncture wounds, which can lead to a variety of conditions such as cellulitis, abscesses, ulcers, and even sinus tract drainage. There will be a need for an estimated 331.5 billion surgical procedures to address the amount of illnesses addressed by surgery

globally. *M. fortuitum* is one of the bacteria which is linked to surgical procedures. Moreover, no mycobacterial culture was performed at the time of diagnosis[40].

It is also important to take into account the need for long-term antibiotic medication when diagnosing non-transplantable mycoses (NTMs) in order to treat infections. Healthcare professionals who treat patients with persistent skin or soft tissue infections, particularly those with compromised immune systems or those who have recently undergone surgery, have to be on the lookout for mycobacterial infections that are spreading quickly. More research is necessary to determine the ideal length of treatment and to compare different regimens.

2.9.3 Blood stream infection

Blood samples from people with autoimmune diseases, cancer, gastrointestinal issues, intravenous drug use, chronic kidney disease, and sickle cell anemia have been shown to contain *M. fortuitum*. *M. fortuitum*-related blood-borne infections can be treated with the use of early species detection in blood and testing for antimicrobial resistance[41]. The IDSA (Infectious Diseases Society of America) has constituted strict diagnostic standards for blood borne infections brought on by a variety of bacteria, including NTM, as well as catheter usage.

The length of the time needed to culture of the bacteria and the fluctuating time it takes for NTM to provide positive findings are significant obstacles within the identification of NTM infections in bloodborne due to catheter use. A combination of at least two active antimicrobials administered for at least four weeks, together with removal of the intravascular catheter for treatment, was described in a case report of blood infection associated to an intravenous catheter[42].

2.9.4 Bone infection

Numerous bone infections, including osteomyelitis and otitis media, have been linked to *M. fortuitum* as a result of medical procedures such trauma, stem cell transplantation, and surgery. Documentation of *M. fortuitum*-caused osteomyelitis in spinal in intravenous drug users is also available. There are additional case reports of *M. fortuitum* co-infections with *M. tuberculosis* and *M. chelonae* in individuals with osteomyelitis. Treatment takes around six weeks for *M. fortuitum* infections in bone and may involve surgery. Dual infections with *M. tuberculosis* and *M. fortuitum*, on the other hand, require lengthier medication therapy, lasting approximately nine months [47].

2.9.5 Pulmonary infection

Most *M. fortuitum* pulmonary infections occur in patients who have had prior lung infections, such as tuberculosis or cystic fibrosis. *M. fortuitum*-caused lung infections can also be attributed to gastro-esophageal infections [45]. 10% of *M. fortuitum* lung infections were mistakenly identified as multidrug-resistant M. TB infections, according an Indian report. One of the common NTM in India, according to epidemiological research of the regularity of non tuberculous mycobacterium among suspects of lung infections, is *M. fortuitum*. An analogous research from Iran has been reported, in which It was demonstrated that *M. fortuitum* was the most common non-therapeutic mushroom found in individuals who may have had TB.

2.9.6 Lymph node infection

Infectious agents such as *M. fortuitum* have been linked to cervical lymphadenopathy in studies. Few case reports identify that lymphadenitis can cause by the *M. fortuitum*, largely because it is associated with a patient's immunosuppressed state. One such case report connected *M. fortuitum* lymphadenitis to weakened immunity resulting from infection with the varicella-zoster virus. In an epidemiological investigation, *M. fortuitum* was identified as one from the many mycobacteria which is responsible for the rapid proliferation of cervico-facial lymphadenitis[46].

2.9.7 Clinical significance of NTM

The three main SGM species are, *M. kansasii, M. marinum*, and *M. avium* complex. *M. intracellulare*, which is part of the *M. avium* complex, is thought to be the most prevalent culprit responsible for infections in NTM [43]. But RGM species, such as *Mycobacterium abscessus* complex (*M. abscessus* subsp. *M. massiliense, M. Bolletii*, and *M. abscessus*), *M. chelonae* complex (*M. chelonae, M. franklinii, M. saopaulense,* and *M. immunogenum*), and *M. fortuitum* complex (*M. fortuitum, , M. brisbanense,* and *M. houstonense, M. neworleansense*) are becoming more and more significant as the primary agents that can cause diseases in humans[46].

Human disorders such skin infections, implant-associated infections, disseminated diseases in immunocompromised people, and superficial lymphadenitis are mostly caused by NTM. Pulmonary infections are responsible for 80–90% of NTM-associated illnesses . Incidence rates for TB and NTM disorders decreased by 81% and 94%, respectively, between 1946 and 2014, according to a thorough investigation of the link between the two of diseases. The majority of

NTM are increasingly emerging as human pathogens, which is the cause of the elevated rate of NTM infection.

Mycobacteria's acid-fast nature makes it impossible to distinguish between different strains of the bacteria by primary staining and microscopy [44]. As a result, techniques for NTM differential diagnosis are being developed more and more to determine the optimal course of medication for the early management of NTM infections and the decrease of morbidity and mortality. The best techniques for differentiating between MTB complex and/or between strains of NTM are smear microscopy, specimen culturing on solid or liquid medium, biochemical tests (niacin reduction and catalase, iron uptake, arylsulfatase test), and molecular techniques like phylogenetic analysis[48].

2.10 Treatment of *M. fortuitum* infections

Tobramycin, amikacin, ciprofloxacin, levofloxacin, imipenem, clarithromycin, cefoxitin, linezolid, and sulfamethoxazole are among the drugs that can harm *M. fortuitum*. Because of acquired mutational resistance to quinolones, *M. fortuitum* infections cannot be treated with quinolone monotherapy (clarithromycin); instead, quinolones must be used in conjunction with another antimicrobial drug. However, *M. fortuitum*'s in-vitro resistance to macrolides is caused by the presence of an inducible erythromycin methylase (erm) gene; this makes it a crucial factor to take into account when choosing the precise anti-mycobacterial medication combination for chemotherapy[49].

Additionally, research is being done on novel drugs for the treatment of *M. fortuitum* infections. Tigecycline has anti-*M. fortuitum* action. However, because of its negative consequences, its clinical usage is still not approved. Disulfiram is active against *M. fortuitum* in a bactericidal manner. Additionally, d-cycloserine has emerged as a potential treatment for *M. fortuitum*, either by itself or in combination with clarithromycin.

2.11 Current status of drugs against NTM

A random clinical trial revealed that over 90% of patients receiving chemotherapy experienced adverse reactions. As a result, patient education is necessary for the management of adversities in order to decrease the risk of treatment and increase the likelihood of treatment completion. Although NTM is widely distributed worldwide, there are currently no vaccines against the disease. The treatment and outcomes of NTM diseases vary depending on the species of NTM.

The therapeutic antibiotic regimen used to treat infections linked to the *M. avium* complex consists of amikacin, ethambutol, macrolides, and rifamycin (also known as rifabutin or rifampicin). For *M. kansasii* infections, the current standard of therapy consists of an isoniazid, ethambutol, and rifampicin regimen. Since rifampicin resistance in *M. kansasii* isolates raises the risk of treatment failure, rifampicin susceptibility testing is necessary for every isolate. Second-line antimicrobials (trimethoprim-sulfamethoxazole, moxifloxacin, amikacin, doxycycline, ciprofloxacin, rifabutin, and linezolid) are evaluated if the tested *M. kansasii* isolates are resistant to rifampicin. At least two active medicines, such as rifampicin combined with ethambutol, doxycycline, clarithromycin, and trimethoprim-sulfamethoxazole, are used in the treatment regimen for *M. marinum* [44].

Novel antibiotics, including clofazimine (a class of antibiotics containing riminophenazine), bedaquiline (a diarylquinoline), delamanid (a novel antibiotic), tedizolid (a compound from the xazolidinone family), omadacycline (a novel tetracycline analog), and tigecycline (a tetracycline derivative), have demonstrated efficacy against a number of non-targeted microorganisms and could serve as valuable alternatives to traditional antibiotic treatment [41].

2.12 Mycobacterial Biofilm formation

The intricate process of mycobacterial biofilm production is essential to the pathogenicity and endurance of mycobacterial species[51]. Mycobacteria connect to surfaces in a sequence that results in the development of biofilm cells through the stacking of aggregates. This process is known as biofilm formation[52]. The biofilm begins to disperse once it reaches a particular thickness, only to re-aggregate at other locations. Mycobacteria are able to form robust colonies that are hard to remove because to this dynamic mechanism. Studies have demonstrated that mycobacteria of both pathogenic and non-pathogenic species are capable of forming biofilms. By serving as barrier layers, these biofilms can help pathogenic mycobacterial species avoid the immune system of their hosts and withstand antibiotic therapy[53]. Developing successful tactics to address mycobacterial infections requires an understanding of the mechanics and distinctive properties of mycobacterial biofilms[54].

Numerous elements, such as the impact of nutrients, ions, and carbon sources on bacterial activity, have been shown in studies to have a role in the production of mycobacterial biofilms. Furthermore, several molecules have been shown to be necessary for the first surface attachment and biofilm formation in mycobacteria, including glycopeptidolipids and mycolic

acids. The versatility and durability of mycobacteria in a variety of situations is demonstrated by their capacity to form biofilms utilizing minimum nutritional sources, such as tap water[55].

2.12.1 *M. fortuitum* biofilm

It has been observed that *M. fortuitum*, a mycobacterial species that grows quickly, may create extensive biofilms in as little as 48 hours. These biofilms are essential to *M. fortuitum 's* pathogenicity and persistence, since they help the bacterium fight antibiotics and elude therapy[56]. *M. fortuitum* forms biofilms by colonizing surfaces and aggregating into thick layers that function as a barrier against external agents such as disinfectants and antibiotics[55].

Research has demonstrated that biofilms constitute an ideal habitat for bacteria, offering them the best available nutrition, less competition from other microorganisms, and defense against environmental pollutants and predators[57]. Mycobacteria's ability to exist in biofilms has been linked to their resistance to germicides, partly because of their propensity to produce biofilms. The distinct structure of the mycobacterial cell wall, some pathogenic mycobacteria's capacity to live inside phagocytic cells, and the quick mutation of target molecules are assumed to be the causes of this resistance[58].

To effectively address mycobacterial infections, it is important to understand the mechanisms behind *M. fortuitum's* biofilm production. Mycobacteria thrive in the protected habitat that biofilms offer them, making treatment and eradication attempts more difficult[59]. To improve our understanding of mycobacterial diseases and create focused strategies to address drug tolerance and antibiotic resistance linked to biofilm formation, more investigation into the dynamics of *M. fortuitum* biofilm development is needed[60]. The biofilm of Mycobacteriosis reveals heightened resistance to common disinfectants as well as common medicines used to treat the disease. *M. fortuitum* biofilm can quickly infect clinical and industrial environments due to its increased resistance to biocides such sodium hypochlorite, sodium hypochlorite, phenol, silver nitrate, hydrogen peroxide, and glutaraldehyde.[57].

Using an eight well chamber slide model and a dynamic flow cell chamber, the study assessed that the architect of *M. fortuitum* biofilm and demonstrated its resistance to the antibiotics tobramycin, gatifloxacin, and amikacin. DNase treatment, on the other hand, was successful in decreasing the bulk of the biofilm, indicating that eDNA was structurally important for the maintenance of the *M. fortuitum* biofilm. Atypical mycobacterial keratitis is mostly caused by *M. fortuitum*, and this is because of the development of biofilms that are becoming more resistant to conventional medications[61].

2.13 Stress related studies on *M. fortuitum*

Numerous research on *M. fortuitum* have examined the bacterium's capacity for adaptation and survival in a range of stressful environments. According to a research, the rimP gene helps *M. fortuitum* survive in detergent- and hypoxic-stressed environments. The rimP gene is engaged in this process, which is essential for the bacterium's survival in various conditions, according to the study, which employed TnphoA random mutagenesis to discover genes relevant for adjusting to acidic stress in *M. fortuitum*. The rimP gene was found to be a major participant in this process by the study, underscoring its importance in the bacterium's capacity to endure a variety of stressful environments. The results highlight the role of the rimP gene in *M. fortuitum*'s environmental adaptability and resilience, providing insight into the processes underlying the bacterium's ability to flourish in the face of stressors such as detergent exposure and hypoxia[62].

An investigation of in vitro stress survival along within vivo infection of an acidic-susceptible mutant of *M. fortuitum* was done [63]. *In vitro* acidic stress adaptation in *M. fortuitum* was studied with the goal of identifying the genes involved. Acid resistance genes are essential for the survival of the bacterium, as evidenced by the results, which demonstrated that the mutant strain of *M. fortuitum* was more vulnerable to acidic stress than the wild-type strain. The mutant strain MT727, which had a mutation in the ribosomal maturation factor expressing gene rimP, was shown to be more vulnerable to acidic stress than the wild-type strain. The study used TnphoA random mutagenesis to discover genes involved for adapting to acidic stress in *M. fortuitum*. The virulent behaviour and growth behaviour of the mutant strain under various in vitro stress conditions, including as heat stress, hypoxia stress, and detergent stress, were also examined in the work. In comparison to the wild-type strain of *M. fortuitum*, the results demonstrated that the mutant strain displayed poor growth behaviour under detergent and hypoxic stress conditions.

The growth, development and survival of mutant *M. fortuitum* strains under stress conditions was examined in comparison to the wild type bacteria. The study discovered that under several stress circumstances, like hypoxia stress, detergent stress, and acidic stress, the recombinant strains of *M. fortuitum* had distinct growth patterns. According to the study's findings, the *M. fortuitum* genome has a number of genes related to survival and stress response, which are essential for the bacterium's ability to persist in a variety of settings[63].

The results of the investigation showed that the *fabG4* knockdown strain has reduced cell surface hydrophobicity, which suggests that *M. fortuitum*'s capacity to induce intracellular infections under stressful circumstances has been lessened. This study clarifies the function of *fabG4* in *M. fortuitum*'s virulence and stress response, offering important new information on possible targets for reducing such infections. The relevance of *fabG4* in *M. fortuitum*'s stress response and survival was further demonstrated by the study's discovery that the knockdown strain had worse survival under in vitro nutritional deprivation stress than the wild-type strain. By shedding light on the processes that allow *M. fortuitum* to flourish in the face of stressors like nutritional scarcity, this work advances our knowledge of possible targets for the management of mycobacterial infections. The work emphasizes the role that *fabG4* plays in *M. fortuitum*'s virulence and stress response, indicating that it may be a useful target for reducing mycobacterial infections. The results of this investigation provide light on the processes that allow *M. fortuitum* to flourish in the face of stresses such as food scarcity and offer important new avenues for the treatment of mycobacterial diseases[64].

2.14 Clinical significance of *M. fortuitum*

Common environmental organisms that are frequently isolated from natural soil, tap water, and surface water are non-tuberculous mycobacteria. Because of this, the occurrence of NTM lung sickness cannot be supported by the isolation of NTM species sample from a respiratory tract alone[65]. Four separate clinical syndromes are used to classify human disease caused by NTM infection: disseminated disease, cutaneous disease, lymphadenitis, and pulmonary disease. Chronic pulmonary illness is the most prevalent localized clinical condition among them[66]. There are people who have NTM infection yet show no signs of lung illness. An infection like this might be a transitory illness or a sign of colonization. As a result, microbiologic, radiographic, and clinical criteria are required for the diagnosis of NTM lung illness[67]. In several nations, *M. fortuitum* is a frequently isolated organism from the respiratory specimens within clinical labs.However, not much research has been done on this organism's clinical value to far. We thus set out to examine the potential clinical relevance of *M. fortuitum* found in respiration specimens[68].

Research indicates that individuals with underlying lung disorders, such as interstitial lung disease, old pulmonary TB, lung cancer, and other non-tuberculous mycobacterial (NTM) pulmonary illnesses, are at an increased risk of developing *M. fortuitum* pulmonary infections. Since the presence of *M. fortuitum* is frequently seen as evidence of colonization or temporary

infection, the majority of patients with the bacteria found in respiratory specimens do not necessarily require long-term antimicrobial medication[65].

Despite ongoing *M. fortuitum* lung illness detection in respiratory tracts, most individuals in a study did not require long-term antibiotic medication. Nonetheless, people with gastric reflux illness may develop a real pulmonary infection, and little is known about the disease's natural course and diagnostic criteria for a true *M. fortuitum* pulmonary infection. A case report of a patient who presented with migratory infiltrates and had a difficult *M. fortuitum* lung infection due to persistent aspiration revealed that the patient experienced refractory pneumonia even after receiving appropriate antibiotic treatment. Following the identification of granulomatous lesions, a remarkable recovery with no aggravation for over 5 years was observed with various antibiotic treatment for *M. fortuitum* [69].

In a different investigation, a young, healthy man had lung mycobacteriosis brought on by *Mycobacterium peregrinum*, a species that is closely linked to *M. fortuitum*. The patient complained of coughing and chest discomfort, and molecular and cultural tests verified the diagnosis. After receiving a round of antibiotics, he made a noticeable recovery.

A patient with COPD (chronic obstructive pulmonary disease) was the subject of a case of *M. fortuitum* pulmonary infection, according to another investigation. The patient had a persistent cough and sputum production; a bronchoscopy and culture confirmed the diagnosis. After receiving a round of antibiotics, the patient made a noticeable recovery. Studies show how important it is to use suitable diagnostic procedures and treatment plans in patients with underlying diseases of lungs like COPD, as well as the clinical relevance of pulmonary infection of *M. fortuitum*. The possibility of medication-induced toxicity and the occasional requirement for surgery make managing an *M. fortuitum* lung infection difficult[70].

2.15 Role of *fabG4*

2.15.1 *Mycobacterium* Species

The 3-oxoacyl-(acyl-carrier-protein) reductase enzyme is involved in the fatty acid production pathway, is encoded by the *fabG4* gene. Through its roles in fatty acid metabolism, cell wall construction, antibiotic resistance, and pathogenicity, the *fabG4* gene in *Mycobacterium* species may be fully understood. In mycobacteria, the elongation cycle of fatty acid production involves the *fabG4* gene. In order to produce long-chain fatty acids, it catalyzes the reduction of 3-ketoacyl-ACP intermediates to 3-hydroxyacyl-ACP. These lipids serve as building blocks

for the synthesis of mycolic acids, which are crucial components of mycobacteria's cell membrane. Maintaining the integrity, permeability, and resistance of cell walls against external stresses like antibiotics and host immune responses requires mycolic acids[71].

In *Mycobacterium* species, the *fabG4* gene adds to the variety of mycolic acid structures. The manufacture of mycolic acids, which are long-chain fatty acids with distinct structural properties present in the cell wall of mycobacteria, is one of *fabG4*'s primary roles. Mycolic acids have a crucial role in the cell envelope's stiffness, hydrophobicity, and impermeability, which shields the cell from external stressors and host immunological reactions[72]. The length, saturation, and branching patterns of the fatty acid chains integrated into mycolic acids are influenced by the activity of *fabG4*, which in turn affects the cell wall's overall design and characteristics. Different types of mycolic acids, including as alpha-, methoxy-, keto-, and epoxy-mycolic acids, provide the cell wall distinct characteristics. The *fabG4* enzyme's activity affects the length and make-up of the fatty acid chains that are integrated into mycolic acids, which in turn affects the chemical and physical characteristics of the cell membrane[73].

Mycobacteria's methods of antibiotic resistance have been linked to the *fabG4* gene. Modifications within the fatty acid and mycolic acid synthesis pathways, such as mutations or overexpression of *fabG4*, can lead to changes in the content and thickness of the cell wall.

These modifications can impact the susceptibility of mycobacteria to antibiotics like isoniazid and rifampicin. Research has indicated that mutations in *fabG4* can result in resistance to various first-line anti-TB medications, underscoring the gene's clinical significance in drug resistance pathways[74].

The *fabG4* gene also affects the pathophysiology and pathogenicity of mycobacteria. The cell wall of mycobacteria, which is abundant in mycolic acids and other lipid components produced by *fabG4* activity, is essential for intracellular survival, host-pathogen interactions, and immune evasion. The capacity of *Mycobacterium* species to initiate infection, elude host defenses, and advance illness can be impacted by disruptions to *fabG4* function or expression.

Furthermore, *fabG4* has been linked to the control of mycobacteria's drug efflux pathways and cell envelope permeability[75]. Variations in *fabG4* expression or activity can impact the cell envelope's barrier qualities by changing the lipid and cell wall component composition. This change in permeability can affect how nutrients, poisons, and antibiotics are absorbed and expelled from the cell, which helps the bacteria survive and adapt to their surroundings. In *Mycobacterium* species, *fabG4* could be involved in the synthesis of virulence factors and

stress responses. By regulating fatty acid synthesis and cell wall remodeling in response to environmental stressors such oxidative stress, food constraint, or exposure to host immunological responses, *fabG4* expression can help bacteria survive and persist in the environment. Furthermore, the synthesis of virulence factors, cell surface proteins, and biofilm formation—all of which enhance mycobacteria's pathogenicity—can be influenced by *fabG4*-mediated fatty acid metabolism.

Research has also indicated a connection between the control of bacterial cell division, growth and *fabG4*. Fatty acids and mycolic acids produced by the *fabG4* pathway are necessary for membrane biogenesis, mycobacteria's capacity to expand their cell walls, and their ability to divide. Modifications in *fabG4* function or expression can result in abnormalities in the bacterial shape, growth impairment, and cell wall, underscoring its significance in preserving cellular integrity and survival[76].

2.15.2 Gene knock-downs

Reducing the expression of one or more genes in an organism is a step in the gene knockdown process. The technique known as "reverse genetics" looks at a gene's sequence in order to discover its function. An antisense oligo, a short DNA molecule whose sequence matches a gene, is used to produce knockdown strains. Transcription is stopped at the gene where the antisense oligos connect[72].

Gene knockout is a technique that includes manipulating a cell's or model organism's genomic DNA to permanently stop a certain gene from being expressed. Gene knockdown methods like as homologous recombination and site-specific nuclease-mediated knockout damage and deactivate target genes [42].

The cells or model organisms that survive the knockout event are unable to express the functional result of the knocked-out gene. Rather than the gene being physically removed from the genome, the event just requires a frameshift mutation that introduces a stop codon at the transcription start site (5' end of the gene). Consequently, transcription ends downstream of the stop codon and the relevant gene is eliminated from the chromosome[76].

2.15.3 Other bacteria

Important roles of *fabG4* gene are that it is also found in a number of other bacterial species. The fatty acid biosynthesis routes, redox homeostasis, cell wall construction, antibiotic resistance, and virulence mechanisms that these bacteria normally exhibit are all mediated by

fabG4. Fatty acid metabolism is one important function of *fabG4* in non-mycobacterial bacteria. The 3-oxoacyl-(acyl-carrier-protein) reductase enzyme, which is encoded by the *fabG4* gene, catalyzes the conversion of 3-ketoacyl-ACP intermediates to 3-hydroxyacyl-ACP during fatty acid elongation. The production of long-chain fatty acids, which are the building blocks of many cellular components such as lipopolysaccharides, signaling molecules, and membrane phospholipids, depends on this enzyme activity[77].

Moreover, *fabG4* in non-mycobacterial bacteria supports cellular metabolism and redox equilibrium. The *fabG4*-encoded enzyme contributes to the preservation of cellular redox equilibrium by using NADPH as a cofactor in reduction processes. The synthesis of energy, biosynthesis, and response to oxidative stress conditions all depend on this function. *FabG4* affects the content and characteristics of the bacterial cell envelope in terms of cell wall construction. *FabG4* has a role in the synthesis of cell wall components and membrane lipids via engaging in fatty acid synthesis pathways. Cell envelope integrity, permeability, and resistance to environmental stressors, including as antibiotics and host immunological responses, can all be impacted by changes in *fabG4* expression or activity. In Gram-positive bacteria, lipoteichoic acids (LTAs) and other components of the cell wall are formed by the biosynthesis of fatty acids, which is facilitated by *fabG4*. LTAs are critical for virulence, host immunological recognition, and cell wall integrity. Gram-negative bacteria require lipopolysaccharides (LPS) for barrier function and outer membrane construction, and *fabG4* is implicated in this process[78].

The presence of *fabG4* in non-mycobacterial bacteria affects the processes behind antibiotic resistance. Changes in fatty acid metabolic pathways, such as *fabG4* mutations or overexpression, can affect the composition and characteristics of bacterial cell walls and membranes, affecting bacteria susceptibility to antibiotics. Research has demonstrated that *fabG4* mutations can provide resistance to antimicrobial drugs that target membrane integrity or cell wall production. Furthermore, *fabG4* may support the synthesis of virulence factors and pathogenicity in bacteria that are not mycobacterial. The synthesis of lipids, particularly those controlled by *fabG4*, is necessary for the creation of poisons, adhesins, biofilms, and virulence factors. The development of illness, host interactions, and bacterial virulence can all be impacted by disruption of *fabG4*-related pathways[73].

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Microbial Strains

Table 1: Microbial strains

Strain	**Sources**
E. coli DH5α	IMTECH (Institute of Microbial Technology), Chandigarh, India
Bacillus Subtilis	IMTECH (Institute of Microbial Technology), Chandigarh, India
Mycobacterium smegmatis MC^2155	Central Drug Research Institute (CDRI), Lucknow, India
M. fortuitum ATCC 6841	Central Drug Research Institute (CDRI), Lucknow, India
Aspergillus niger	IMTECH (Institute of Microbial Technology), Chandigarh, India

3.1.2 Media

Table 2: Media

Nutrient Agar	HIMEDIA
Nutrient Broth	HIMEDIA
Potato Dextrose Agar	HIMEDIA
Luria Broth	HIMEDIA
Middlebrook 7H9	HIMEDIA

3.1.3 Chemicals

Table 3: Chemicals

Crystal Violet	Loba Chemie
Gram's Iodine	HIMEDIA
Safranin	HIMEDIA
Basic fuschin	HIMEDIA
Methylene Blue	Fisher Scientific
Lactophenol Cotton Blue	Loba Chemie
Glycerol	Merck

Tween 80	SRL
Agarose	HIMEDIA
Antibiotic Powder of Ampicillin	HIMEDIA
Sodium Chloride	Merck
SDS	BioRad
Saturated Phenol	SRL
Chloroform	SRL
Isomyl	SRL

3.1.4 Instruments

Table 4: Instruments

Weighing Balance	Citizon
Autoclave	REFLITECH
Incubator	Thermo Scintific
Incubator shaker	Macflow Engineering
Laminar Air Flow	Rescholar
pH meter	Eutech
Centrifuge	Eppendorf
Vortex mixer	REMI
Water Bath	NSW INDIA
Light microscope	OLYMPUS
4°C storage	Allied Frost
-20°C storage	Blue Star

3.2 Methods

3.2.1 Different Microbiological techniques

3.2.1.1 Simple Streaking

1. The nutrient agar containing petri plate was labelled properly with the name of microorganism, and date.
2. The sterilization of inoculation loop was done on the flame of a Bunsen burner.
3. When the loop got cool an isolated colony was picked by loop from cultured plate.
4. The loop was dragged gently on the nutrient agar plate in a zig-zag pattern.
5. The loop was again sterilized until it became red hot.
6. The plates were kept inverted in an incubator for 24 hours at 37°C.

3.2.1.2 Quadrant Streaking

1. The nutrient agar containing petri plate was labelled properly with the name of microorganism, and date.
2. The sterilization of inoculation loop was done on the flame of a Bunsen burner.
3. When the loop got cool an isolated colony was picked by loop from cultured plate.
4. A smear was prepared at one end of the plate.
5. Apply the inoculum throughout the first quadrant by waving it back and forth.
6. The loop was sterilized again.
7. The plate was rotated 90 degrees and other streaks were made from the end of the previous streak.
8. The same process was repeated three more times.

3.2.2 Gram staining

1. A grease-free, spotless slide was taken.
2. One drop of normal saline was placed on the slide and smear was prepared with a full loop of sample.
3. Slide was kept for air drying and fixed with heat properly.
4. Crystal violet dye was poured on slide and slide was kept for about 30 seconds to 1 minute and then rinsed with normal water.
5. Gram’s iodine mordant dye was poured on slide and kept for 1 minute and again rinsed with water.

6. After 10 to 20 seconds of washing with 95% alcohol, the slide was once more rinsed with water.
7. Counterstain safranin dye was poured and kept for about 40 seconds and washed with tap water.
8. Slide was kept for air drying properly.
9. After that slide was observed under the light microscope.

3.2.3 Fungal staining

1. A grease-free, spotless slide was taken.
2. One drop of Lactophenol Cotton Blue was poured on the centre of glass slide.
3. With the help of inoculating loop the fungus was taken from cultured plate.
4. Cover slip was used to cover the slide.
5. After that, light microscope was used to observe slide.

3.2.4 Ziehl-Neelsen staining

1. A grease-free, spotless slide was taken..
2. One drop of normal saline was placed on the centre of slide and smear was prepared with full loop of sample.
3. The slide was fixed with heat properly.
4. After the slide get dried, it was saturated with carbol fuschin and put on hot plate for 5 minutes.
5. Slide was washed with decolorizer (3% conc. HCl in 96% Ethanol) and then rinsed with water.
6. Cell was counterstained with Methylene Blue for about 45 seconds.
7. Slide was washed with water.
8. Slide was kept for air drying.
9. After that slide was observed under the light microscope.

3.2.5 Isolation of microorganisms from Water sample

1. Sample was taken in sterilized falcon tubes from JUIT campus from tap water.
2. Different Eppendorf tubes was taken and filled with 900µl of Luria broth in each tubes.
3. 100µl sample was taken from the neat and transferred to the one tube and all the tubes were serially diluted simultaneously, for example: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}.

4. From each tubes including neat 100µl of sample was spread on different plates contating nutrient agar.
5. Plates were kept in an incubator at 37℃ for 24 hours.
6. After 24 hours, colony was counted from plates and CFU was calculated.

3.3 Different Molecular biology techniques

3.3.1 Genomic DNA isolation from *E. coli*

1. *E. coli* sample was inoculated in Luria broth and kept for incubation for 24 hours at 37℃.
2. After 24 hours the *E. coli* culture was transferred to the Eppendorf tubes and centrifuged for five minutes at 9,500×g.
3. The Supernatant was discarded and the pellet was kept in Eppendorf tubes.
4. 400µl of SDS lysis buffer was added to the pellet and mixed by pipetting.
5. After mixing the tubes were incubated at 65℃ for thirty minutes in water bath.
6. After incubation 400µl of Phenol and Chloroform (1:1) was added into the tubes.
7. Tubes were centrifuged at 16,060×g, for ten minutes at Room temperature.
8. The upper layer (Aqueous layer) was transferred to the fresh Eppendorf tubes.
9. To the tubes intended for the precipitation of DNA, an equivalent volume of cold isopropanol was introduced.
10. Tubes were kept at -20℃ for 18 hours.
11. Tubes were again centrifuged at 13,684×g for fifteen minutes at 4℃.
12. The supernatant was discarded and the DNA pellet was rinsed with 500µl of 70% ethanol.
13. Eppendorf tubes were centrifuged again at 9,500×g for five minutes (washing with ethanol was repeated for 2 times).
14. The supernatant was discarded and DNA pellets remained there for air drying by tilting the tubes.
15. The pellet was dissolved in autoclaved distilled water.
16. After that tubes were stored in ice for 1 hour.
17. The 0.6% Agarose was prepared for gel electrophoresis.
18. After homogenization of agarose 3µl Ethidium bromide was added to the agarose.
19. The gel was kept in an electrophoresis chamber and 1X TAE was added.

20. The DNA sample was loaded in wells of agarose gel and 100 V of current was provide to gel for run.
21. The bands of genomic DNA were visualized by observing the gel by using Gel Doc system.

3.3.2 Plasmid DNA isolation

1. *E. coli* containing pUC 19 culture was inoculated in luria broth and kept for incubation for 24 hours at 37℃.
2. After 24 hours the *E. coli* containing culture was transferred to the Eppendorf tubes and centrifuged at 9,500×g for five minutes.
3. The pellet was separated from the supernatant and tubes were inverted on tissue paper.
4. 300µl of Solution I (GTE) was added and mixed by tapping.
5. 600µl of Solution II (SDS+ NaOH) was added to the tubes and mixed by slowly tilting the tubes.
6. Tubes were incubated for five minutes at 37℃.
7. 450µl of Solution III (Potassium acetate + Glacial acetic acid) was added and tubes were incubated within the ice for forty-five minutes.
8. Tubes were centrifuged at 4,656×g, for fifteen minutes and supernatant was stored in new tubes.
9. An equal volume of chilled isopropanol was added to the supernatant.
10. Tubes were kept at -20℃ for 18 hours.
11. Tubes were again centrifuged at 13,684×g for fifteen minutes.
12. After removing the supernatant, 70% ethanol was used to wash the pellet.
13. For ten minutes, tubes were centrifuged at 9,500×g.
14. After being cleaned with 96% ethanol, the pellet was centrifuged once more for ten minutes at 9,500×g.
15. After discarding the supernatant, the DNA pellets were left in the tubes to dry naturally by tilting them.
16. Autoclaved distilled water was used to dissolve the pellet.
17. After that tubes were stored in ice for 1 hour.
18. The 1% Agarose was prepared for gel electrophoresis.
19. After homogenization of agarose 3µl Ethidium bromide was added to the agarose.
20. The gel was kept in an electrophoresis chamber and 1X TAE was added.

21. The DNA sample was loaded in wells of agarose gel and 100 V of current was provide to the gel for run.
22. The DNA bands were visualized by observing the gel by using Gel Doc system.

3.3.3 Restriction Digestion

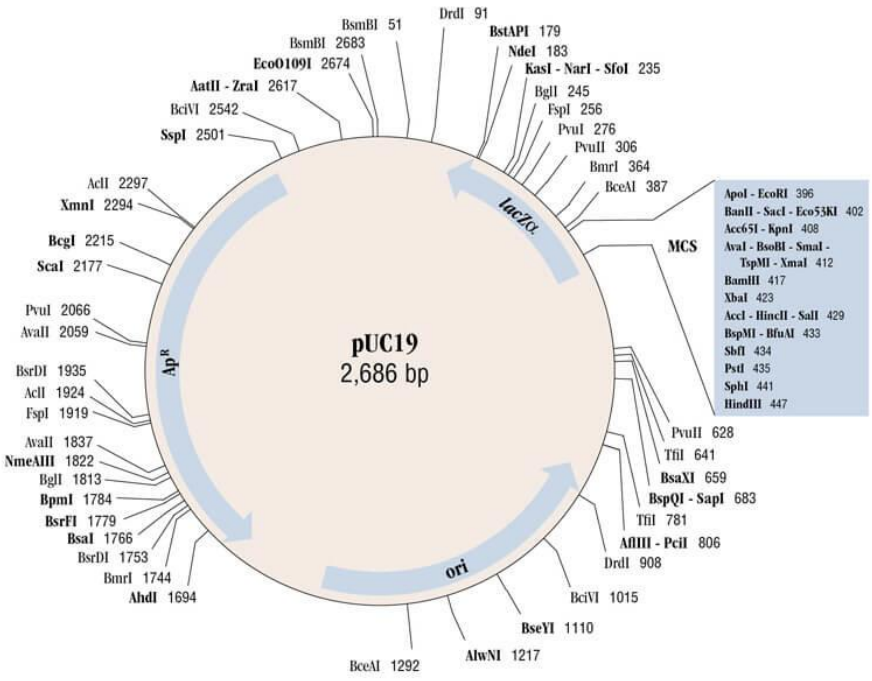


Fig 4: Restriction profile of pUC19[79].

1. Autoclaved vials were taken.
2. Water, Buffer, DNA, and Enzymes were added to the vials in the following concentration:

Table 5: Restriction digestion mixture.

Compositions	HinD III	EcoRI	Pvu II	HinDIII and EcoRI
Water	15.6 µl	15.6 µl	15.6 µl	14.2 µl
Buffer	2 µl	2 µl	2 µl	2 µl
DNA	2 µl	2 µl	2 µl	3 µl
Enzyme	0.4 µl	0.4 µl	0.4 µl	0.4 µl + 0.4 µl
Total = 20 µl				

3. Tubes were incubated at 37℃ for 1 hour.
4. The 1% Agarose was prepared for gel electrophoresis.
5. After homogenization of agarose 3µl Ethidium bromide was added to the agarose.
6. The gel was kept in an electrophoresis chamber and 1X TAE was added.
7. The sample was loaded in wells of agarose gel and 100 V of current was provide to the gel for run.
23. The DNA bands were visualized by observing the gel by using Gel Doc system.

3.3.4 CFU counting of *M. fortuitum*

1. *M. smegmatis* culture was grown in LBGT(Luria Broth with 2% Glycerol and 0.5% Tween 80) media for 3 days at 37℃ under shaking conditions at 200rpm..
2. Different Eppendorf tubes were taken and filled with 900µl of Tween – normal saline (TNS) in each tubes.
3. 100µl sample was taken from the neat and transferred to the one tube and all the tubes were serially diluted simultaneously, for example: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}.
4. From each tubes including neat 100µl of sample was spread on different plates containing NAT (Nutrient agar with 0.05% Tween 80).
5. Plates were kept in an incubator for 3 days at 37℃.
6. After 24 hours colony was counted from plates and CFU was calculated.

3.3.5 Isolation of genomic DNA from *M. fortuitum*

1. *M. fortuitum* culture was inoculated in 100ml LBGT media and incubated at 37℃ for 3 days.
2. Following that, the culture was put into falcon tubes and centrifuged for fifteen minutes at 9,500×g.
3. 2ml of TES buffer (Tris-EDTA-NaCl) were added to the falcon tubes after the supernatant was disposed of.
4. For one hour, the tubes were incubated at 80°C.
5. Following incubation, 2 milligram per mililtre of lysozyme was added to the tubes, carefully mixed, and incubated for 1 hour at 37°C.
6. The tubes were filled with 1.5% Sodium Dodecyl Sulphate and 100 micrograms per milliliter of Proteinase K. They were then incubated for one hour at 50°C.
7. 1 ml of saturated phenol was added to each tube after incubation.

8. Following that, tubes were centrifuged for 20 minutes at 9,500×g.
9. The upper layer (aqueous) was divided and transferred to fresh, sterile tubes.
10. Chloroform: Isomyl (24:1) was added to the tubes, and they were centrifuged for 20 minutes at 9,500×g.
11. The top (aqueous) layer was then moved to other tubes, and after adding more chloroform and isopropyl (24:1), the mixture was centrifuged for 20 minutes at 9,500×g.
12. Equal volumes of isopropanol were introduced to the new tubes following the separation of the top (aqueous) layer.
13. The tubes were kept at -20°C for a whole night.
14. The tubes were centrifuged for 20 minutes at 9,500×g the next day.
15. After removing the supernatant, the pellet was centrifuged for ten minutes at 9,500×g after being cleaned with 70% ethanol.
16. After discarding the supernatant, the DNA pellets were left in the tubes to dry naturally by tilting them.
17. Autoclaved distilled water was used to dissolve the pellet.
18. After that tubes were stored in ice for 1 hour.
19. The 0.6% Agarose was prepared for gel electrophoresis.
20. After homogenization of agarose 3µl Ethidium bromide was added to the agarose.
21. The gel was kept in an electrophoresis chamber and 1X TAE was added.
22. The DNA sample was loaded in wells of agarose gel and 100 V of current was provided to the gel for run.
24. The DNA bands were visualized by observing the gel by using Gel Doc system.

3.3.6 Amplification of *fabG4* gene by doing PCR

Primers used for amplification in this study

Table 6: Primers used for amplifiation

S.No.	Primer name	Primer Sequence	Amplicon size (bp)
Primers for amplification of the *fabG4* gene (1354 bp) of *M. fortuitum*			
1	MF*fabG4*_FWD	CGCGGATCCGCGCTAGGCTCCCAGCATGGCCT	**1354**
	MF*fabG4*_REV	CGCGGATCCGCGGTGGCTTCCGACCTGTTCTCC	

1. Autoclaved PCR vials were taken.
2. DNA Template, Forward and reverse primer, Master mix and Nuclease free water were added to the PCR vials. For PCR amplification the reaction mixture was prepared as follows:

Table 7: Reaction mixture for PCR

DNA template	8 µl
Fwd. Primer	2 µl
Rev. Primer	2 µl
Master mix	24 µl
Nuclease free water	4 µl
Total = 40 µl	

3. From the 40 µl , 10 µl was aliquot in 4 PCR vials and the following conditions were used for PCR amplification.

Table 8: Conditions for PCR

Conditions	**Temperature**	**Time (min)**	
Initial denaturation	95°C	5	
Cyclic Denaturation	95°C	1	**30 cycles**
Annealing	60°C	1	
Cyclic Extension	72°C	1:45	
Final Extension	72°C	8	
Hold	4°C	∞	

1. After that 1.2% Agarose was prepared for gel electrophoresis.
2. After homogenization of agarose 3µl Ethidium bromide was added to the agarose.
3. The gel was kept in an electrophoresis chamber and 1X TAE was added.
4. The amplicons were loaded in wells of agarose gel and 100 V of current was provided to the gel for run.
5. The bands were visualized by observing the gel by using Gel Doc system.

3.4 Study of wild type *M. fortuitum* and mutant *M. fortuitum fabG4* knockdown strain under different stress conditions:

3.4.1 Acidic Stress

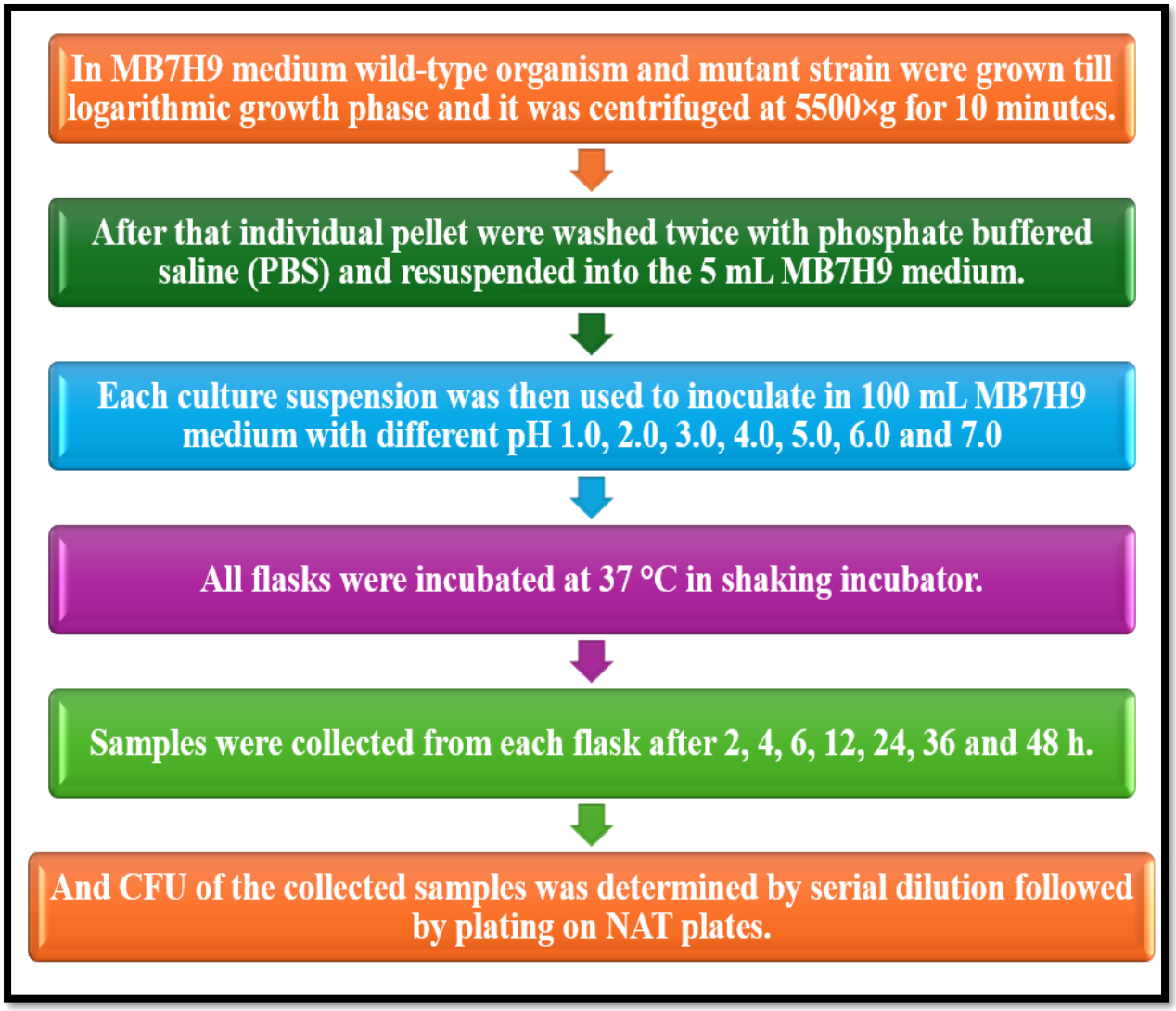


Fig 5: Flow chart showing the detailed protocol employed for study of survival and growth of *M. fortuitum under* acidic conditions [Protocol adapted from Poonam et. al[56] with minor modification].

3.4.2 Hypoxic Stress

Fig 6: Flow chart showing the protocol employed for study of survival and growth of *M. fortuitum under* hypoxic conditions [Protocol adapted from Poonam et. al [63] with minor modification].

3.4.3 Nutrient starvation

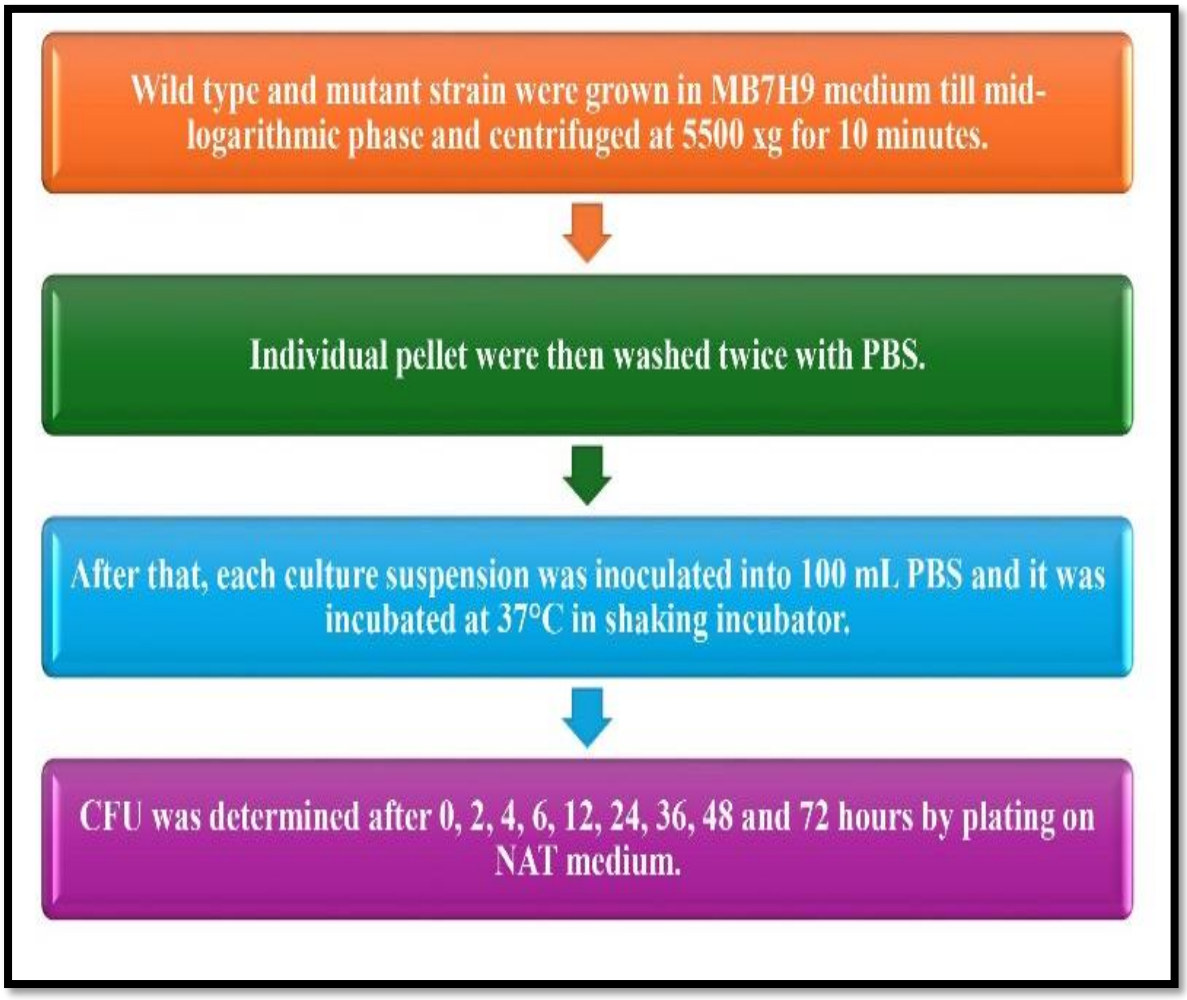


Fig 7 : Flow chart showing the protocol employed for study of survival and growth of *M. fortuitum under* Nutrient starvation conditions [Protocol adapted from Poonam et. al [62] with minor modification].

3.4.4 Detergent Stress

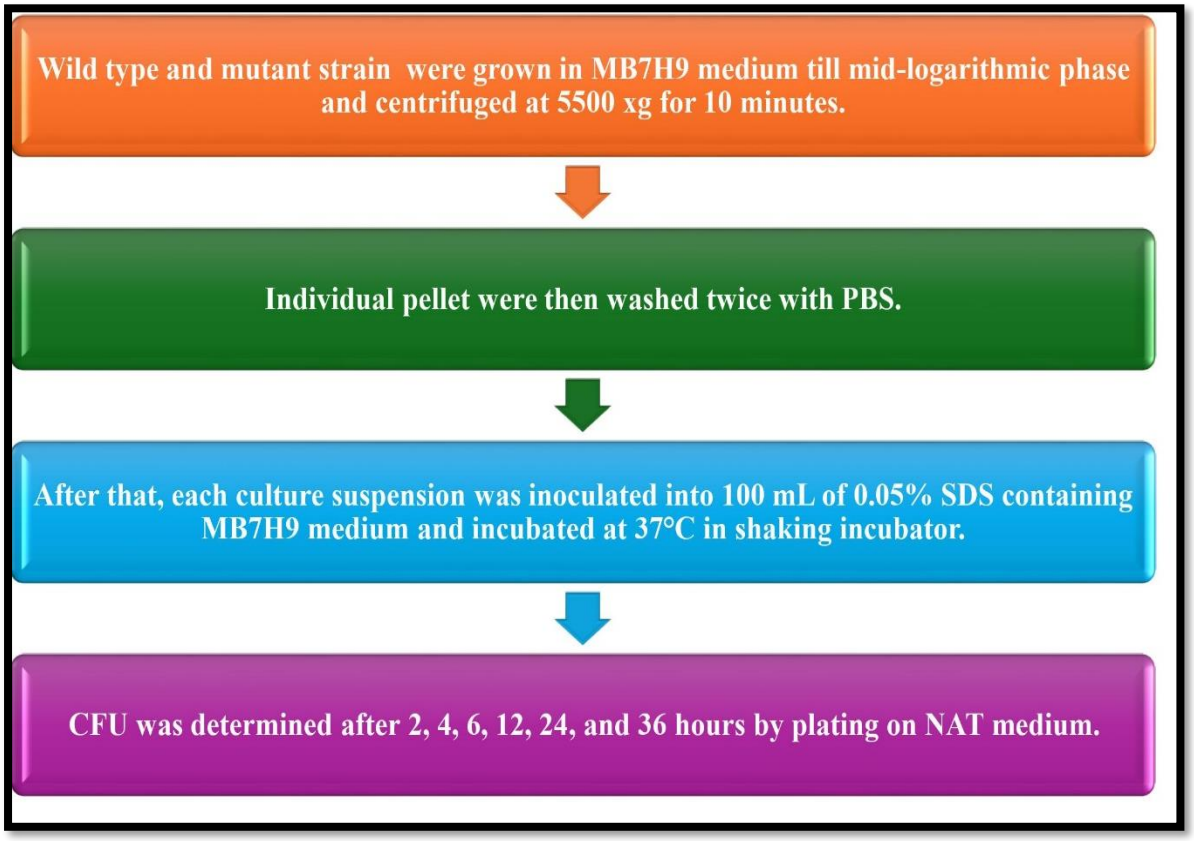


Fig 8 : Flow chart showing the protocol employed for study of survival and growth of *M. fortuitum under* Detergent conditions [Protocol adapted from Poonam et. al [56] with minor modification].

3.4.5 Oxidative stress

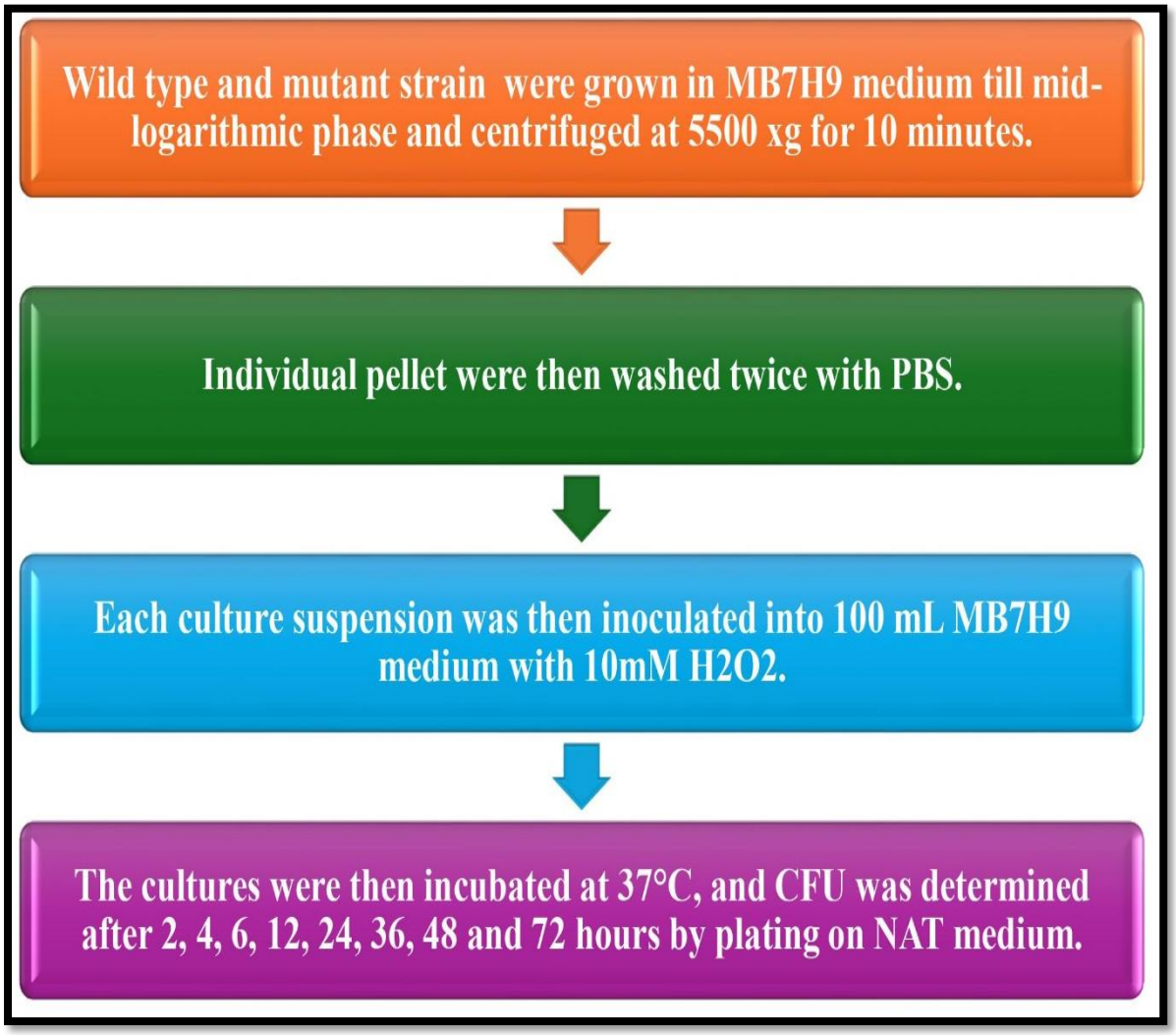


Fig 9 : Flow chart showing the protocol employed for study of survival and growth of *M. fortuitum under* oxidative conditions [Protocol adapted from Poonam et. al [63] with minor modification].

3.5 Statistical Analysis

The experiments were performed in duplicates and readings were taken separately. The standard deviation or standard error mean bars were used to display the data as the mean of the measurements.

The formula for calculating Standard deviation:

Standard deviation (SD) $\boldsymbol{\sigma}$ = $\sqrt{\frac{\sum_{i=1}^{n}(x_i-\bar{x})^2}{n-1}}$

Variance = σ^2

The formula for Standard Error calculations:

Standard error (SE) $\boldsymbol{\sigma\bar{x} = \frac{\sigma}{\sqrt{n}}}$

Here: $\boldsymbol{\bar{x}}$ = mean of sample

n = size of sample

MS Excel was used to evaluate Statistical significance.

CHAPTER 4

RESULTS

4 Results

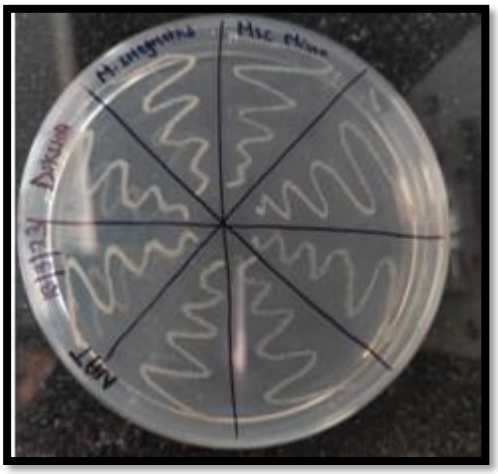
4.1 Basic Microbiological Techniques

4.1.1 Simple Streaking

Cultures of *E. coli, B. subtilis,* and *M. smegmatis* were streaked on NA (Nutrient agar) or NAT (Nutrient agar with 0.05% Tween 80) media to increase the amount of inoculum for short term preservation of the cultures. Representative images of culture plates [Fig:10] are shown below.

(a)

(b)

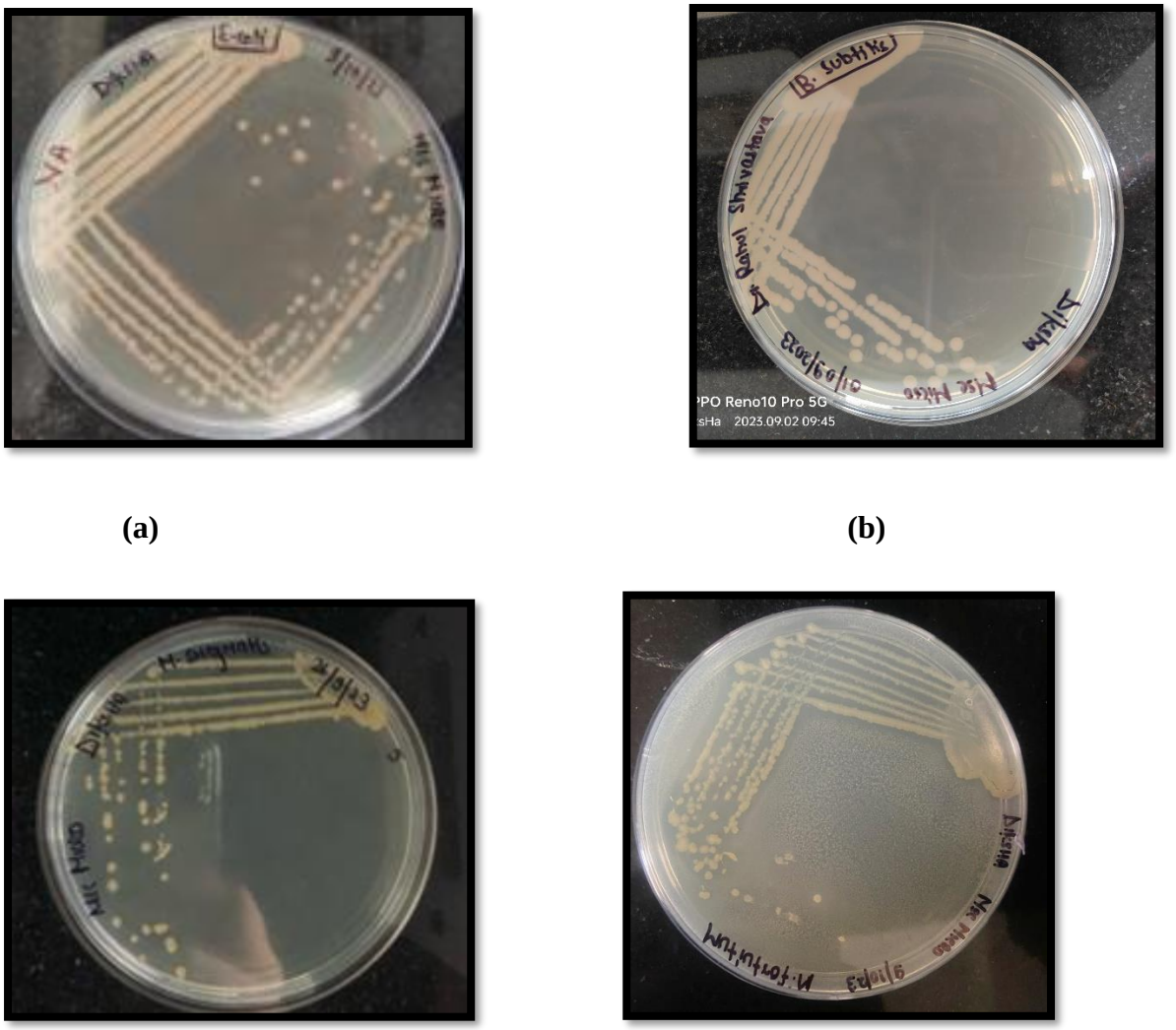


(c)

Fig 10 : (a) Simple streaking of *E. coli* and (b) Simple streaking of *B.subtilis* and (c) Simple streaking of *M. smegmatis*

4.1.2 Quadrant Streaking

Quadrant streaking of *E. coli, B. subtilis, M. smegmatis,* and *M. fortuitum* was done to obtain isolated colonies. And these colonies were used for further experiments. Representative images of culture plates [Fig:11] are shown below.



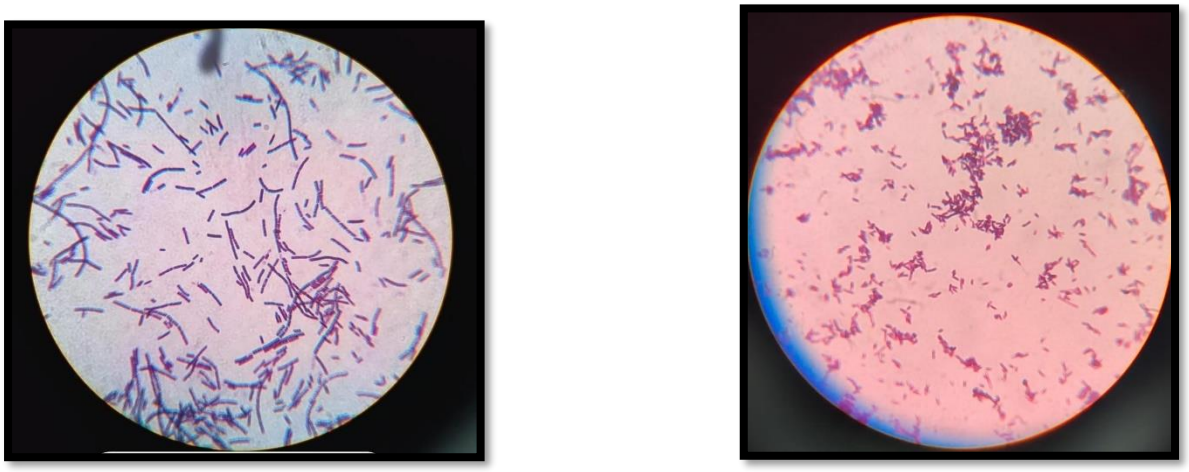
(a) **(b)**

(c) **(d)**

Fig 11 : (a) Quadrant Streaking of *E. coli* and (b) Quadrant streaking of *B. subtilis* and (c) Quadrant Streaking of *M. smegmatis* (d) Quadrant streaking of *M. fortuitum*

4.2 Gram Staining

Gram staining was done to confirm that the colonies we have isolated by streaking contained pure cultures of *E. coli* or *B. subtilis*.



(a) **(b)**

Fig 12: The mage shows that (a) Gram staining of *Bacillus subtilis* and (b) Gram staining of *E. coli*.

4.3 Fungal Staining

The staining of fungus was done to learn about fungal staining technique used for preliminary identification of any fungus.

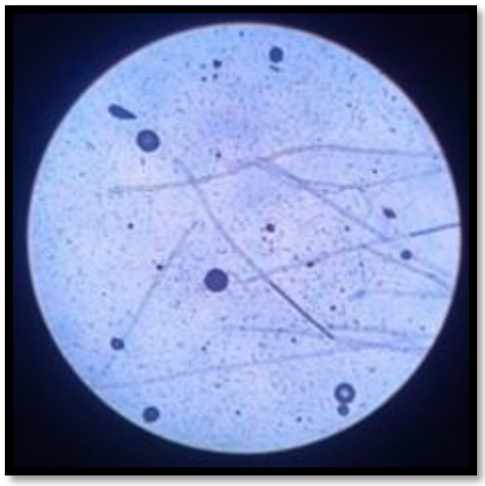
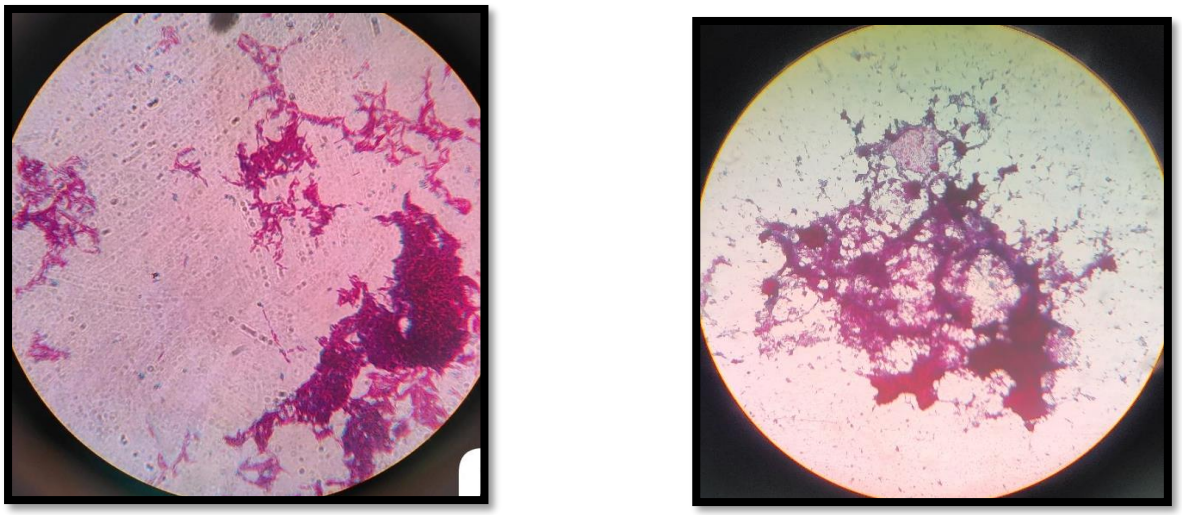


Fig 13 : Fungal Staining of *Aspergillus niger*.

4.4 Ziehl-Neelsen staining

Ziehl- Neelsen staining was done to confirm that the colonies we have isolated by streaking were taken from pure cultures of *M. smegmatis* and *M. fortuitum*.



(a) **(b)**

Fig 14 : (a) Ziehl- Neelsen staining of *M. smegmatis* and (b) Ziehl – Neelsen staining of *M. fortuitum*

4.5 CFU count of water sample

Tap water was taken from the JUIT campus and CFU count of water sample was done to determine number of cells were present in the tap water. Serial dilution of samples was made and 100 µL of each dilution was plated in duplicate, Fig: 15 shows representative plates obtained after incubation of plates. No. of colonies obtained in the study has been shown in Table:9.

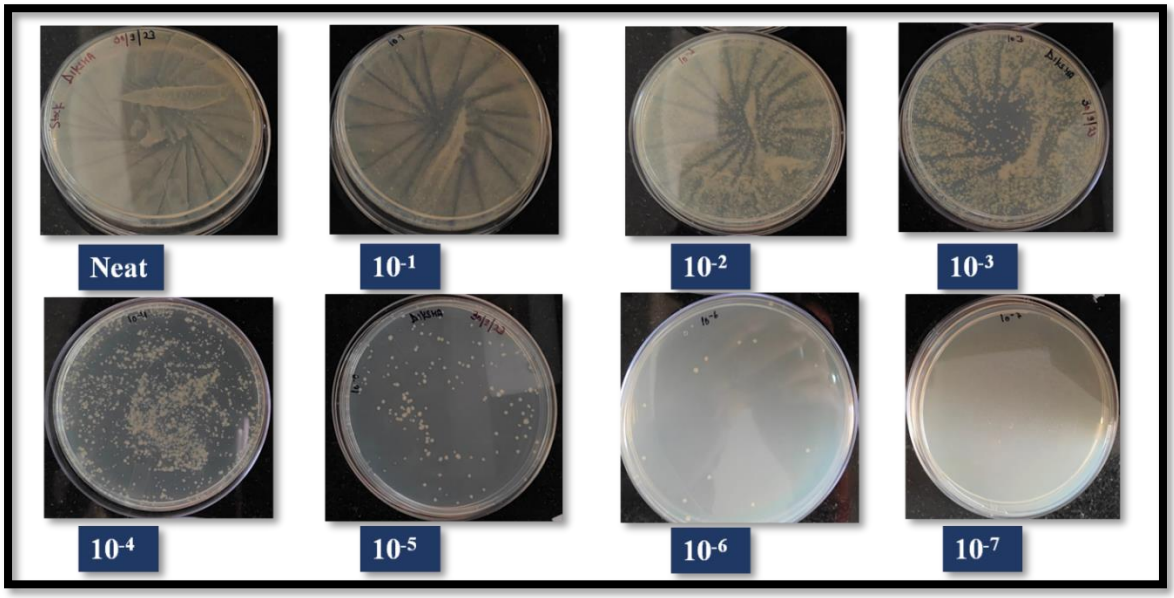


Fig 15 : Petri plates showing colonies obtained on spreading of different dilution of water sample.

Table 9: Dilutions and number of colonies obtained.

Dilutions	No. of colonies
Neat	Not countable
10^{-1}	Not countable
10^{-2}	Not countable
10^{-3}	Not countable
10^{-4}	Not countable
10^{-5}	113
10^{-6}	25
10^{-7}	0

$$\text{CFU/mL} = \frac{113 \times 10^{-5}}{0.1} = 1.13 \times 10^{8}\ \text{CFU/ml}$$

4.6 Molecular techniques

4.6.1 Genomic DNA isolation from *E. coli*

Overnight-grown *E. coli* culture was used to isolate its genomic DNA. And genomic DNA was run on 0.8% of agarose gel [Fig:16].

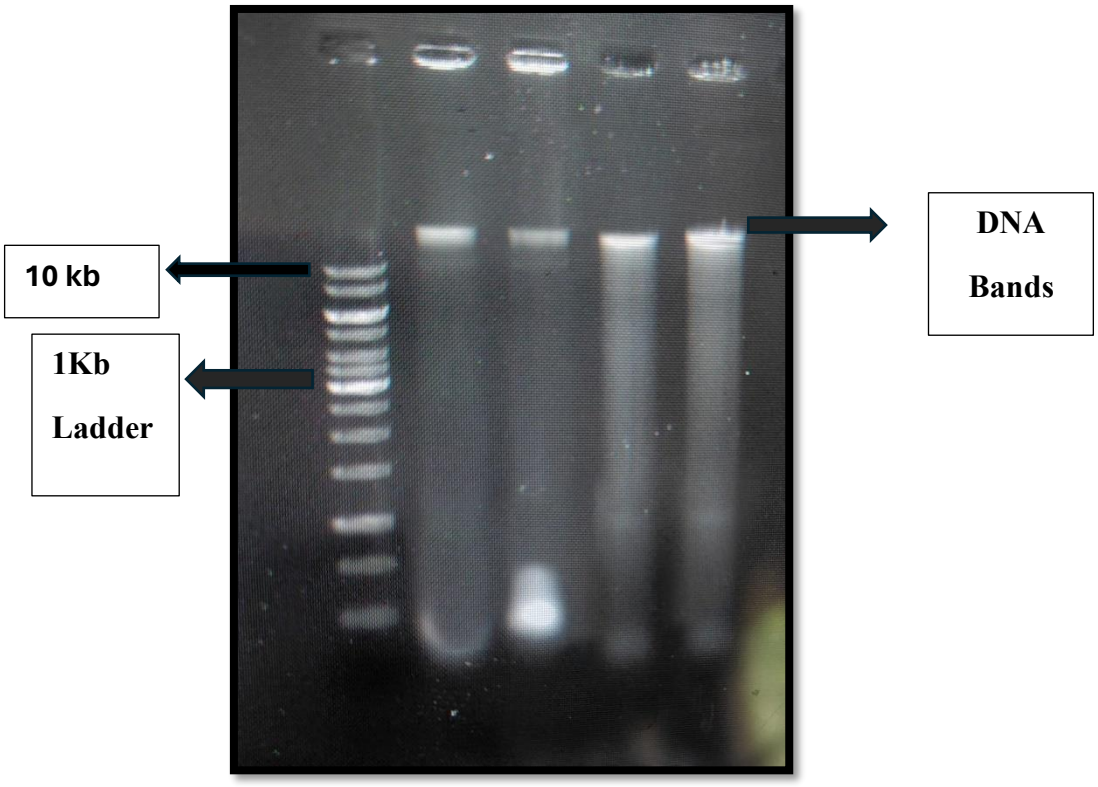


Fig 16 : Genomic DNA of *E. coli.* This image of agarose gel shows that in lane 1 there is 1kb DNA ladder (GeneRuler) (Size of fragments in bp) (from bottom to top; 250, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 5000, 6000, 8000, 10000) and in Lane 2-5 there is DNA bands of more than 10kb.

4.6.2 Plasmid DNA isolation

E.coli culture containing pUC-19 plasmid as used to isolate the plasmid, which may be used for molecular biology studies. Fig: 17 shows different form of pUC plasmid isolated.

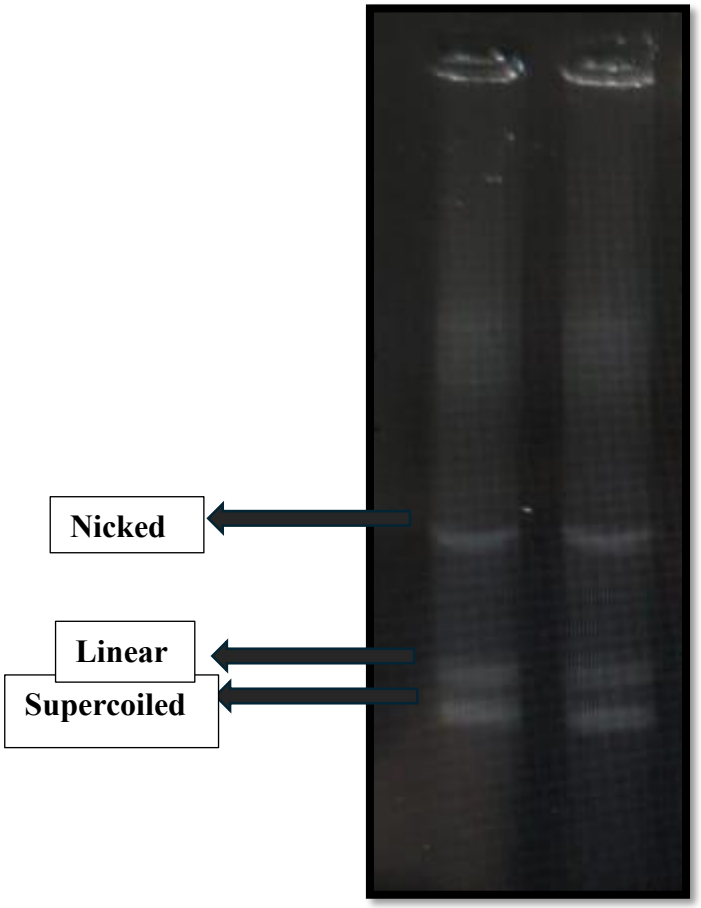


Fig 17 : Plasmid DNA isolation. The figure shows that in Lane 1 and 2 there is plasmid DNA and showing 3 bands of nicked, linear, and Supercoiled DNA.

4.6.3 Restriction Digestion

Restriction digestion of pUC- 19 plasmid was done by using single cutter (HindIII or EcoRI) and double cutter (PvuII) restriction enzymes. Double digestion was also performed with the enzyme: EcoRI and HindIII. After digestion all the samples were run on agarose gel has shown in Fig:18.

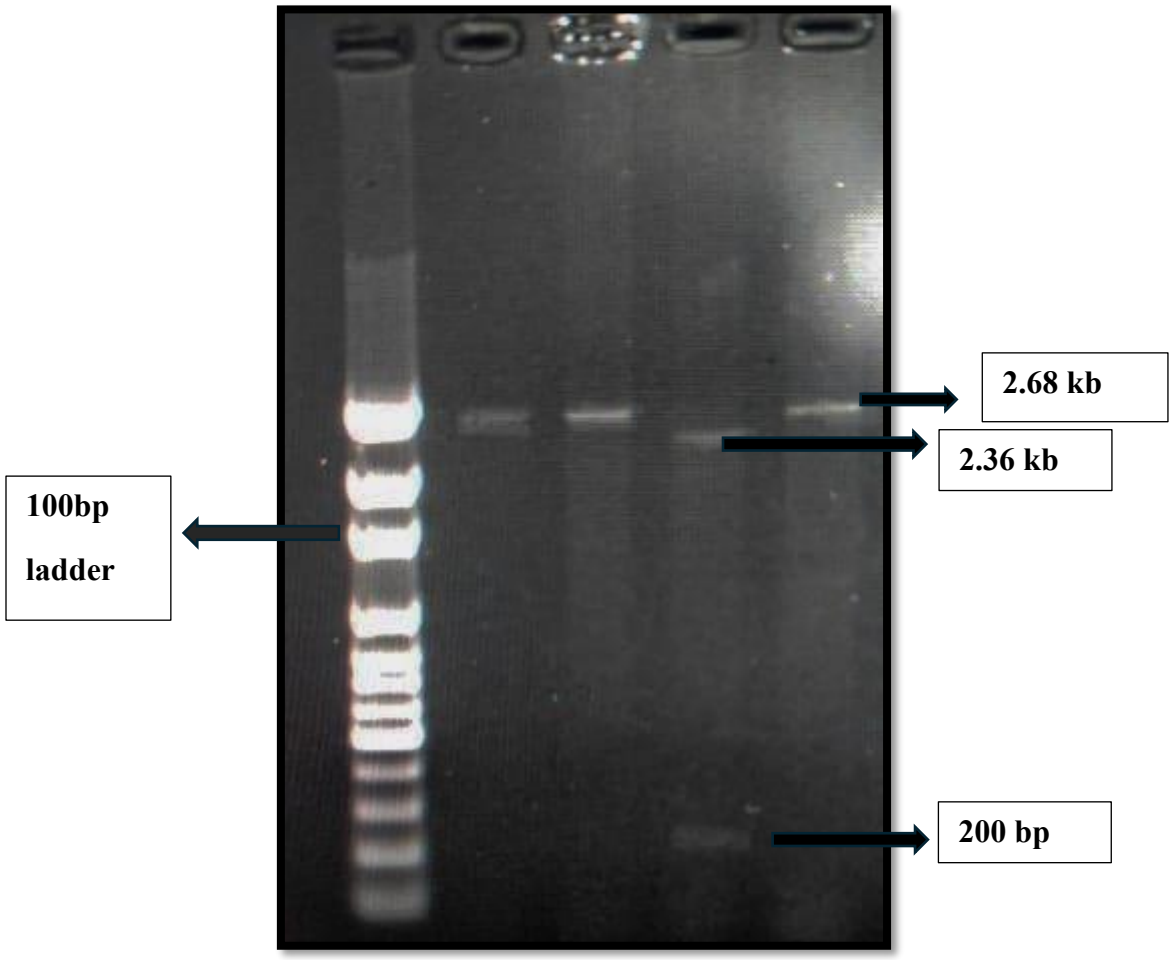


Fig 18 : Restriction digestion of pUC 19. The bands in figure shows that the restriction digestion of pUC 19 with different enzymes. In lane 1 = 100bp ladder (GeneRuler) (Size of fragments in bp) (from bottom to top; 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1500, 2000, 3000), In lane 2 digested with HindIII (2.68kb), In Lane 3 digested with EcoRI (2.68kb), Lane 4 digested with PvuII (2.36kb and 222bp) and in Lane 5 there is double digestion with HindIII and EcoRI(2.6kb and 52bp)

4.6.4 CFU counting of *M. fortuitum*

CFU count of *M. fortuitum* was done to know that how many colonies are present in 1 ml of culture. Broth culture were serially diluted till 10^{-7} dilutions and 100 µL of each dilution was plated in duplicate, Fig: 19 shows representative plates obtained after incubation of plates. No. of colonies obtained in the study has been shown in Table:10.

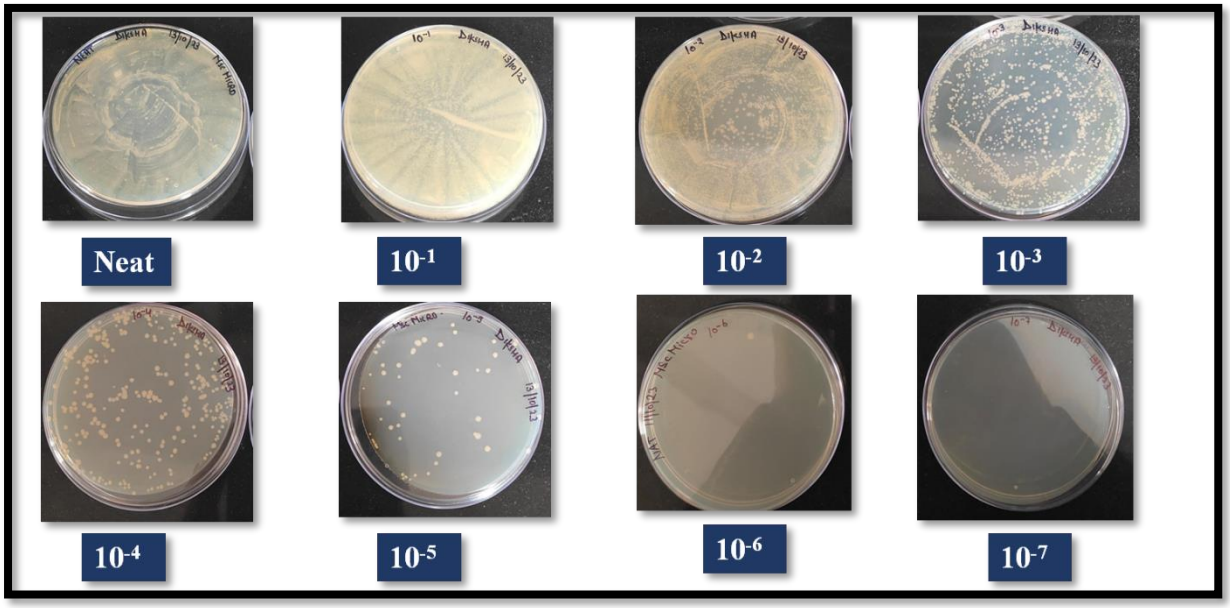


Fig 19: CFU counting of *M. fortuitum*. Image shows that all the dilutions are plated on Petri plates and colonies were observed and counted.

Table 10: Dilution and number of colonies obtained.

Dilutions	No. of colonies
Neat	Not countable
10^{-1}	Not countable
10^{-2}	Not countable
10^{-3}	Not countable
10^{-4}	174
10^{-5}	59
10^{-6}	3
10^{-7}	0

$$\text{CFU/mL} = \frac{174 \times 10^{-4}}{0.1}$$

$$= 1.74 \times 10^{7} \text{CFU/ml}$$

4.6.5 Isolation of genomic DNA from *M. fortuitum*

Isolation of DNA from *M. fortuitum* was done and genomic DNA was run on 0.8% agarose gel [Fig:20]. Genomic DNA was employed for amplification of the gene (*fabG4)*.

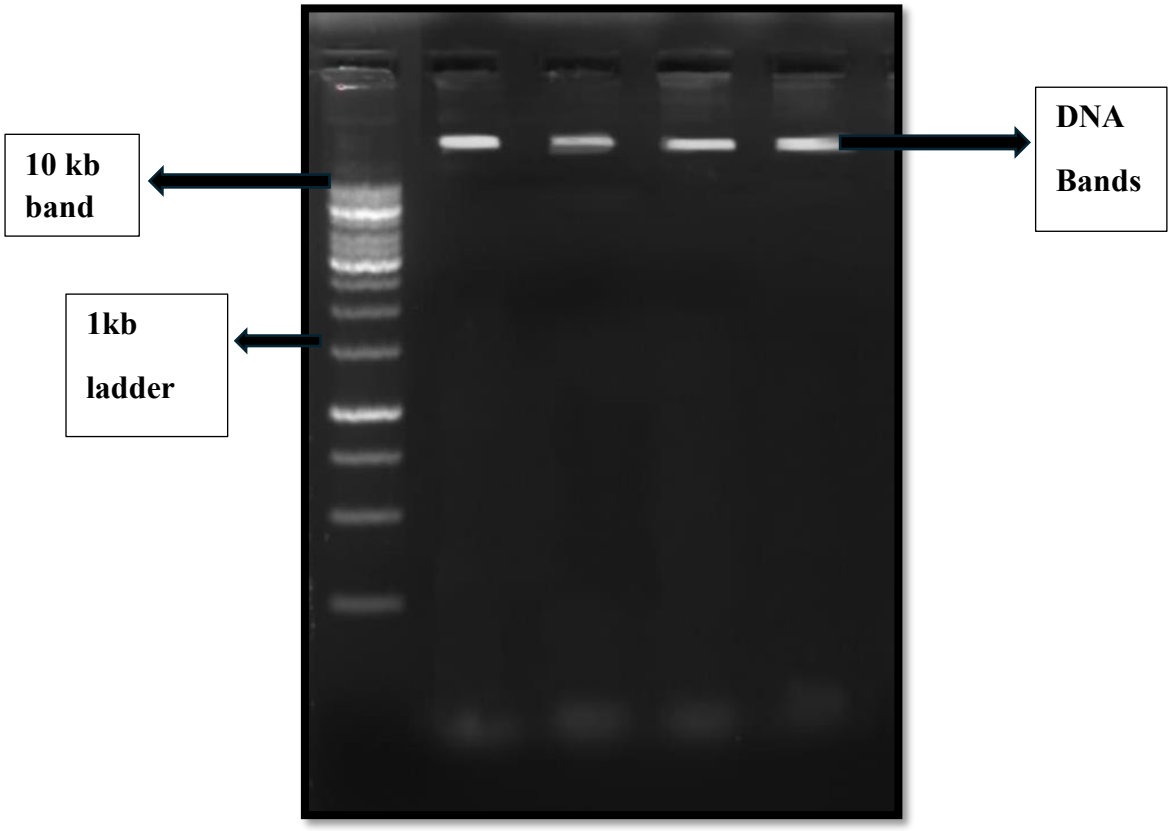


Fig 20 : Genomic DNA of *M. fortuitum.* The figure of agarose gel shows that in the Lane 1 there is 1kb of DNA ladder (GeneRuler) (Size of fragments in bp) (from bottom to top; 250, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 5000, 6000, 8000, 10000) and in Lane 2,3,4,5 DNA bands of *M. fortuitum*

4.6.6 Amplification of *fabG4* gene by PCR

After the isolation of genomic DNA from *M. fortuitum* the gene (*fabG4)* was amplified by PCR and the amplicon was run on agarose gel. Results of the amplification are shown in Fig: 21.

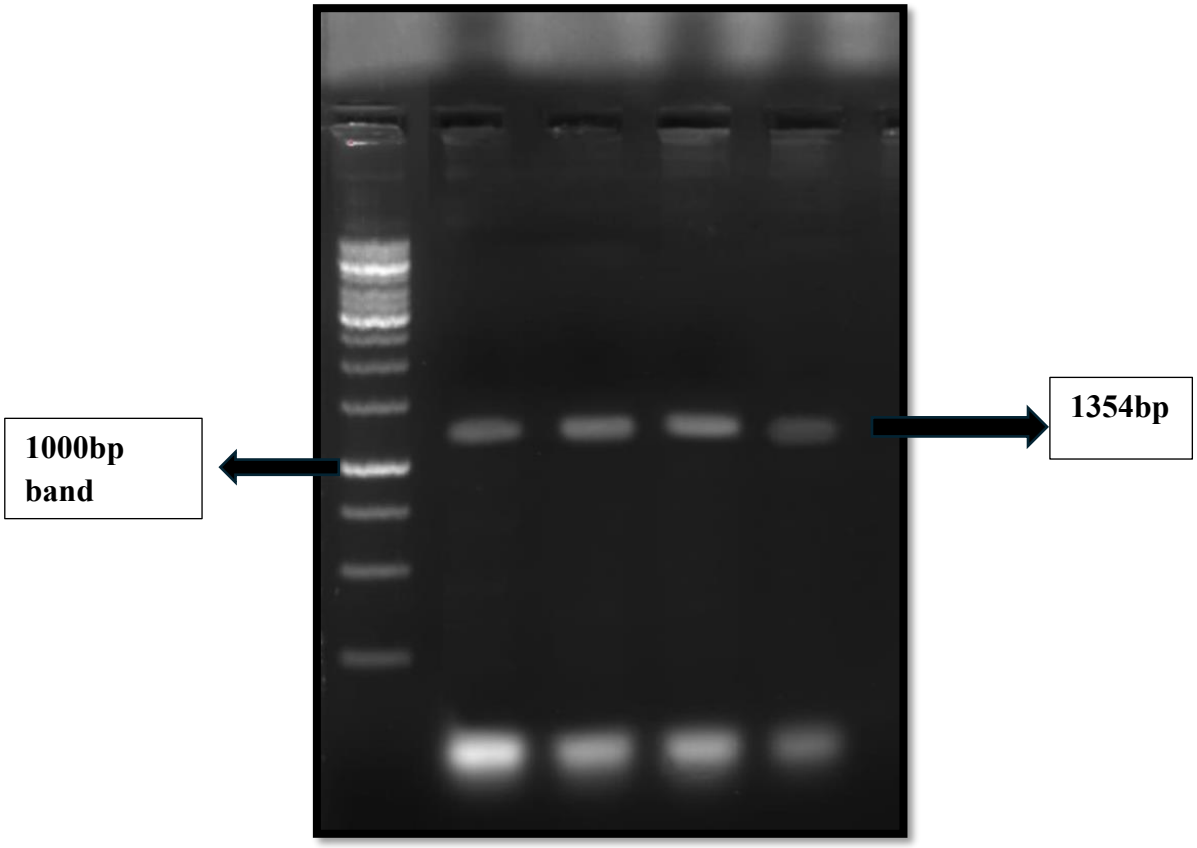


Fig 21 : **Amplification of *fabG4* gene from *M. fortuitum* DNA**. Figure shows that in the lane 1 there is a 1Kb DNA ladder (GeneRuler) (Size of fragments in bp from bottom to top; 250, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 5000, 6000, 8000, 10000) and in Lane 2,3 ,4,5 there is a band of amplification of *fabG4* (1354 bp) gene from *M. fortuitum* by doing PCR

4.7 Study of wild type *M. fortuitum* and mutant *M. fortuitum fabG4* knockdown strain under different stress conditions:

4.7.1 Acidic Stress

Mutant (*fabG4*) strain and Wild type strain of *M. fortuitum* were inoculated in different pH media as shown in Fig:22 and samples were collected at different time points and plated on NAT plates to determine CFU.

(a) Mutant strain of *M. fortuitum* were inoculated in conical flasks containing MB7H9 medium with different pH range from 1.0 – 7.0

(b) Wild type strain of *M. fortuitum* were inoculated in conical flasks containing MB7H9 medium with different pH range from 1.0 – 7.0

Fig 22: Growth of wild type and mutant after 48 hours. Mutant and wild type strain of *M. fortuitum* were inoculated in conical flasks containing MB7H9 medium with different pH range from 1.0 – 7.0. In this image we can observe that the turbidity in pH 1.0 – 4.0 was very low after 48 hours.And in pH 5.0- 7.0 both wild type and mutant showed significant growth.

pH-1.0

Mutant and wild type strain of *M. fortuitum* were inoculated in MB7H9 medium of pH-1.0. The growth of both wild type and mutant strain constant decline after 6 hours. However in case of mutant a steep decline was observed. After 6 hours no survival was seen as shown in Fig:23.

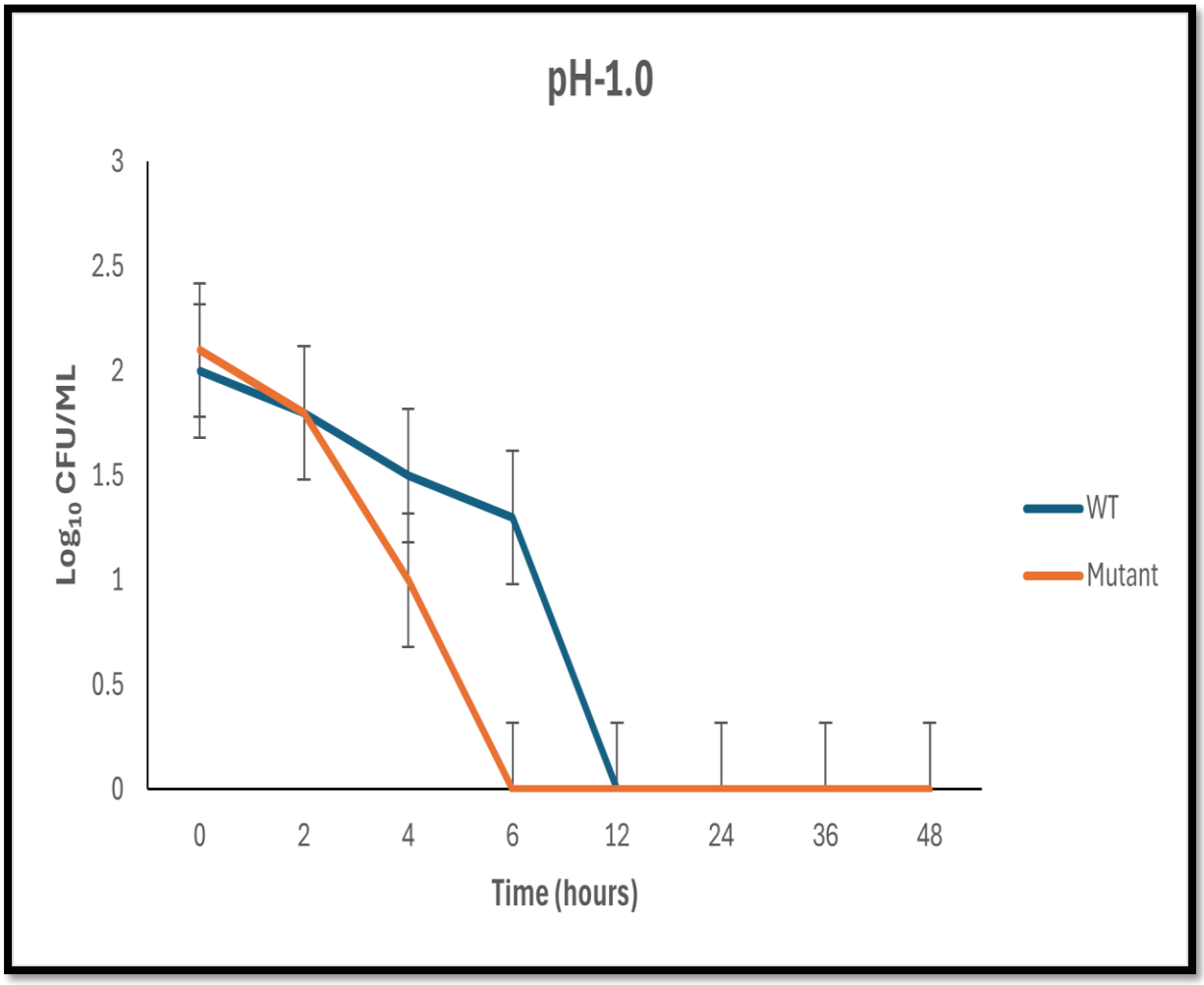


Fig 23: Survival and growth of wild type (WT) *M. fortuitum* and *M. fortuitum fabG4* gene mutant knockdown strain at pH- 1.0. Figure shows that at pH-1.0 growth of WT organism declined after 6 hours, however mutant growth is suppressed faster in just 2 hours. Growth of both WT and mutant strain show constant decline in growth just after inoculation.

pH- 2.0

Mutant and wild type strain of *M. fortuitum* were inoculated in MB7H9 medium of pH-2.0. The growth of both wild type and mutant strain constant decline after 4 hours. However in case of mutant a steep decline was observed. After 6 hours no survival was seen in mutant and in wild type no survival seen after 12 hours as shown in Fig: 24.

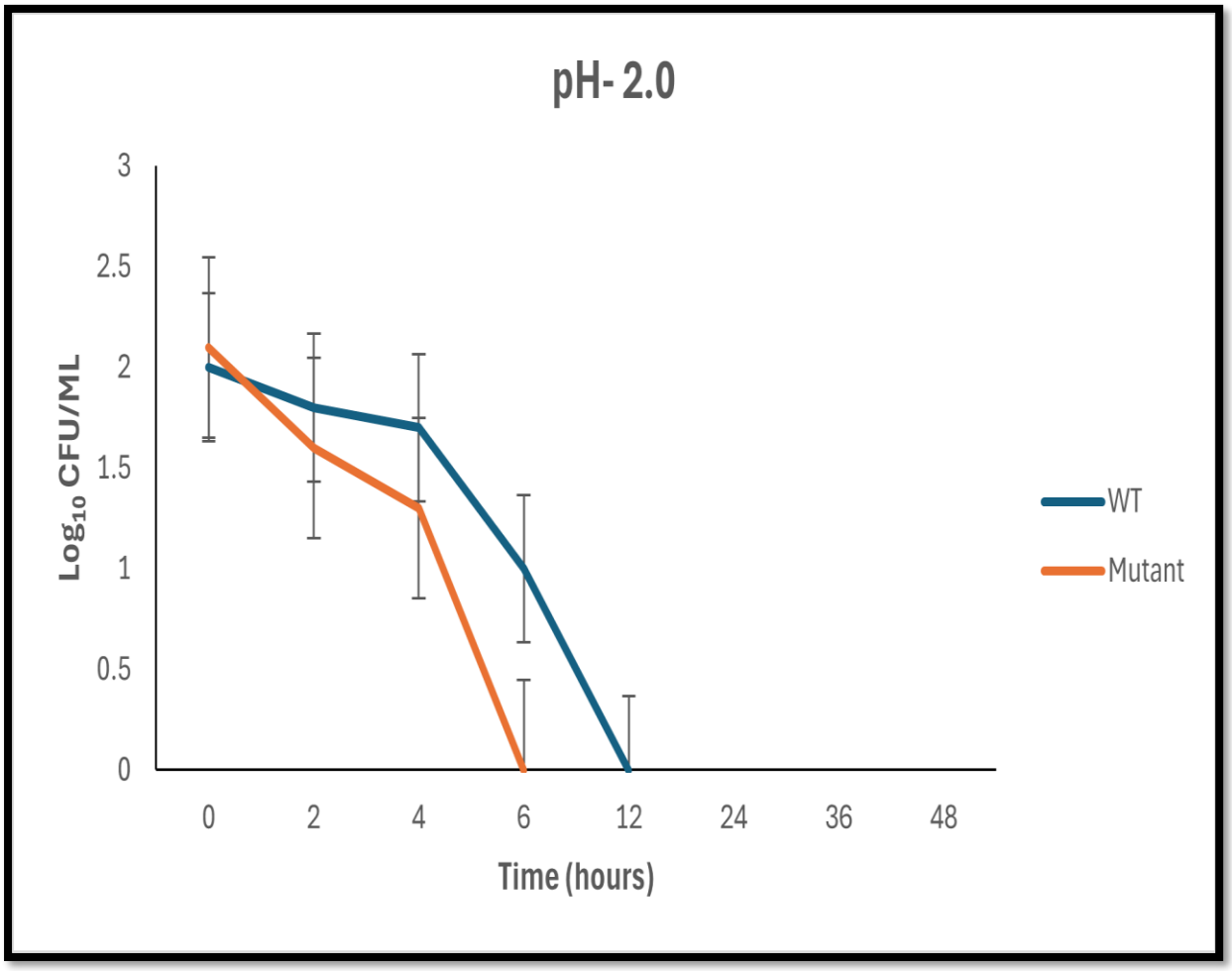


Fig 24 : Survival and growth of *M. fortuitum fabG4* gene mutant knockdown strain at pH- 2.0 Figure shows that at pH 2.0 the growth of the WT organism slows down after 6 hours, but the mutant exhibits a drop even sooner after 4 hours. Growth of both WT and mutant strain show constant decline in growth just after inoculation.

pH-3.0

Mutant and wild type strain of *M. fortuitum* were inoculated in MB7H9 medium of pH-3.0. The growth of both wild type and mutant strain constant decline after 6 and 12 hours. However in case of mutant a steep decline was observed. After 12 hours no survival was seen in mutant and in wild type no survival seen after 24 hours as shown in Fig: 25.

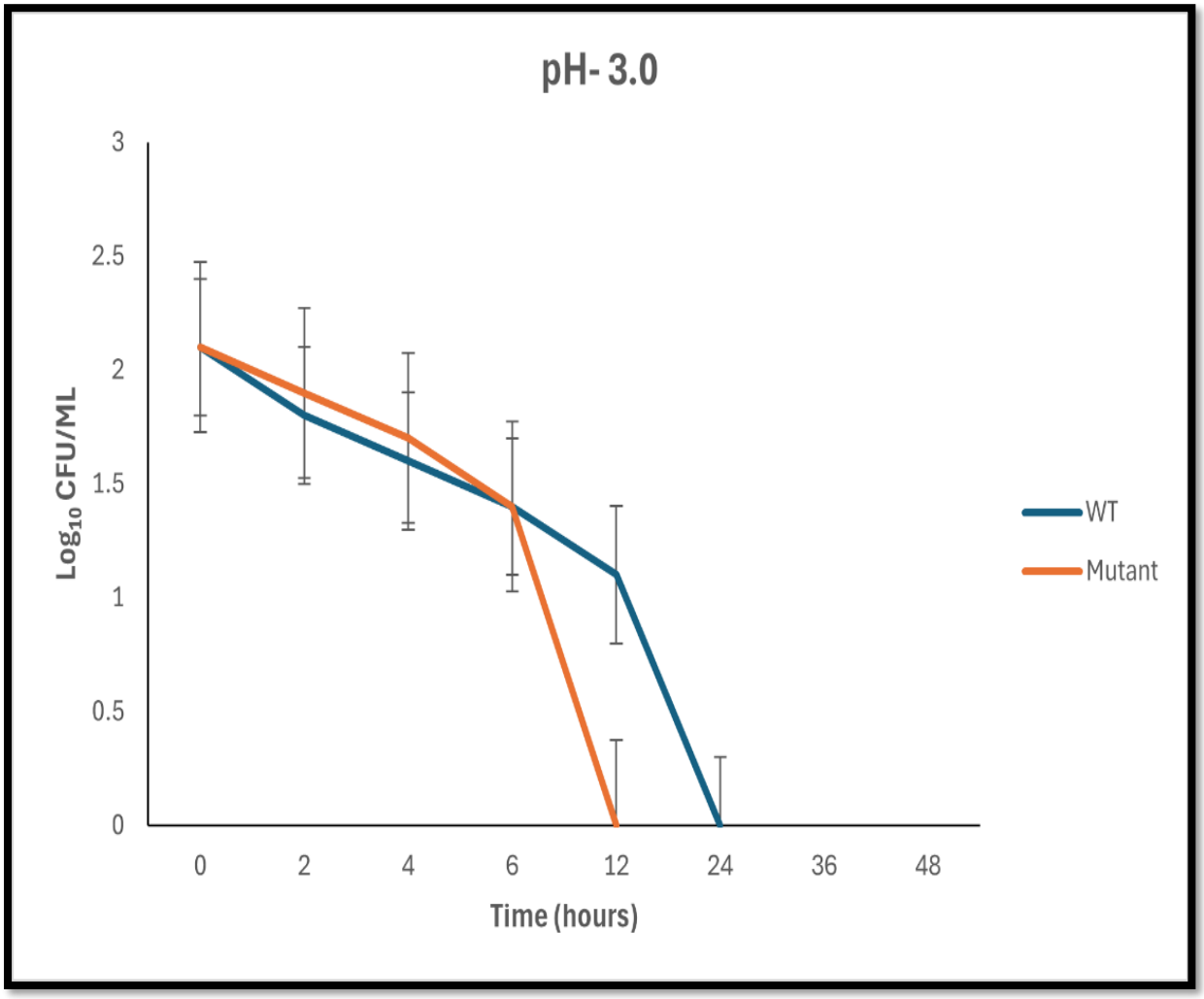


Fig 25: Survival and growth of *M. fortuitum fabG4* gene mutant knockdown strain at pH- 3.0 Figure shows that at pH- 3.0 the growth of the WT organism slows down after 12 hours, but the mutant exhibits a drop at 6 hours.

pH-4.0

Mutant and wild type strain of *M. fortuitum* were inoculated in MB7H9 medium of pH-4.0. However in case of mutant a steep decline was observed. The growth of both wild type and mutant strain constant decline after 36 hours as shown in Fig: 26.

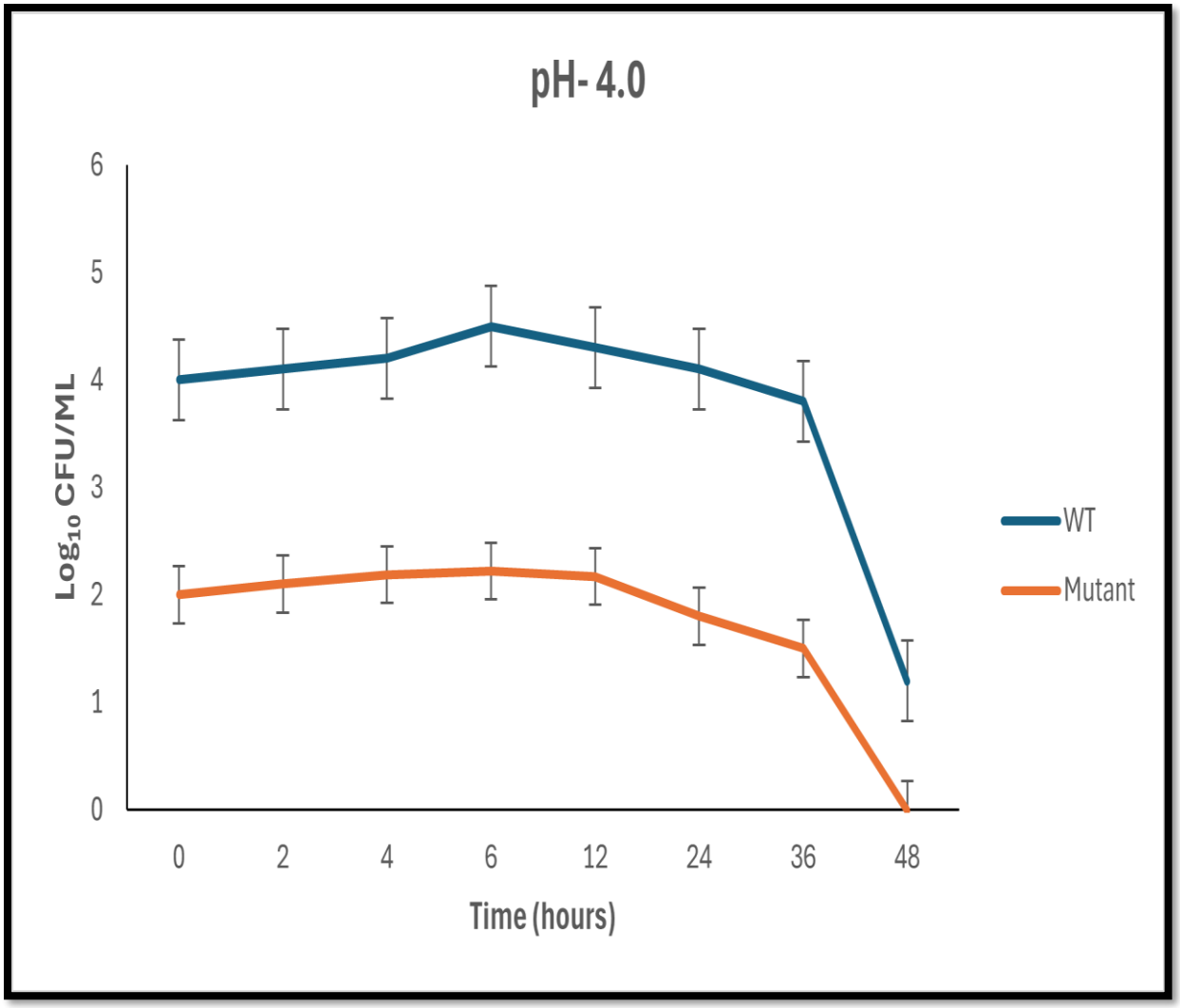


Fig 26: Survival and growth of *M. fortuitum fabG4* gene mutant knockdown strain at pH- 4.0 Figure shows that At pH-4.0, Growth of the mutant and WT both declines after 36 hours. After 48 hours no survival of mutant was observed while the WT showed more than 2-log decrease in its CFU.

pH – 5.0

Mutant and wild type strain of *M. fortuitum* were inoculated in MB7H9 medium of pH-5.0. The growth of both wild type and mutant strain show increase after 24 hours as shown in Fig: 27.

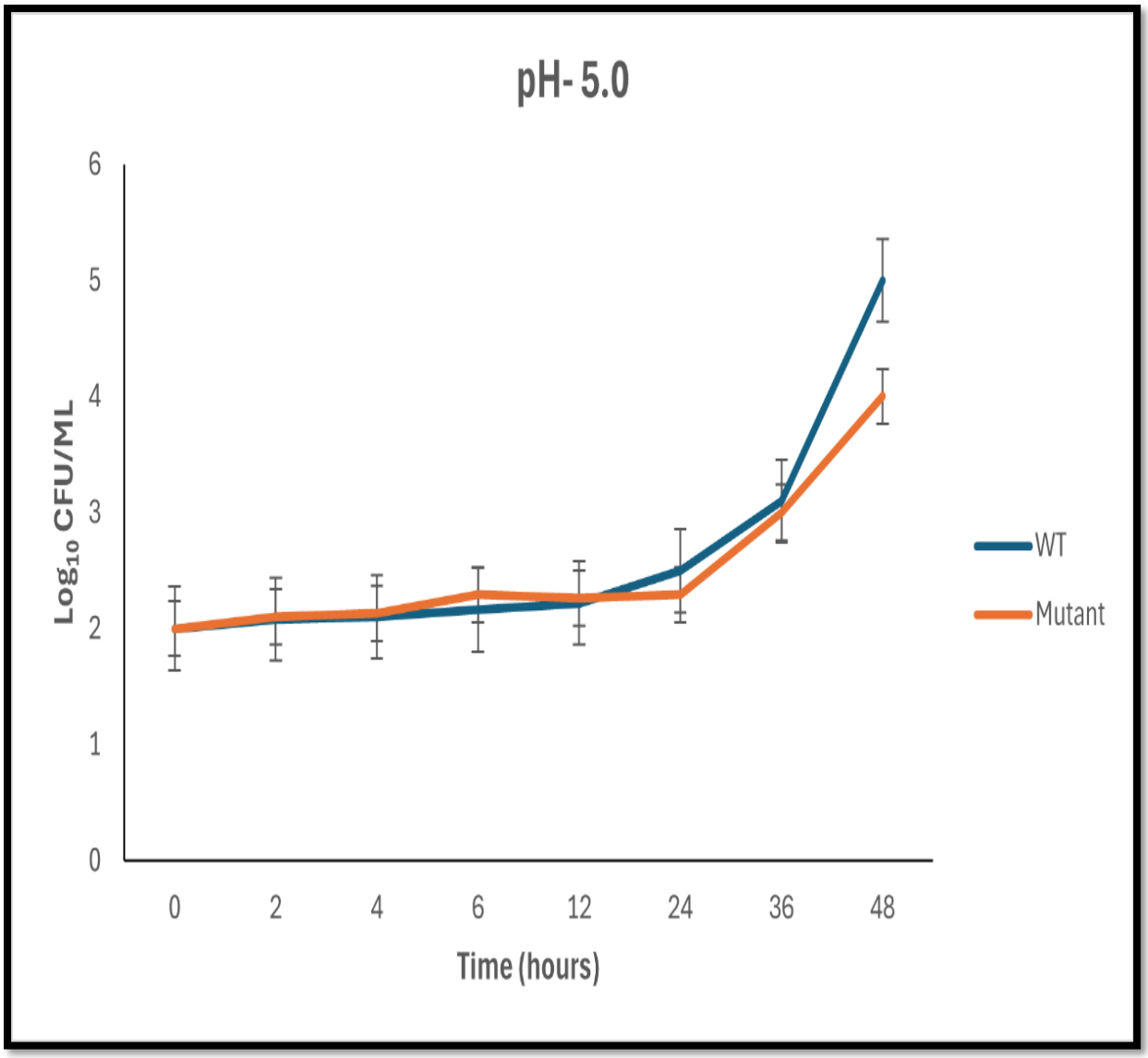


Fig 27: Survival and growth of *M. fortuitum fabG4* gene mutant knockdown strain at pH- 5.0 Figure shows that the growth of both WT and mutant increased after 24 hours. The mutant showed lesser increase of around 2-log from 24h to 48h, in comparison to the WT which showed more than 3-log increase in in its CFU during the period

pH-6.0

Mutant and wild type strain of *M. fortuitum* were inoculated in MB7H9 medium of pH-6.0. The growth of both wild type and mutant strain show increase after 24 hours as shown in Fig: 28.

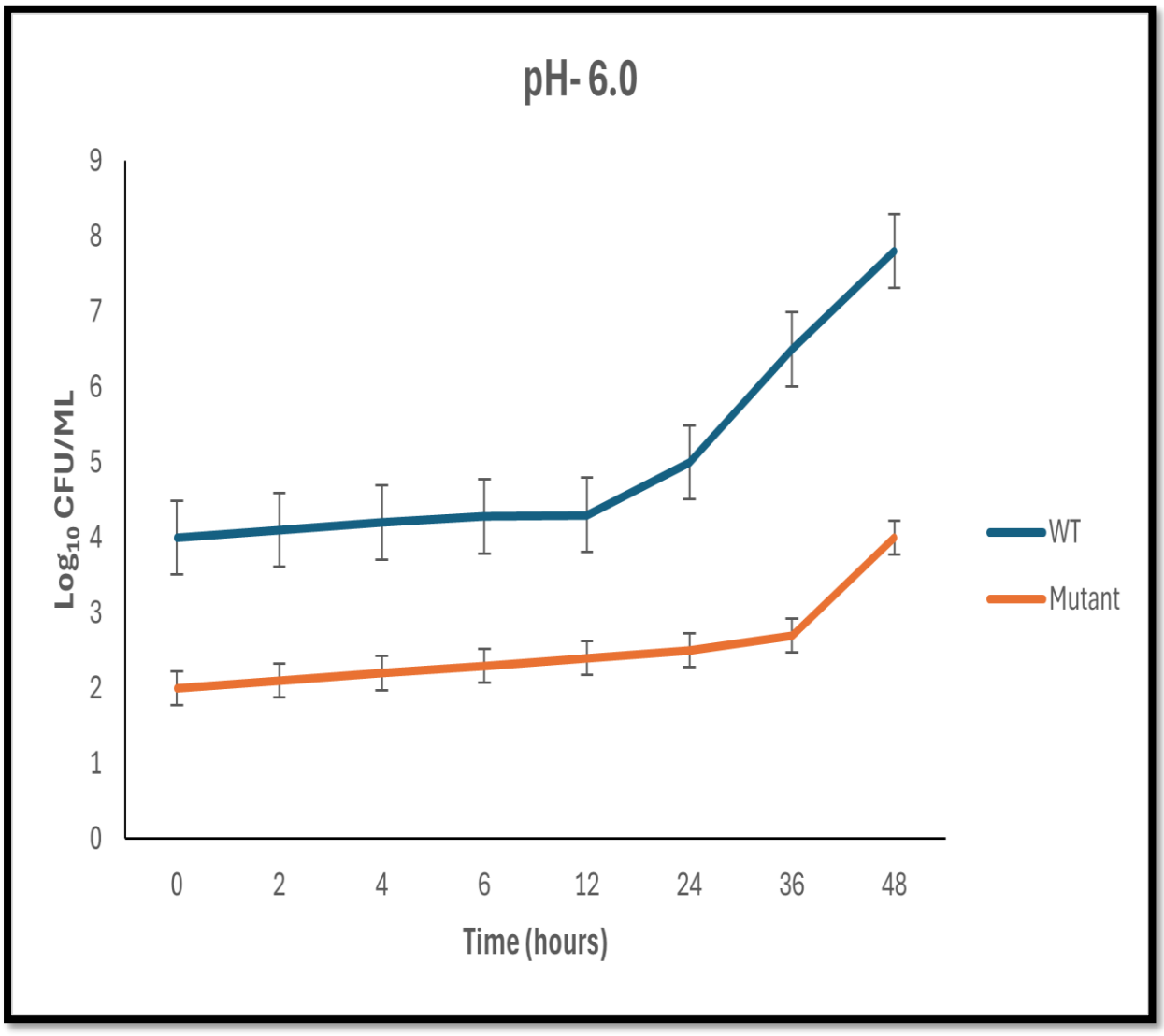


Fig 28: Survival and growth of *M. fortuitum fabG4* gene mutant knockdown strain at pH- 6.0 Figure shows that the growth of both WT and mutant increased after 24 hours while the WT showed more than 2-log decrease in its CFU.

pH- 7.0

Mutant and wild type strain of *M. fortuitum* were inoculated in MB7H9 medium of pH-7.0. The growth of both wild type and mutant strain show increase after 24 hours as shown in Fig: 29.

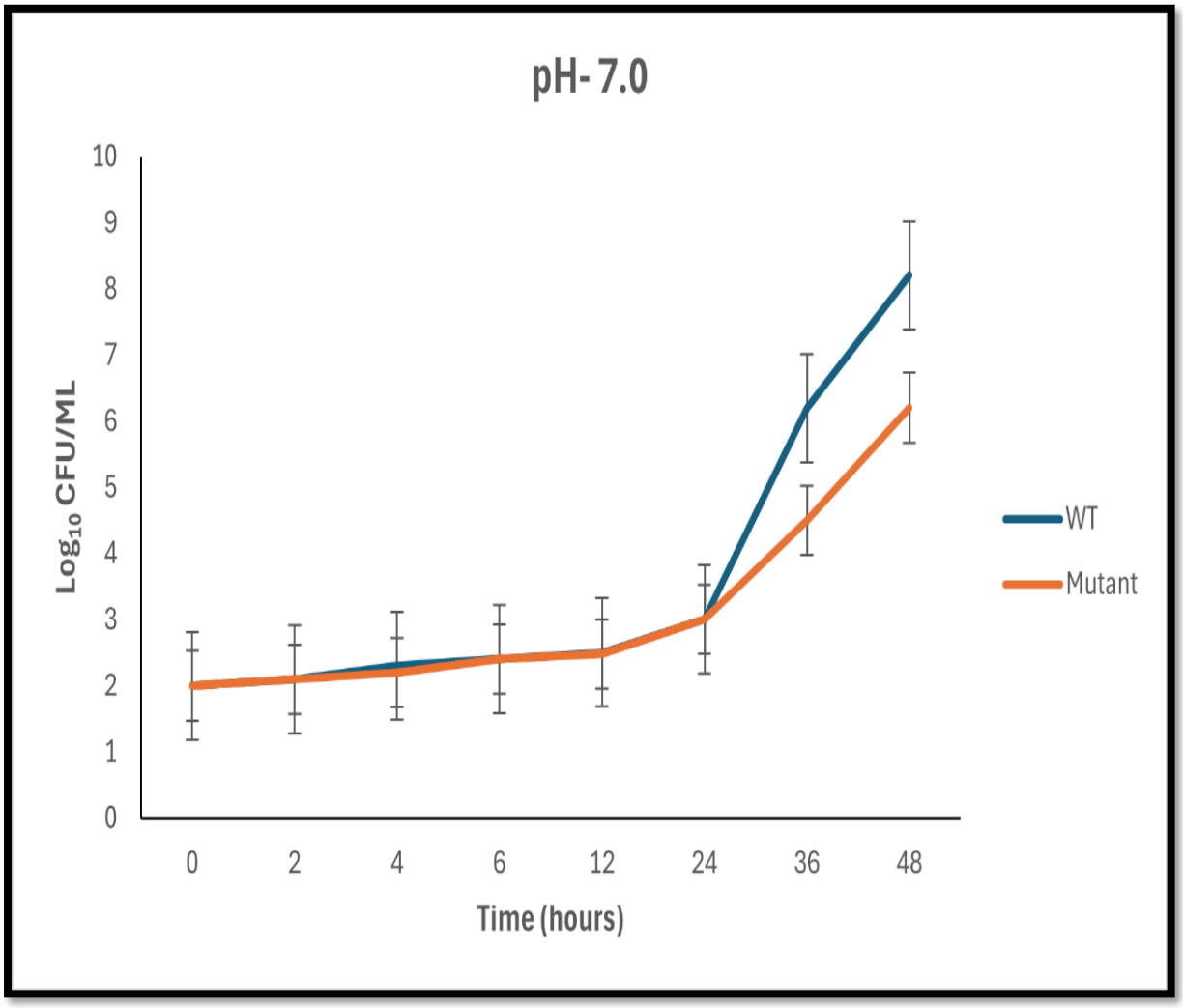
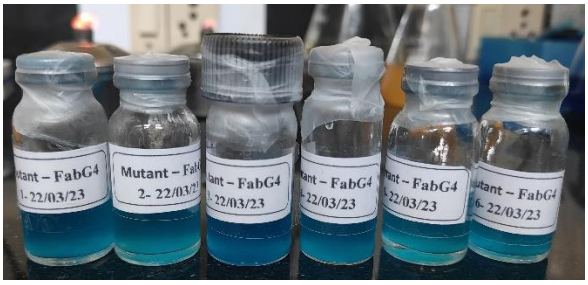


Fig 29: Survival and growth of *M. fortuitum fabG4* gene mutant knockdown strain at pH- 7.0 Figure shows that the growth of both WT and mutant equally increased after 24 hours.

4.7.2 Hypoxic Stress

Seed culture was grown in MB7H9 media and 1.5µg/ml Methylene Blue (as Oxygen indicator) was added in cultured media and aliquot in vials and covered with rubber caps. Vials were kept at 37℃ without shaking. After 6 days the colour change from blue to colorless was observed after 6 days as shown in Fig: 30 and samples were collected at different time points and plated on NAT plates to determine CFU.

0 hour (WT)



0 hour (MT)

6 days (WT)

6 days (MT)

12 days (WT)

12 days (MT)

21 days (WT)

21 days (MT)

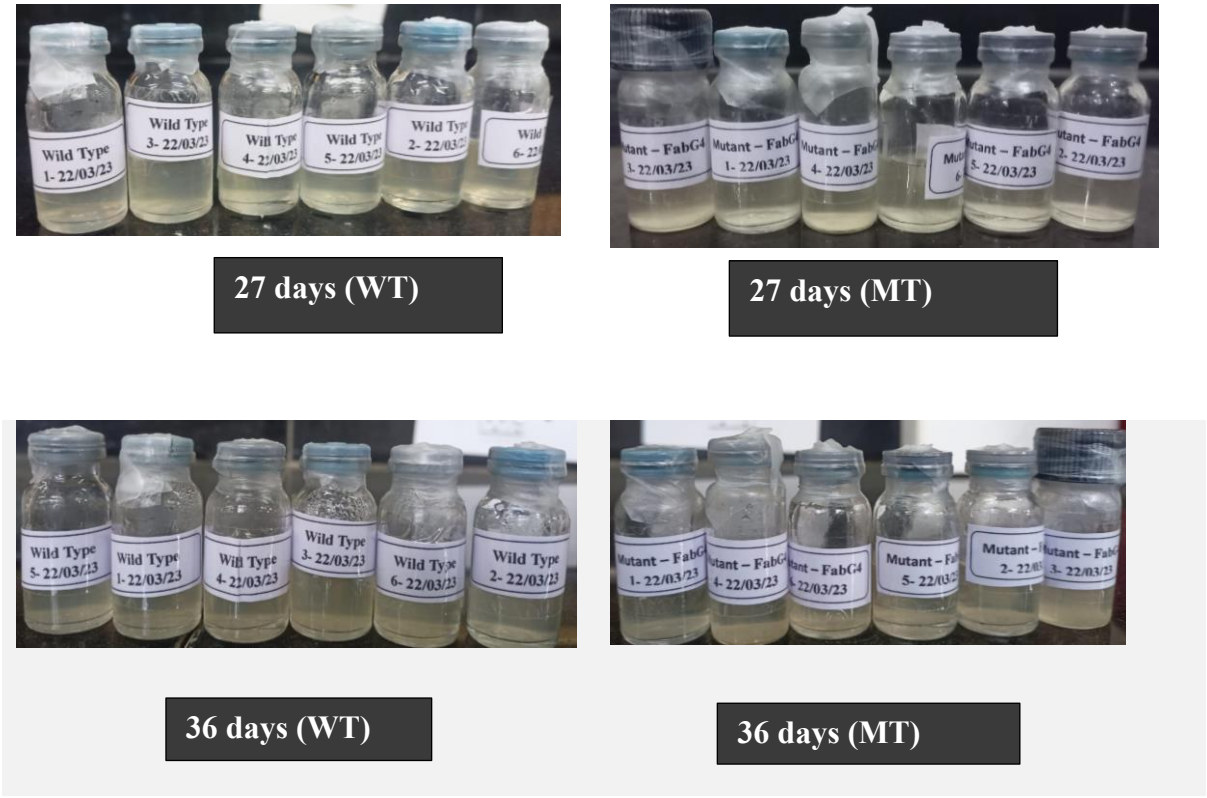


Fig 30: Hypoxic conditions. Image shows that Methylene Blue was added to the vials as oxygen indicator. After 6 days it became colorless due to the absence of oxygen and both wild type and mutant strains were in hypoxic conditions.

Mutant and wild type strain of *M. fortuitum* were inoculated in MB7H9 medium and 1.5µg/ml Methylene Blue (as Oxygen indicator) was added in cultured media. The growth of mutant strain was decline after 15 days.Whereas in case of wild type it shows a constant growth. However after 33 days no survival was seen in case of mutant strain as shown in Fig:31.

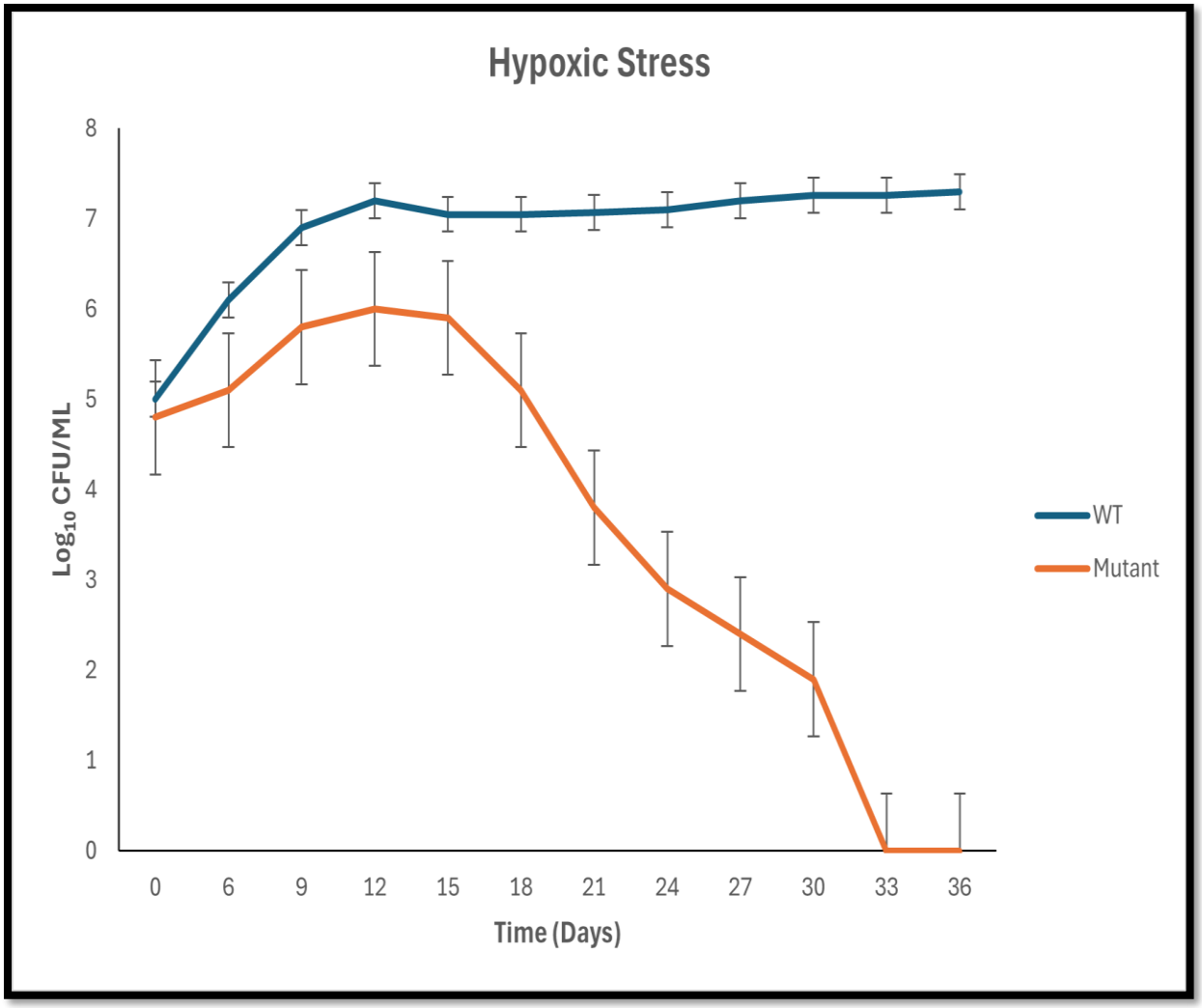


Fig 31: Survival and growth of *M. fortuitum fabG4* gene mutant knockdown strain under hypoxic stress
Figure shows that Mutant growth slows down after 15 days under low oxygen (hypoxic) stress, but wild type growth was remains constant for 36 days. There was no survival was observed in mutant strain after 33 days.

4.7.3 Nutrient Starvation

For nutrient starvation stress, 1X PBS was prepared and inoculated with Mutant (*fabG4*) strain and wild type strains of *M. fortuitum* and incubated at 37℃ under shaking conditions at 180 rpm as shown in Fig:32 and samples were collected at different time points and plated on NAT plates to determine CFU.

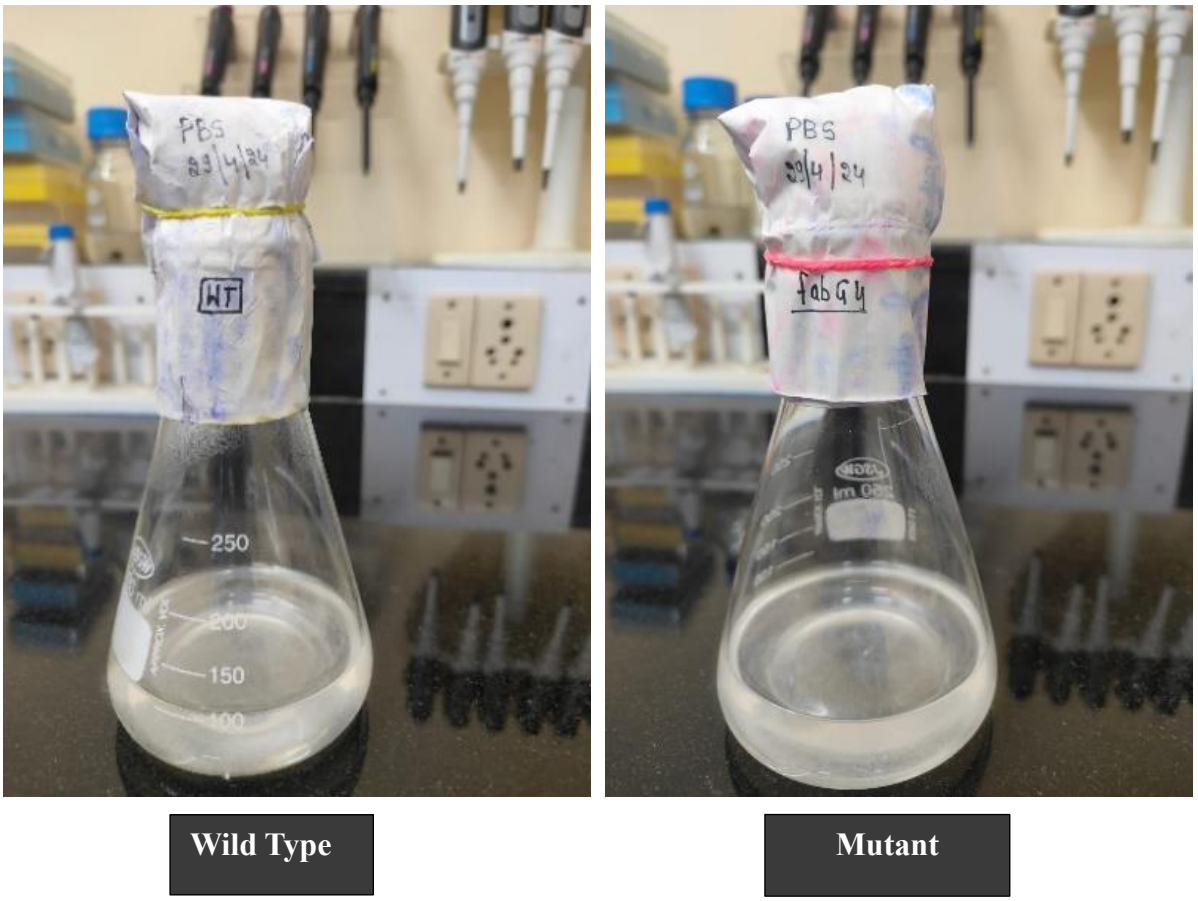


Fig 32 : Growth of wild type and mutant after 72 hours. Mutant and wild type strain of *M. fortuitum* were inoculated in conical flasks containing 1X PBS buffer. In this image we can observe the turbidity after72 hours.

Mutant and wild type strain were grown in 1X PBS buffer. After 4 hours CFU of mutant strain was observed to decline till 72 hours while the growth of wild type remained constant till 72 hours. At 72 hours the growth of mutant showed a decrease of about 2.5 log in its CFU in comparison to the initial CFU while in contrast the WT showed an increase in its CFU of about 0.7-0.8 Log as shown in Fig: 33.

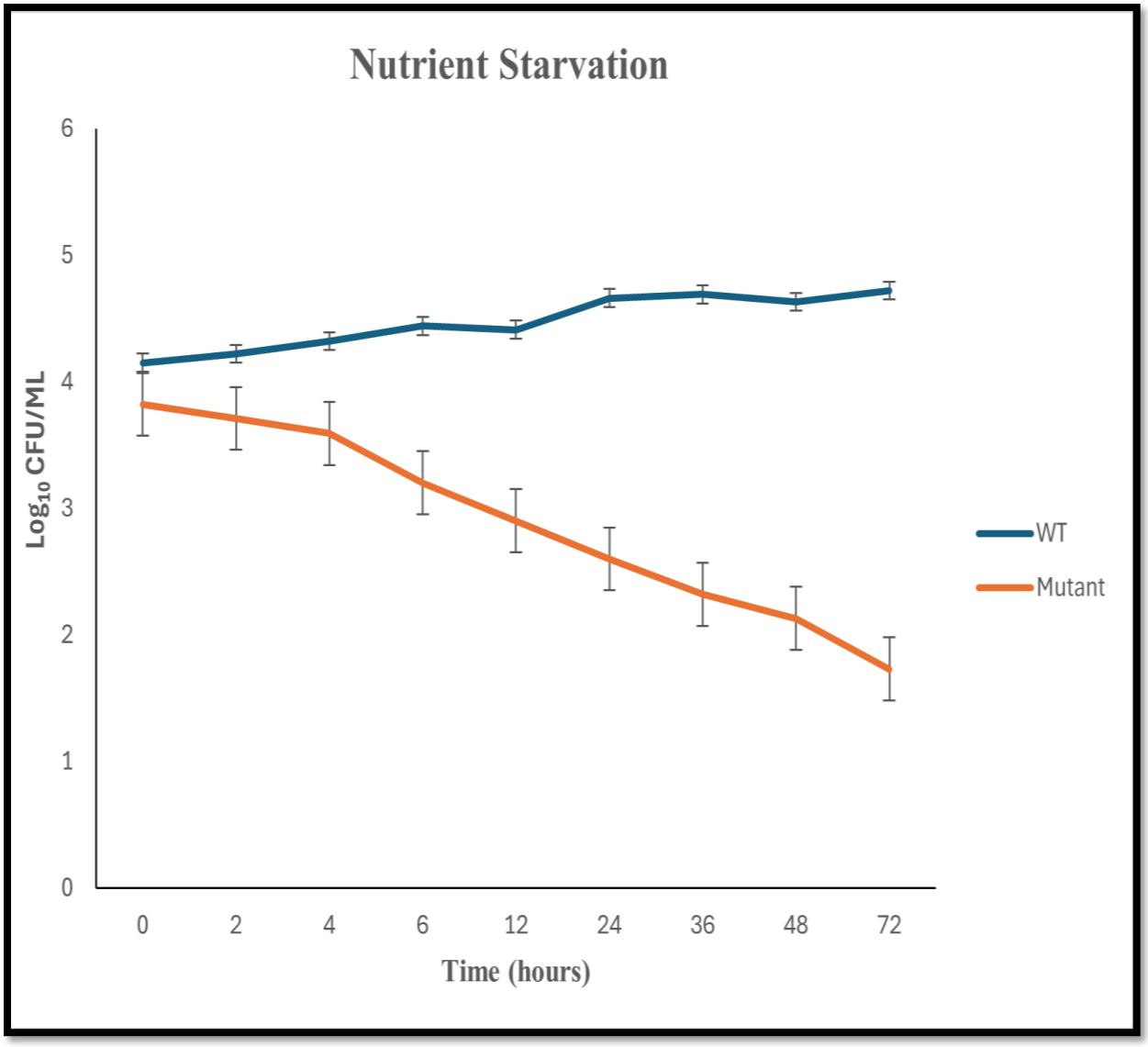


Fig 33: Survival and growth of *M. fortuitum fabG4* gene mutant knockdown strain under nutrient starvation stress Figure shows that mutant growth start declining after 4 hours and the growth of wild type remains constant till 72 hours.

4.7.4 Detergent Stress

MB7H9 media was prepared and 0.05% SDS was added and inoculated with Mutant (*fabG4*) strain and wild type strains of *M. fortuitum* and incubated at 37℃ under shaking conditions at 180 rpm as shown in Fig:34 and samples were collected at different time points and plated on NAT plates to determine CFU.

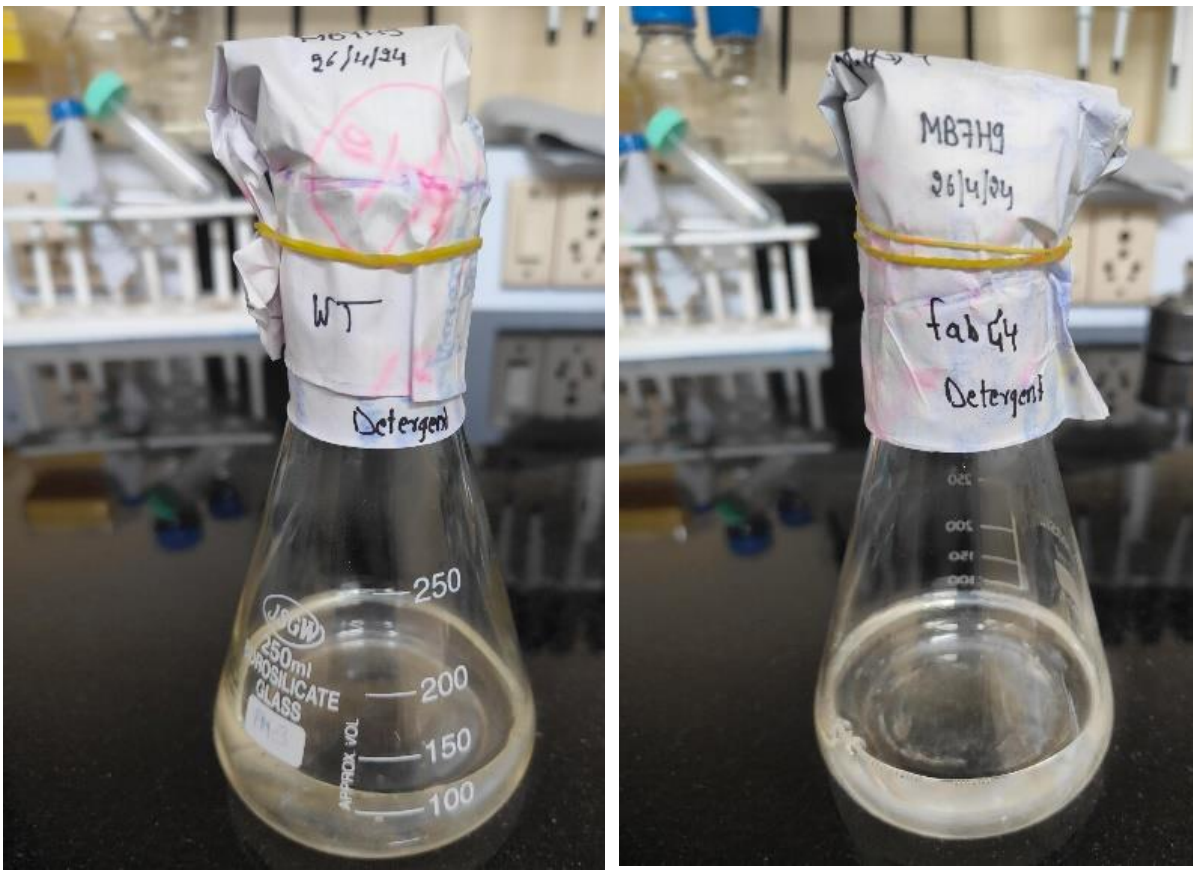


Fig 34: Mutant and wild type strain of *M. fortuitum* were inoculated in conical flasks containing MB7H9 medium and 0.05% SDS. In this image we can observe the turbidity after 72 hours.

Mutant and wild type strain were grown in MB7H9 and 0.05% SDS was added. After 6 hours the increase in growth of mutant was observed. Whereas in case of wild type showed increase after 12 hours as shown in Fig:35.

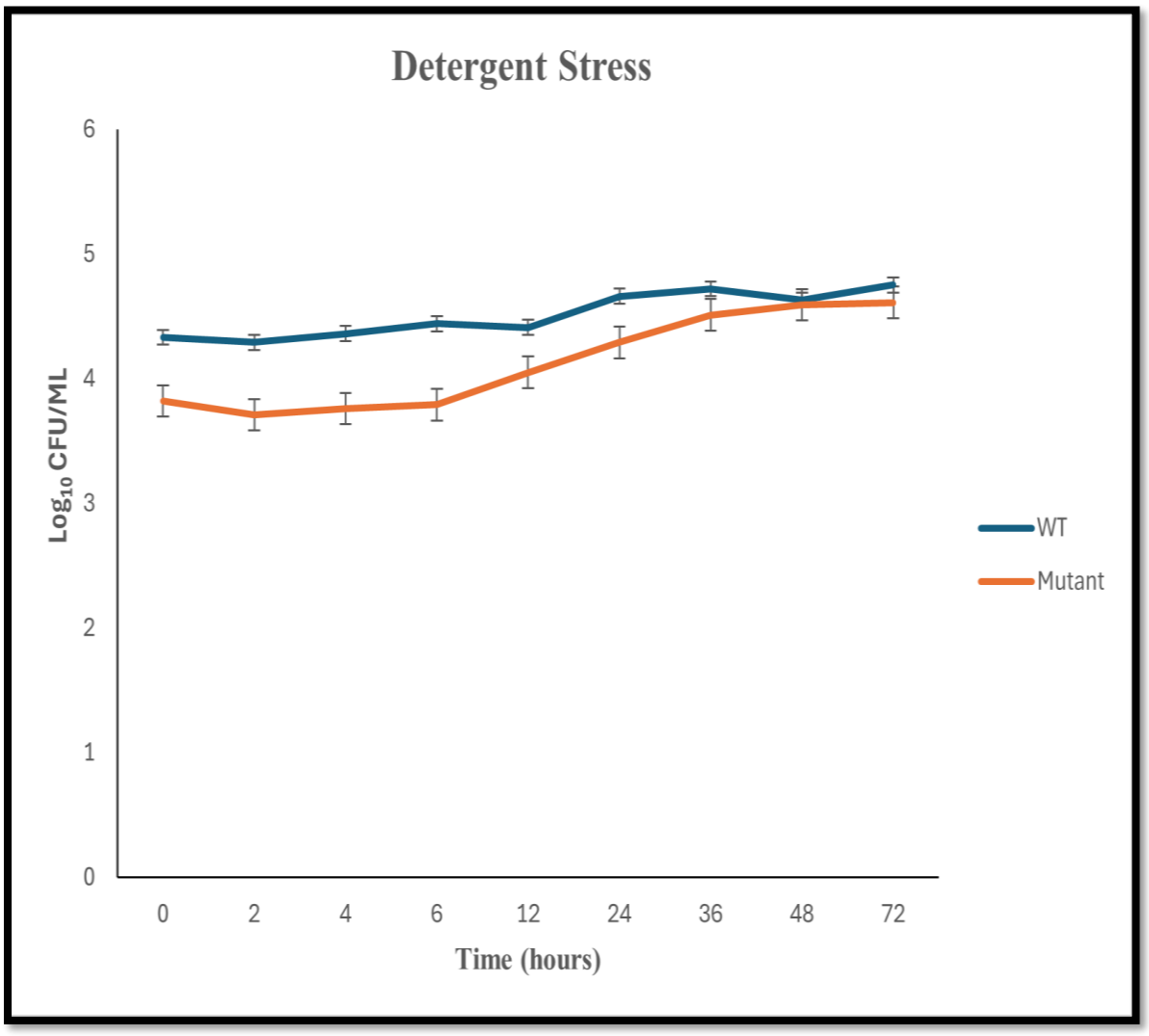


Fig 35 : Survival and growth of *M. fortuitum fabG4* gene mutant knockdown strain under Detergent stress
Figure shows that there was no difference observed in growth of both mutant and wild type strains. Both the strains wild type and mutant showed similar growth till 72 hours.

4.7.5 Oxidative Stress

MB7H9 media was prepared and 10mM H_2O_2 was added and inoculated with Mutant (*fabG4*) strain and wild type strains of *M. fortuitum* and incubated at 37℃ under shaking conditions at 180 rpm as shown in Fig:36 and samples were collected at different time points and plated on NAT plates to determine CFU.

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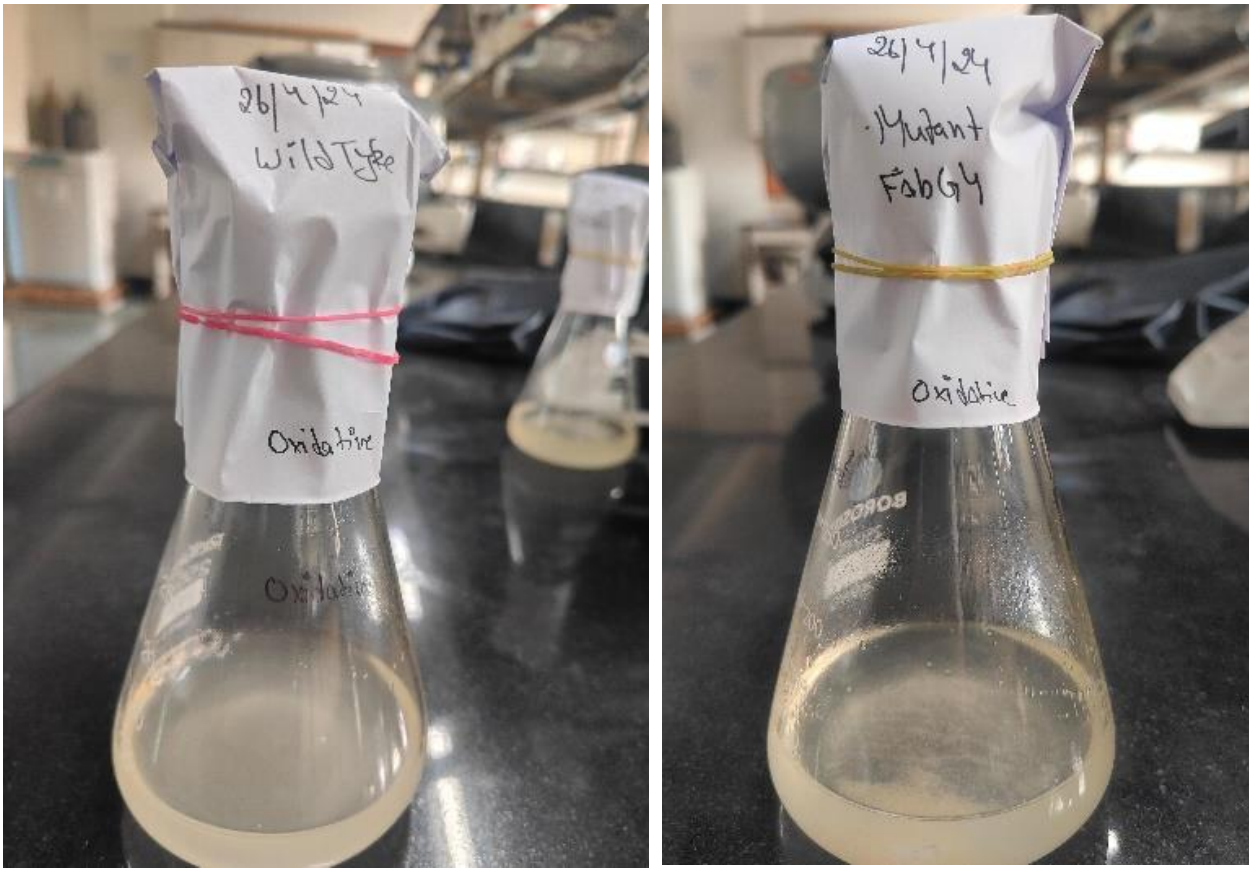

Wild Type **Mutant**

Fig 36: Mutant and wild type strain of *M. fortuitum* were inoculated in conical flasks containing MB7H9 medium and and 10mM H_2O_2. In this image we can observe the turbidity after 72 hours.

Mutant and wild type strain were grown in MB7H9 and 10mM H_2O_2 was added. After 4 hours the decline in growth of mutant was observed. Whereas in case of wild type there was increase in growth after 4 hours. However, after 72 hours the mutant showed a decline of about 1.5log in CFU in contrast to the WT which showed increase as shown in Fig: 37.

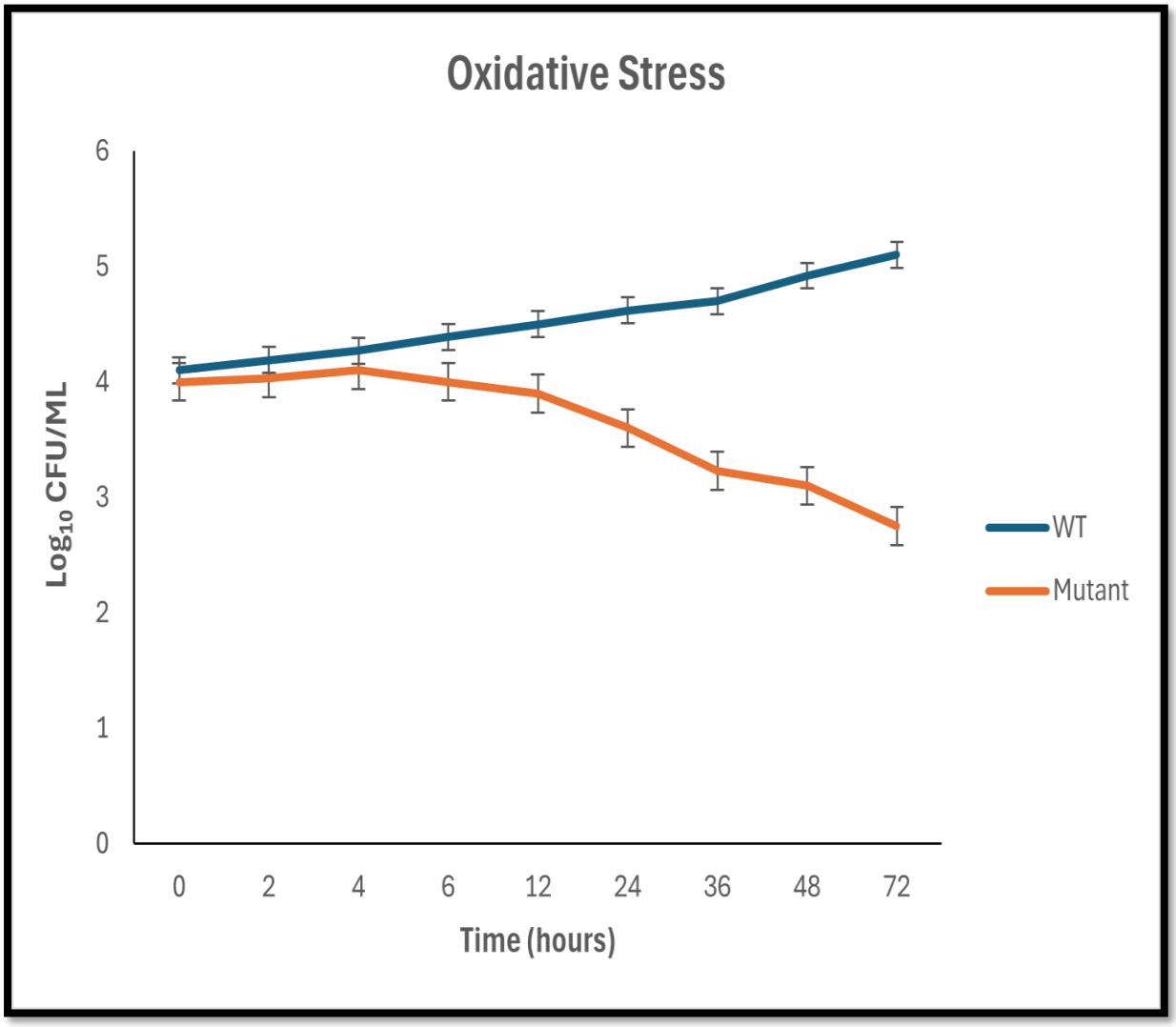

Fig 37: Survival and growth of *M. fortuitum fabG4* gene mutant knockdown strain under oxidative stress
Figure shows that After 4 hours, mutant growth starts to slow down, whereas wild type growth was observed at constant rate till 72 hours.

CHAPTER 5

DISCUSSION AND CONCLUSION

5 Discussion and Conclusion

NTM are well-known pathogens that are widely dispersed in the environment and that may infect both immunocompetent and immunocompromised people, resulting in pulmonary illnesses, infections of the skin and soft tissues, and widespread infections. With an increasing population, longer life expectancies, and a larger pool of immunocompromised people, the current global dynamic environment creates a setting where an increase in NTM illnesses is anticipated in the near future.

M. fortuitum is a nontuberculous mycobacterium (NTM) that is developing quickly and has gained interest due to its potential clinical importance in a variety of settings. Due to its widespread presence in the environment it is frequently found in dust, water, and soil—it has the potential to be an opportunistic pathogen. *M. fortuitum* can cause a variety of infections in clinical settings, including as catheter-related infections, skin and soft tissue infections, pulmonary infections in people with underlying lung diseases, can spread illnesses in individuals whose immune systems are compromised.

M. fortuitum is an opportunistic pathogen that is a member of the nontuberculous mycobacteria (NTM) group. Because of its important role in pathogenesis, it has become more and more popular in recent years. It is important to comprehend *M. fortuitum*'s involvement for a number of reasons, especially when considering human health and illness.

Because *M. fortuitum* may cause a variety of illnesses in humans, it has a noteworthy role in pathogenesis. These infections can range in severity from mild ones like infections of the skin and soft tissues to more serious ones like lung illness, disseminated infections, and infections brought on by medical operations. This wide range of infections highlights *M. fortuitum*'s flexibility and versatility as a pathogen, which makes it an intriguing research topic.

This study looked at the role of the *fabG4* gene in *M. fortuitum*, a bacterium that is well-known for its ability to thrive in harsh environments and cause opportunistic illnesses. The main goal of this study was to determine how *fabG4* affects the bacterium's ability to survive under different stressors, such as oxidative stress, detergent stress, nutritional deprivation, hypoxia stress, and acidic stress.

The results showed that *fabG4* plays a critical role in *M. fortuitum*'s ability to withstand acidic conditions. With comparison to the wild type strain, the mutant strain *fabG4* showed a much quicker fall in growth at lower pH values like pH 1.0-3.0. This implies that under acidic stress,

fabG4 helps to preserve the integrity and functioning of the cell membrane. Both strains showed comparable growth trends at pH 4.0, which is a somewhat acidic environment. This suggests that different processes may be at work at this pH level. It's interesting to note that after 24 hours, both the mutant and wild type strains showed growth at higher pH values (pH 5.0-7.0), which may indicate the existence of compensating mechanisms or a threshold wherein *fabG4* is no longer essential for survival. These results emphasize the role that *fabG4* plays in acidic stress tolerance, especially at lower pH values.

Significant differences in survival under hypoxic (low oxygen) circumstances were noted by the research. After 15 days, the *fabG4*-deficient mutant strain showed a decrease in growth, but the wild type strain continued to thrive. This implies that *M. fortuitum*'s capacity to adapt and endure in settings with low oxygen supply is dependent on *fabG4*.

Similar to this, when exposed to nutritional shortage, the wild type strain showed prolonged life whereas the mutant showed a sharp fall in growth. This suggests that *fabG4* may be implicated in mechanisms pertaining to the uptake or use of nutrients under hunger stress.

The reaction to detergent stress was different from the others. In the presence of detergent, both the mutant and wild type strains showed growth, indicating that *fabG4* may not be essential for this particular stress response.

In contrast, the wild type strain continued to thrive under oxidative stress conditions, but the mutant strain showed a reduction in growth after 4 hours. This discovery raises the possibility that *fabG4* plays a crucial role in *M. fortuitum*'s defense systems against oxidative damage.

In the current study we found that that *fabG4* is essential for the bacteria to survive under hypoxic stress (low oxygen), acidic stress (pH 1.0 -3.0), and nutrient starvation. In these circumstances, mutant strains with a non-functional *fabG4* showed a much lower viability than wild-type strains. However, it seems that the *fabG4* gene is less important for oxidative and detergent stress resistance. While only the wild type grew under oxidative stress, both wild-type and mutant strains showed similar growth under detergent stress conditions.Homologues of MF*fabG4* may be used as drug target for related pathogenic mycobacteria.

CHAPTER 6

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6 References

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CHAPTER 7

APPENDIX

7. Appendix

7.1 Growth media for bacterial culture

Media was prepared in Milli-Q water and autoclaved at 15 psi (1.05 kg/cm2) for 20 min [on liquid cycle].

7.1.1 LB medium Luria-bertani medium) [pH = 7.4]

Tryptone	10g
Yeast extract	5g
NaCl	10g

Components were dissolved in 1000 mL Milli-Q water. Solid media was prepared by adding 1.5 % agar

7.1.2 LBGT (LB broth with glycerol and Tween 80) [pH = 7.4]

LB Broth	1000 ml
Glycerol	0.5 % (v/v)
Tween 80	0.15 %(v/v)

7.1.3 Middlebrook 7H9 (MB7H9) broth [pH = 7.4, Final volume make-up = 450 mL, using Milli-Q water]

MB7H9 broth base	2.35 g
Glycerol	0.5 % (v/v)
Tween 80	0.1 % (v/v)

7.1.4 Nutrient medium (broth) [pH = 7.4]

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

Components were dissolved in 1000 mL Milli-Q water. Solid media was prepared by adding 1.5 % agar.

7.1.5 Nutrient Agar Tween 80 (NAT) [Agar = 1.5 % (w/v)]

Nutrient Broth	13 g / 1000 mL Milli-Q water
Agar	1.5 % (w/v)]
Tween 80	0.05%(v/v)

7.2 Reagents for Ziehl-Neelsen (acid-fast) staining

7.2.1 Basic fuchsin (primary stain)

Basic fuchsin	3 g
Phenol	5 % (v/v)
Ethanol (96 %)	10 mL

5 % phenol was added to 3g basic fuchsin (dissolved in 10 mL of 96 % ethanol). The volume was adjusted to 100 mL using Milli-Q water. The solution was filtered through Whatman filter paper

7.2.2 Acid alcohol (decolourizer)

3 mL HCl (concentrated) was added to 97 mL 96% ethanol.

7.2.3 Malachite green (counter stain)

Malachite green	0.25 g
Milli-Q water	100 mL (final volume)

The mixture was stirred to dissolve the solute, and filtered through Whatman filter paper.

7.3 Antibiotic

Antibiotic were sterilized by filtration through 0.22 µm filter (Millipore).

Name of Antibiotic	**Stock Solution**	**Working Concentration**
Ampicillin	20 mg/ml in dH_20	100 µg/ml

7.4 Reagents and Buffers

7.4.1 1X Phosphate Buffered saline (PBS) (pH 7.4; Final volume make up = 1000 ml, using Milli- Q water)

KH_2PO_4	0.24 g (1.8mM)
Na_2HPO_4	1.42 g (10mM)
NaCl	8.0 g (1.37 mM)
KLC	0.2 g (2.7 mM)

7.4.2 Tris-HCl buffer

Tris salt was dissolved in distilled water to prepare Tris-HCl buffer of desired strength. The pH was adjusted using concentrated HCl. 10 mM Tris-HCl (pH 8.0) was used for bacteriological work.

7.4.3 Ethylene diamine tetra acetic acid (EDTA)

0.5 M solution of disodium salt of EDTA was prepared in Milli-Q water. The pH was adjusted to 8.0 using NaOH pellets, and the stock was stored at 4°C.

7.4.4 Normal saline [8.5 g NaCl was dissolved in 1000 mL Milli-Q water]

7.4.5 Tween normal saline (TNS) [Final volume make-up = 1000 mL, using Milli-Q water]

NaCl	9g
Tween 80	0.1% (v/v)

7.5 Reagents and buffers for isolation of genomic DNA (gDNA) from M. fortuitum

7.5.1 Tris-EDTA saline (TES) buffer

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

7.5.2 Lysozyme [Storage at -20°C]

50 mg/mL lysozyme was dissolved in Milli-Q water, and sterilized using a 0.22 µm filter (Millipore).

7.5.3 Proteinase K [Storage at -20°C]

20 mg/mL proteinase K was dissolved in Milli-Q water, and sterilized using a 0.22 µm filter (Millipore).

7.5.4 Buffered phenol

Molten phenol was equilibrated once with 1M Tris-HCl (pH 8.0), and subsequently with 0.1M Tris-HCl (pH 8.0), till pH showed up in the range 7.8 - 8.0. It was submerged in 10 mM Tris-HCl (pH 8.0), and stored in a dark bottle (away from direct light), at 4°C.

7.5.5 Chloroform: isoamyl alcohol (24:1)

24 mL chloroform was mixed with 1 mL isoamyl alcohol to obtain chloroform: isoamyl alcohol solution of ratio 24:1. The solution was stored in a dark bottle, at 4°C.

7.6 Lysis buffers for isolation of plasmid DNA from *E. coli*

7.6.1 ALS-I: Glucose Tris-EDTA buffer (GTE) [Storage at 4°C]

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

A mixture containing 25 mM Tris-HCl and 10 mM EDTA (prepared using the standard stocks) was sterilized by autoclaving for 20 min at 15 psi. 50 mM glucose (sterilized using a 0.22 µm filter (Millipore) was added to the autoclaved mixture (of Tris-HCl and EDTA) to prepare the desired buffer.

7.6.2 ALS-II: NaOH-SDS mixture [prepared freshly before use]

NaOH	0.2 N (diluted from 10 N stock)
SDS	1.0 % (w/v)

7.6.3 ALS-III: Acetate mixture [Final volume make-up = 1000 mL, using Milli-Q water; Storage at 4°C]

5 M potassium acetate	60 mL (3 M w.r.t potassium and 5 M w.r.t acetate
Glacial acetic acid	11.5 mL

7.7 Agarose gel Electrophoresis

7.7.1 TAE buffer (50X) [Final volume make-up = 1000 mL, using Milli-Q water]

Tris-base	242 g
Glacial acetic acid	57.1 mL
0.5 M EDTA (pH 8.0)	100 mL

7.7.2 Gel loading dye (6X) [Final volume make-up = 10 mL, using Milli-Q water; Storage at 4°C]

Bromophenol blue	0.25 % (w/v)
Xylene cyanol	0.25 % (w/v)
Sucrose	40 % (v/v)

CHAPTER 8

PUBLICATION

8 Publications

1. Study role of *fabG4* and *hadC*genes in *M. fortuitum* under stress conditions
   Diksha Suman, Sourav Kumar, Rahul Shrivastava*
   Abstract published at Proceedings of "National Conference on Environment Sustainability – Scientific, Economic and Social Implications" organized by ROYAL COLLEGE OF ARTS SCIENCE & COMMERCE, Mumbai on February 10 2024.

2. Biogenic Silica in Ocean and Terrestrial Environment
   Diksha Suman, Sourav Kumar, Ashok Kumar Nadda, Rahul Shrivastava*
   Book chapter submitted and accepted for publication to Royal Society of Chemistry for Book entitled "Biogenic Silica"

3. Role of Oral Microbiome in Prognosis and Diagnosis.
   Sourav Kumar, **Diksha Suman**, Rahul Shrivastava*
   Book chapter submitted and accepted for Book entitled "Oral Microbiome: Hygiene and Health".

4. Oral microbiome as tool for Diagnosis and Prognosis of Disease
   Sourav Kumar, **Diksha Suman**, Rahul Shrivastava*
   Abstract submitted and accepted at National Conference on Environment Sustainability – Scientific, Economic and Social Implications organized by ROYAL COLLEGE OF ARTS SCIENCE & COMMERCE, Mumbai on February 10 2024.