ANALYZING THE ROLE OF *MYCOBACTERIUM FORTUITUM HADC GENE* UNDER STRESS CONDITIONS

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

Master of Science

In

Microbiology

By

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Under the supervision

of

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To

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May, 2024

DECLARATION

I hereby declare that the work presented in this report entitled "**Analyzing the role of *Mycobacterium fortuitum hadC* gene under 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 proficiency stream.

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

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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 MSc project report entitled **"Analyzing the role of *Mycobacterium fortuitum hadC* gene under stress conditions"** which is being submitted by **Sourav Kumar (225112004)** in fulfilment for the award of Master of Science in Microbiology by the Jaypee University of Information Technology, is the record of candidate's own work carried out by him 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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LIST OF ABBERIVATIONS

M. fortuitum	***M. fortuitum***
M. smegmatis	***Mycobacterium smegmatis***
FAS - II	**Fatty acid synthase -II**
PCR	**Polymerase Chain Reaction**
NTM	**Non-Tuberculosis *Mycobacterium***
TB	**Tuberculosis**
MTB	***Mycobacterium tuberculosis***
M. lepare	***Mycobacterium lepare***
CMN	**Corynebacterium, *Mycobacterium* & Nocardia**
SSTIs	**Skin & Soft tissue Infections.**
MAC	***Mycobacterium* avium complex**
M. kansasii	***Mycobacterium Kansasii***
M. xenopi	***Mycobacterium xenopi***
M. abscessus	***Mycobacterium abscessus***
ATS	**American Theoraic Society**
IDSA	**Infectious Diseases Society of America**
RGM	**Rapidly Growing Mycobacteria**
MF	***M. fortuitum***
BP	**Base pair**
ICNP	**International Code of Nomenclature of Prokaryotes**
ATCC	**American Type of Culture Collection**
CIED	**Cardiac Implanted Electronic Device**
pH	**Potential of Hydrogen**
E. coli	***Escherichia coli***
S. pneumoniae	***Streptococcus pneumoniae***
S. aureus	***Staphylococcus aures***
P. aeruginosa	***Pseudomonas aeruginosa***
pUC	**Plasmid of University of California**

LBGT	**Luria Broth Glycerol Tween**
NAT	**Nutrient Agar Tween**
Nacl	**Sodium chloride**
Etbr	**Ethidium Bromide**
SDS	**Sodium Dodecyl Sulphate**
CFU	**Colony Forming Unit**
TAE	**Tris – Acetate - EDTA**
°C	**Degree Celsius**
ml	**Mili Liter**
µl	**Micro liter**
mg	**Micro gram**
hrs	**Hours**

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ABSTRACT

M. fortuitum is a fast-growing, pathogenic, non-tuberculous bacteria which can infect bones and joints as well as the skin. It is an immobile, rod-shaped, gram-positive bacteria that is mostly found in water and soil. *M. fortuitum* is highly adaptable to different environments, and it survives in a variety of often difficult conditions. In this study, *hadC* was investigated in relation to survival and viability in *M. fortuitum* under different types of stress. The process of fatty acid production is essential for the mycobacterial cell membrane to develop. For the biosynthesis of fatty acid and mycolic acid *hadC* are important gene. The fatty acid synthase type-II (FAS-II) enzyme 3-hydrooxyl-ACP dehydratase (*hadC*) is important for pathogenesis and virulence in *Mycobacterium* species. The knockdown strain of *M. fortuitum* and *hadC* gene constructed in our lab was used for current study. To gain an insight into their role of *hadC* under stress conditions, the knockdown strain was studied under various *in vitro* stress conditions including nutrient starvation, acidic, hypoxic stress, detergent stress and hypoxic stress. This study showed that the *hadC* gene was not essential for its survival under acidic, detergent, hypoxic and nutrient starvation stress. But under oxidative stress mutant showed a slight decline as compared to wild type. This study emphasizes the roles that *hadC* plays 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, *hadC*, Knockdown, *in vitro*.

CHAPTER –1

INTRODUCTION

1. Introduction

Mycobacteria classified as nontuberculous mycobacteria (NTM) do not belong to the *Mycobacterium leprae* or *Mycobacterium tuberculosis* complex. Soil, tap water, and natural water sources are among the environmental elements that may contain NTM. Genus *Mycobacterium* belong to the phylum Actinobacteria and *M. leprae* and the *M. tuberculosis* (MTB) complex are found in this genus. Members of the MTB complex cause both extrapulmonary and pulmonary tuberculosis, whereas *M. leprae* causes leprosy [1]. The genus "*Mycobacterium*" contains 200 more species that are referred to as atypical or nontuberculous mycobacteria (NTM).

Although NTM does not cause tuberculosis, they can induce diseases in both immunocompromised and immunocompetent individuals that are similar to those caused by MTB. *M. fortuitum* is found in soil, water, and even home plumbing systems, As an aerobic, rod-shaped bacterium. It is a member of the *Mycobacterium* genus. *M. fortuitum* is a fast-growing non-tuberculous mycobacterium (NTM) that has drawn more attention from the scientific and medical communities since it may infect people with a range of illnesses, regardless of their immune system status. Because NTM prevalence varies geographically and reporting methods and diagnostic criteria have changed over time, it is challenging to pinpoint the exact number of NTM cases worldwide.

Nonetheless, research indicates a notable rise in diseases linked to NTM, particularly in developed countries. The true frequency of NTM infections was understated prior to the 1980s because they were commonly disregarded and incorrectly identified as tuberculosis (TB) or other respiratory diseases. As awareness increased and diagnostic tools became more advanced, the number of confirmed NTM cases increased over time [2]. Even though *M. fortuitum* is widely distributed, it is frequently regarded as an opportunistic pathogen that mostly affects those with weakened immune systems or underlying medical disorders.

One of *M. fortuitum's* most important traits is its capacity to grow quickly—usually in a matter of days—as opposed to *M. tuberculosis*, which grows more slowly and is the causal agent of tuberculosis [3]. A wide range of clinical symptoms, including skin and soft tissue infections, respiratory disorders, and widespread illness, have been linked to *M. fortuitum*. Among the most often reported manifestations are infections of the skin and soft tissues, including cellulitis, osteomyelitis, and abscesses. It's interesting to note that *M. fortuitum* has also been linked to

respiratory tract infections, including pneumonia, in both immunocompetent and immunocompromised people.

The growing frequency of *M. fortuitum* infections and its intrinsic resistance to certain antimicrobial treatments highlight the need of comprehending the molecular processes governing its survival and adaptability in a variety of environmental niches and stresses. Its adaptability to a variety of settings shows that it has built-in defences against different types of stress. Researchers have studied the effects of stress on both mutant & wild type strains of *M. fortuitum* in order to comprehend these mechanisms [4].

The bacterium's response to a variety of stresses has been examined in these investigations, including exposure to detergent stress, low oxygen availability (hypoxic stress), nutrient deprivation (nutrient starvation), acidic environments (acid stress), and the harmful effects of reactive oxygen species (oxidative stress). Researchers want to pinpoint the genes and physiological pathways essential for *M. fortuitum*'s resilience by comparing the survival rates and physiological responses of wild type and mutant strains under these conditions. The development of tailored treatments to fight infections brought on by an opportunistic pathogen may benefit from this knowledge.

1.1 Objectives:

- To investigate the role of the *M. fortuitum* gene *hadC* in stress response.
- ´To analyse the effect of *hadC* knockdown on *M. fortuitum* growth and survival under various stress conditions, acidic stress, oxidative stress, and hypoxic stress & detergent stress.

Chapter –2

REVIEW OF LITERATURE

1. **Review of Literature**

2.1 *Mycobacterium*

Mycobacterium is one of the earliest known genera. Originally, a collection of microorganisms that developed as mould-like pellicles on liquid medium were referred to by the generic term *Mycobacterium* [1]. The genus *Mycobacterium* in the *Actinomycetales* order and *Mycobacteriaceae* family includes Mycobacteria. Generally lacking aerial hyphae, galactose, arabinose, and meso-diaminopimelic acid are found in the cell walls of mycobacteria, which are aerobic, fast-acting, rod-shaped actinomycetes that sporadically branch. Their guanine and cytosine (GC) deoxyribonucleic acid (DNA) base ratios range from 62 to 70 mol%. [1], [2].

A wide range of microorganisms can be classified as actinomycetes based on their morphology or ecology. The species that are part of the group *Corynebacterium, Mycobacterium*, and *Nocardia* [CMN] are easily separated from mycobacteria; this group also contains the genera *Rhodococcus, Gordona, and Tsukamurella* [3]. Mycobacteria are capable of producing mycolic acids, which are high molecular weight β-hydroxy fatty acids with a long α-side chain. Members of the Mycobacteriaceae family are known as mycobacteria. The heightened lipid content of these organisms is typified by an abundance of waxes known as mycolic acids.

The organism resists acid alcohols' ability to decolorize it because of these mycolic acids. The term "acid-fast" describes these cells [4]. If an acid-fast stain, such Ziehl-Neelsen or modified Kinyoun stains, is used, the cells frequently take the shape of beaded rods. It is very important to distinguish between mycobacteria and partly acid-fast species of the genera *Nocardia or Rhodococcus*. There are three types of species in the genus *Mycobacteria:* saprophytes, opportunistic pathogens, and pathogens [5], [6], [7]. *Mycobacterium* species has NTM (Non-Tuberculosis Mycobacterium) and MTB (*M. tuberculosis*). One is responsible for causing tuberculosis and NTM is responsible for causing other infections such as skin, eyes etc.

2.2 MTB (*M. tuberculosis*)

The bacteria that cause tuberculosis (TB), *M. tuberculosis*, is extremely sophisticated and adaptive and has coexisted with humans for thousands of years. These bacteria can withstand a variety of popular antibiotics and endure severe environments because of its special cell wall structure, which is rich in lipids [8]. It mostly affects the lungs, though it can affect other regions of the body as well. The bacteria are spread from person to person by airborne particles, which are frequently expelled into the atmosphere when an infected person talks, sneezes, or

coughs. The germs may enter the host's lungs by inhalation, where they may stay dormant or develop into an active case of tuberculosis [9].

It is important to distinguish between latent tuberculosis infection and active tuberculosis illness; the former is very symptomatic and infectious. Medical history, physical examination, skin, blood, and imaging tests are all used in the diagnosis of tuberculosis (TB). Sputum cultures or molecular diagnostics are frequently needed for confirmation in order to detect the presence of *M. tuberculosis* DNA [10]. A course of various antibiotics lasting at least six months is usually required for treatment; prominent first-line medications include isoniazid, rifampin, ethambutol, and pyrazinamide. Treatment and public health initiatives are faced with serious obstacles due to the advent of extensively drug-resistant TB (XDR-TB) and multidrug-resistant TB (MDR-TB).

A multimodal strategy is needed to manage tuberculosis (TB) worldwide [8], [11]. This includes administering the Bacille Calmette-Guérin (BCG) vaccine, improving living circumstances through public health programs, and lowering the risk of transmission in high-risk groups. Even with improvements in detection, management, and avoidance, tuberculosis (TB) continues to rank among the world's leading infectious disease deaths, especially in poor nations and among communities where HIV incidence is high [12].

2.2 NTM (Non-Tuberculosis mycobacterium)

Species that do not cause TB are known as non-tuberculous mycobacteria (NTM), which are distinct from the *M. tuberculosis* complex [13]. Non-Tuberculosis Mycobacterium are essentially free-living, naturally occurring creatures. As of now, around 140 NTM species have been discovered. Pulmonary infections are the most common (65–90%) of the several mycobacterial illnesses they may cause [14].NTM has historically been categorized based on pigment formation and growth rate. The slow growers, Types I, II, and III, are categorized based on their pigment formation, and take seven days or longer to grow [15].

Bacteria are classified as photochromogens (type I) if they create pigment only when exposed to light, scotochromogen (type II) if they produce pigment in the dark, and non-photochromogens (type III) if their pigmentation is weak. Even while rapid growers (type IV) grow faster than seven days, they nonetheless develop more slowly than the majority of other bacteria [16]. NTM are opportunistic pathogens, which may be found in both natural and anthropogenically related situations. Opportunistic pathogens are microorganisms that can

become pathogenic following a disturbance of their host, such as a past illness, co-infection, immunodeficiency, or age [17].

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 illnesses linked to NTM. Incidence rates for TB and NTM illnesses decreased by 81% and 94%, respectively, between 1946 and 2014, according to a thorough analysis [18]. The expanding appearance of most NTM as human pathogens is the reason for the increased rate of NTM infection.

There are now more reported NTM species than ever before, which makes it more difficult to correctly identify them using both molecular and traditional methods that look at phenotypic features [19], [20]. Major NTM species associated with human disease: see Table 1.

Table 1: Major *Mycobacterium* species along with the infections they cause to humans.

S.No	Species	Infections
1	*Mycobacterium intracellulare*	Gastrointestinal infections.
2	*M. fortuitum*	Skin, bone infections and soft tissue
2	*Mycobacterium kansasii*	Skin and Pulmonary disease.
3	*Mycobacterium paratuberculosis*	Johne's disease.
4	*Mycobacterium scrofulaceum*	Tissue infection.
5	*Mycobacterium simiae*	Pulmonary infections
6	*Mycobacterium ulcerans*	Buruli ulcer
7	*Mycobacterium interjectum*	Lymphadenitis in children and pulmonary involvement in adults.
8	*Mycobacterium immunogenum*	Hypersensitivity pneumonitis.

9	***Mycobacterium chelonae***	**Musculoskeletal, pulmonary infections.**
10	***Mycobacterium marinum***	**Nodular granulomatous disease**
11	***Mycobacterium* neoaurum**	**Urinary tract infections, skin infections**
12	***Mycobacterium wolinskyi***	**Skin and soft tissue infections (SSTIs).**
13	***Mycobacterium septicum***	**Surgical-site infections**
14	***Mycobacterium thermoresistible***	**Causes diseases in patients with HIV**

2.3 Clinical significance: NTM

A type of environmental microbes known as non-tuberculous mycobacteria (NTM) can cause skin and soft tissue infections, disseminated sickness, lung ailments, and lymphadenitis in humans. [21]. The individual species and the patient's underlying health condition determine the therapeutic importance of NTM. Non-tuberculous mycobacteria (NTM) are mycobacteria other than *M. leprae* and *M. tuberculosis* complex [22]. Four unique clinical syndromes are recognized for 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 [23].

NTM can result in a persistent infection in lung illness that is frequently mistaken for TB. The NTM species *M. kansasii, M. avium complex (MAC), M. xenopi, and M. abscessus* are most frequently linked to pulmonary illness. It can be difficult to determine the clinical significance of NTM isolated from respiratory samples since certain species may be colonists rather than pathogens [24]. Nonetheless, several radiographic and clinical characteristics can be used to assess the clinical importance of NTM isolates. For example, fibrocavitary lesions, weight loss, fever, and nodular bronchiectatic lesions on chest CT are significantly associated with NTM-PD [23].

Research indicates that diseases linked to (NTM) are increasing. A rising percentage of mycobacterial diseases in both industrialized and developing nations are caused by NTM. Non-tuberculous mycobacteria are common environmental organisms that are often isolated from soil, tap water, and surface water [25]. Consequently, there is insufficient evidence to support the existence of NTM lung illness based only on the isolation of NTM species from a respiratory sample. There are people who have NTM infection yet show no signs of lung illness. An infection of this kind might be a sign of colonization or a passing illness.

Therefore, to diagnose NTM lung sickness, several criteria related to clinical, radiographic, and microbiologic aspects must be met [26]. The Infectious Diseases Society of America (IDSA) and the American Thoracic Society (ATS) most recently updated the diagnostic criteria for non-tubercular lung disease (NTM) in 2007 [27]. 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 increased by 81% and 94%, respectively, between 1946 and 2014, according to a thorough investigation of the link between the two diseases. The majority of NTM are increasingly emerging as human pathogens, which is the cause of the elevated rate of NTM infection [28].Among the rapidly growing mycobacteria (RGM) is *M. fortuitum*. These bacteria are characterized by their in vitro resistance to antimycobacterials and their capacity to establish colonies in less than one week, setting them apart from other NTM. In several nations, in clinical laboratories, *M. fortuitum* is a commonly isolated organism from respiratory specimens [29], [30].

2.4 *M. fortuitum*

Eighty years ago, *M. fortuitum* was initially isolated from frogs. Da Costa Cruz identified *M. fortuitum*, a potentially new species of mycobacteria with fast growth, from a skin abscess in 1938[15], [31]. Infrequent cases of disease caused by this fast-growing *Mycobacterium* were documented throughout the following 20 years. *M. fortuitum* belongs to the Runyon group IV and is a fast-developing nontuberculous mycobacterium (NTM) [32]. It is commonly isolated from both respiratory and non-respiratory tissues, and it has therapeutic significance for both humans and animals [33]. The *M. fortuitum* complex is recognized for its high-level drug

resistance indicators and consists of opportunistic pathogens that are present in dust, soil, and water.

The *M. fortuitum* is one of the main causes of pulmonary NTM illnesses and can infect the central nervous system. Additionally, it makes immunocompromised people more susceptible to widespread infections. Mycobacteriosis, bronchiectasis, and cystic fibrosis are risk factors for lung infections caused by *M. fortuitum*[38]. The tight relationship between *M. fortuitum* CT6 and MF GZ001 was confirmed by phylogenetic analysis and average nucleotide identity (ANI) study. Although *M. fortuitum* infections are uncommon, they have the potential to spread diseases, such as lung infections and brain tumors. Strong pathogenicity is displayed by *M. fortuitum*, which has been associated with many virulence genes, including antigen 85 complex clusters*, nar, sigma,* and *mce* [39].

These genes may encode putative virulence factors linked to the lipid and protein metabolism, suppression of apoptosis, mycobacterial cell envelope, gene control, and nitrate reductase activity. The invasion and persistence of mycobacteria in the host are caused by the *mce* operons, which are found in several types of mycobacteria [40]. Because *M. fortuitum* has a high degree of innate resistance and the ability to build biofilms on surfaces, which increases bacterial persistence, treatment options for this illness are complicated. Multidrug treatment is advised by guidelines, although none of the medications now in use depend on inhibiting the production of mycolic acid, which is a verified mechanism of action for the first-intent drug INH and the second-intent medicine NITD-916 [41].

More study is needed to establish the optimum form of administration, length of therapy, and whether surgery is always necessary for such infections, as the best course of action and results are not well recognized. Drug resistance in *M. fortuitum* is well recognized, and this resistance stems from several pathways, including intrinsic and mutational resistance to antibiotics. Upon examination of its drug resistance mechanisms, *M. fortuitum* has been discovered to be resistant to several first-line TB drugs [38], [42], [43].

2.5 Taxonomy

M. fortuitum is a species of bacteria that is a member of the Actinobacteria phylum and genus *Mycobacterium* [44]. Study published the species' original description in 1938, and the International Code of Nomenclature of Prokaryotes (ICNP) officially adopted it in 1980. 6841, also known as CCUG 20994, ATCC 6841, CIP 104534, DSM 46621, NCTC 10394, IFO 13159, NBRC 13159, and JCM 6387, is the type of strain of *M. fortuitum*. The species' 16S rRNA gene sequence, AJ536039, places it in the phylum Actinobacteria according to genome-based taxonomy [45], [46], [47].

- **Name**: *M. fortuitum* (Approved Lists 1980) [45]
- **Type strain**: 6841; DSM 46621; ATCC 6841; CIP 104534; CCUG 20994; JCM 6387; IFO 13159; NBRC 13159; NCTC 10394 [46].

2.6 Epidemiology

In the global context, there has been a noticeable rise in NTM infection cases in recent years. The rise in NTM infection sources, vulnerable population, advancements in laboratory detection methods, and public knowledge of NTM illnesses are all responsible for the increase. Due to the rising incidence of NTM infections worldwide, the epidemiology of non-tuberculous mycobacteria (NTM) and *M. fortuitum* is a complicated and multidimensional subject of study that has attracted a lot of attention recently. There have been reports of NTM infections globally, including those brought on by *M. fortuitum*, with certain places showing a higher incidence than others.

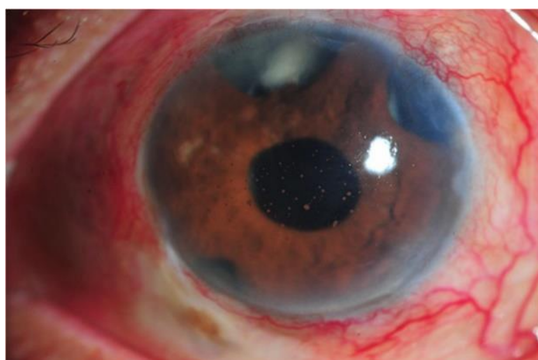
Although studies from Australia, North America, China, India, Africa, the Middle East, and Europe indicate a worldwide distribution of these diseases, the epidemiological research of NTM infections is still in its early phases. All age groups are affected by NTM infections, such as *M. fortuitum*, but middle-aged and older people are more likely to experience them—65.5% of cases occur in people over 45 yrs. The mid-fourth decade of life is usually when patients with *M. fortuitum* infections associated with cosmetic and reconstructive surgery are younger. Distribution of Species and Drug Sensitivity The species distribution of NTM isolates varies by geographical area; worldwide, *M. avium complex (MAC*) is the most frequent cause of NTM lung illness.

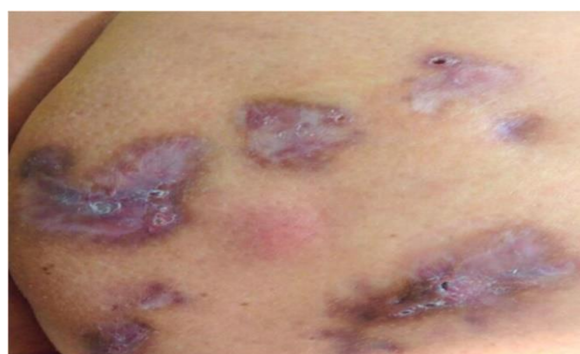
At least 50 strains of *M. fortuitum* have been discovered; it belongs to the Runyon group IV of fast developing NTM and is found in both soil and water. Since NTM isolates frequently exhibit resistance to several antibiotics, treatment of these organisms might be difficult, raising serious concerns about their drug sensitivity. NTM can tolerate a broad range of ambient temperatures and are widely distributed in the environment. They may be found in soil, vegetation, and water systems. Human living overlaps with the natural habitats of non-timber marine mammals (NTM), which include brackish and marshy waterways, municipal water distribution systems, and potting soil. This creates the potential for human infection.

Certain groups may be more vulnerable to NTM infections due to behavioural variables like medical tourism and biologic ones such underlying structural flaws and abnormalities of the gastrointestinal tract. Health outcomes and regional economic growth may be favourably correlated with the prevalence of NTM infection. Worldwide, NTM illness incidence and mortality have been rising significantly. NTM is spreading through person-to-person contact and has developed a high level of antibiotic resistance. The areas where NTM infections are more prevalent are predicted to grow because of climate change.

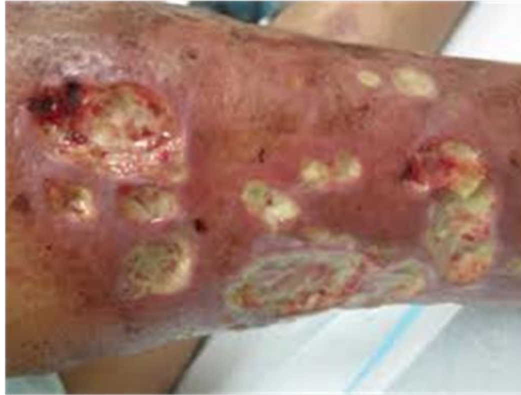
2.7 Infection caused by *M. fortuitum*

It is a wide variety of illnesses that can be caused by *M. fortuitum*. It can also cause skin infection and soft tissue infection. Some representative pictures of *M. fortuitum* infections are as shown in Fig: 1 below:

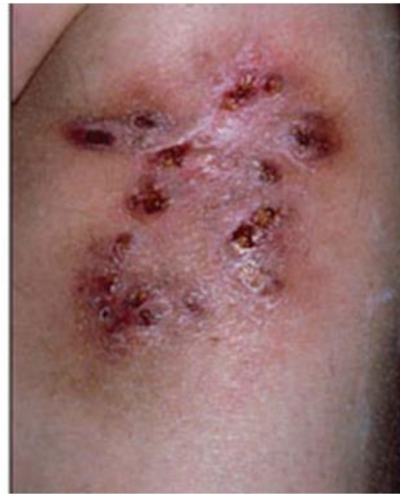




(a) (b)



(c)



(d)

Fig1: (a) eye infection caused by *M. fortuitum,* (b), (c), (d) tissue and skin infections of *M. fortuitum.*

2.7.1 Skin Infection

A fast-growing nontuberculous *Mycobacterium* that can cause a variety of skin and soft tissue infections is called *M. fortuitum*. These infections are frequently linked to intrusive operations such as surgery, injections, trauma, or other treatments. Immunocompetent people usually develop a single abscessed lesion in the injured area 4-6 weeks after the triggering event; in 20–30% of instances, this lesion may heal on its own. Nonetheless, without a certain history of prior trauma, immunocompromised people are more prone to develop diffused, numerous subcutaneous nodules [10].

Soft tissue and skin infections caused by *M. fortuitum* can present with a variety of clinical presentations, such as nodules, non-healing ulcers, and persistent sinus discharges. Treatment-resistant and prone to recurrence, these infections are frequently subacute or chronic. Depending on the patient's immunological condition and the length of the lesions, the histology results might vary. Culture is usually necessary for diagnosis since the organisms are readily missed on regular histological inspection. In certain areas, like Korea, *M. fortuitum* is the most

prevalent fast-growing mycobacterium that causes infections of the skin and soft tissues. The upper and lower extremities were the most frequently involved areas in a survey of 26 cases in Korea [37]. Medical treatments such as fat grafting, injections, acupuncture, and liposuction were frequently the source of infection.

2.7.2 Eye infection.

It is well recognized that *M. fortuitum* causes infections of the eyes, especially keratitis and corneal ulcers. It might be difficult to properly detect and treat certain illnesses. Patients who have eye infections caused by *M. fortuitum* often exhibit discomfort, ulceration, stromal inflammation, and conjunctival hyperemia. The confirmation of *M. fortuitum* eye infections is achieved by means of corneal scrapings culture and acid-fast staining, both of which are crucial in determining the bacterium's existence [12]. Trauma or other types of eye injuries can result in *M. fortuitum*-caused eye infections.

Patients may get keratitis or corneal ulcers because of these events; these conditions can be dangerous and need to be treated very once to avoid complications and additional eye damage. *M. fortuitum* infection can also cause nonpruritic papular eruption, which appears 1-2 weeks following tattoo operations [13]. This shows the variety of ways that this bacterium can harm the eye and surrounding tissues. *M. fortuitum* presents a serious concern when it comes to eye infections, particularly when the illness is linked to trauma or surgical treatments.

To properly treat an infection, prompt and precise diagnosis is essential for starting the right course of treatment, which frequently entails a combination of antibiotic medication and, in some circumstances, surgical intervention. *M. fortuitum*-caused eye infections need specialist treatment and careful observation to minimize complications and guarantee the best possible results for the patient.

2.7.3 Implanted device Infection

There have been accounts of *M. fortuitum* leading to infections in cardiac devices, including infections in pacemakers. These infections need to be treated very away since they might be fatal [14]. Rapidly growing mycobacteria (RGMs)-induced post-implantation cardiac implanted electronic device (CIED) infections typically show early start, localized redness, swelling, and spontaneous drainage at the implant site, frequently without systemic symptoms. The thorough removal of the contaminated device and the use of the proper antibiotics are typically required for the treatment of such illnesses.

Patients undergoing surgery, especially those requiring the installation of medical equipment, are more susceptible to *M. fortuitum* infection in implanted devices. Bacteria can enter the body through contaminated water and equipment, resulting in illness [23].

2.8 Treatments for *M. fortuitum* infections

Tobramycin, Amikacin, Ciprofloxacin, Levofloxacin, Imipenem, Clarithromycin, Cefoxitin, Linezolid, and Sulfamethoxazole are among the drugs that might harm *M. fortuitum*. Since *M. fortuitum* has developed a mutational resistance to quinolones, treating *M. fortuitum* infections with quinolones alone—clarithromycin—needs to be combined with another antimicrobial medication. The reason for *M. fortuitum*'s in-vitro resistance to macrolides, on the other hand, is the presence of an inducible erythromycin methylase (*erm*) gene. For this reason, the precise combination of anti-mycobacterial medications for chemotherapy must be considered.

Within 4-6 months, a combination of an aminoglycoside (amikacin) and β-lactam (imipenem or cefoxitin) or quinolone (L-ofloxacin or ciprofloxacin) is used to treat serious problems caused by *M. fortuitum* [41]. *M. fortuitum*-induced lung infections can be cured in 12 to 24 months with oral or intravenous administration of minocycline, sulfamethoxazole, and moxifloxacin.[47]. Nonetheless, the ATS advises routine susceptibility testing for RGM to linezolid, amikacin, cefoxitin, fluoroquinolones, clarithromycin, tobramycin, and imipenem because to the rising NTM drug resistance [48].

Additionally, research is being done on novel drugs for the treatment of *M. fortuitum* infections. Tigecycline has anti-*M. fortuitum* action [49]. 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 [50].

2.10. Biofilm formation.

An arrangement of bacteria submerged in an extracellular polymeric substance (EPS) matrix that they self-produce and often adhere to surfaces is called a biofilm [54]. Biofilms may grow on a variety of surfaces, such as natural aquatic systems, industrial or potable water system pipework, living tissues, and medical equipment [55]. In terms of gene expression and metabolic activity, the microorganisms within a biofilm are distinct from their counterparts that are planktonic, or freely floating. Free-floating bacteria attach themselves to a surface to start

the biofilm development process [56]. Next, they produce extracellular polymeric substances (EPS) that help the microbes cling together and build intricate three-dimensional structures.

The bacteria are protected by the EPS matrix against desiccation, antibiotics, the host immune system, and predators. Biofilms are made up of one or more species of microorganisms, such as bacteria, fungus, and protists, and may be found in a variety of habitats, including manmade and natural surfaces [57], [58]. Fossil records from 3.25 billion years ago contain biofilms, demonstrating the organisms' lengthy evolutionary history and capacity for environmental adaptation [59]. Numerous elements, such as surface characteristics, nutrition availability, fluid dynamics, and the presence of other microbes, affect the development of biofilms [55], [56]. Human actions can also have an impact on biofilms.

For example, the usage of medical equipment, water treatment, and food preparation can all lead to circumstances that encourage the creation of biofilms [58].A frequent mycobacterial species found in soil and other aquatic habitats is *M. fortuitum*, which grows quickly. Biofilm development may have a role in this species' resistance to antimicrobial drugs and mechanism of transmission, making it difficult to eliminate and linked to illness [60]. Using a modified Robbins device, the production of thick biofilms by *M. fortuitum* on surfaces was examined. The results showed that this species could build biofilms on surfaces in as little as 48 hours [61].

The rapid colonization rate and the large number of sessile organisms that have been collected indicate that *M. fortuitum* can form biofilms, which might be a novel way for mycobacteria to avoid antibiotic treatment [62]. The organization inside a biofilm protects bacteria from predators and hazardous substances like chemical disinfectants and antibiotics. It also lessens competition from other microbial species. Mycobacterial resistance to antibacterial agents such as antibiotics and disinfectants is known to be influenced by several factors, including the ability of certain pathogenic mycobacteria to survive inside phagocytic cells, the unique structure of the mycobacterial cell wall, and the rapid mutation of target molecules [63].

To sum up, *M. fortuitum* is a mycobacterial species that grows quickly and can create thick biofilms on surfaces in less than 48 hours, indicating that it produces biofilms easily [64], [65]. *M. fortuitum*'s capacity to build biofilms in a variety of nutrient-rich and low-nutrient environments suggests that this capacity is independent of nutrient circumstances or the presence of other species [63], [66]. Bacteria inside a biofilm are shielded from predators and

harmful agents such as chemical disinfectants and antibiotics by the organization within the biofilm, which also reduces competition from other microbial species.

Mycobacteria's resistance to germicides may be attributed to their capacity to form biofilms, indicating that biofilm development is a new way by which mycobacteria evade antimicrobial therapy [65].

2.11. Stress Related Studies: *M. fortuitum*

The clinical importance and stress-tolerant nature of *M. fortuitum* have led to several studies on the topic of stress. Studies examined the effects of hydrogen peroxide on the transcription and expression of genes related to oxygen reactive species detoxification in *M. fortuitum* cultures. After 30 minutes of treatment with 20 mM hydrogen peroxide, the study found that the transcription levels of the target genes increased. However, the circumstances studied did not have any effect on the bacteria's survivability. Comparing *M. fortuitum* to other mycobacterial species, this shows that it is more resistant to stress caused by hydrogen peroxide. An indwelling pleural catheter-related case of *M. fortuitum* infection was documented in another investigation; the illness was well treated with antibiotics and catheter removal.

The study also examined the literature on *M. fortuitum* thoracic empyema and discovered that *M. fortuitum* is eliminated by TNF-a-mediated endoplasmic reticulum stress, indicating that ER stress might be a possible target for *M. fortuitum* infections. In a mouse infection model, MT727, an acid-susceptible mutant of *M. fortuitum* with a mutation in the ribosomal maturation factor expressing gene *rimP,* demonstrated reduced virulence and persistence, according to one research. Additionally, the study discovered that MT727 exhibited aberrant growth behavior in both hypoxic and detergent stress settings, indicating that *rimP* is involved in both stress conditions' survival [67].

Another study looked at the development and endurance of recombinant *M. fortuitum* strains in acidic, alkaline, and oxidative environments. The strains had differing growth and survival rates under the various stress settings, according to the study. Certain strains fared better under acidic and alkaline stress, while others fared better under oxidative stress [67], [68]. The results of a transcriptional analysis of *M. fortuitum* cells treated with hydrogen peroxide demonstrated that the transcription levels of the oxidative stress response genes, *kat GII* and *sod A*, were greater. On the other hand, under other stress circumstances, including acidic or alkaline stress, the study did not uncover any appreciable alterations in the transcription of these genes.

In vitro, *M. fortuitum* is subjected to acidic stress. An acid-susceptible mutant of *M. fortuitum*, MT727, has been discovered to exhibit reduced virulence and persistence in a mouse infection model. This mutant has a mutation in the ribosomal maturation factor expressing gene *rimP*. Additionally, under detergent and hypoxic stress conditions, the study discovered that MT727 had abnormal growth behavior, indicating that *rimP* is involved in survival under these circumstances. *M. fortuitum* is also subjected to hypoxic stress in vitro; under these circumstances, it has been observed that mutant MT727 exhibits aberrant growth behavior in contrast to the wild-type strain.

M. fortuitum is also subjected to detergent stress in vitro; under these conditions, it has been discovered that mutant MT727 exhibits aberrant growth behaviour in contrast to the wild-type strain. *M. fortuitum* is subjected to heat stress in vitro, and it has been discovered that while it can survive at temperatures as high as 45°C, its growth rate diminishes with rising temperatures. In summary, the research indicates that *M. fortuitum* displays variable virulence and growth behaviour in response to several stressors, and some genes, including *rimP* and those pertaining to the oxidative stress response, are crucial for survival in various environments. Immunologic investigations on a patient with a chronic infection caused by *M. fortuitum* showed normal levels of T cells, T inducers, and T suppressors, indicating that the infection was not brought on by an immune system malfunction.

The possible contribution of immunologic stress linked to the persistent infection to the pathophysiology of the illness was not examined in the study, though. The *M. fortuitum subsp. fortuitum* MF GZ001 clinical strain was the subject of a comparative genomic analysis that identified high-level treatment resistance markers [69]. According to these results, *M. fortuitum* appears to have a strong pathogenicity linked to several virulence genes, such as antigen 85 complex clusters, *mce, nar*, and *sigma*. These genes may encode putative virulence factors linked to the lipid and protein metabolism, suppression of apoptosis, mycobacterial cell envelope, gene control, and nitrate reductase activity.

All *Mycobacterium* species have the *mce* operons, can cause the invasion and persistence of mycobacteria in the host. *Mce4* is associated with the process of cholesterol digestion. The type I complex protein of NADH dehydrogenase in the mycobacterial membrane is encoded by the *nuoG* gene of *M. fortuitum* and is believed to represent a developing virulence factor. The performance of *M. fortuitum fabG4* knockdown mutants in a nutrient-starved environment was

examined in a different investigation. The findings demonstrated the necessity of the *fabG4* gene for nutrient-starved environment survival, underscoring the significance of this gene in *M. fortuitum*'s stress response [70].

Additionally, a study on the *fabG4* knockdown of *M. fortuitum* revealed that the knockdown strain had decreased cell surface hydrophobicity, which suggests a reduction in the capacity of *M. fortuitum* to cause intracellular infections. Additionally, the study discovered that the knockdown strain's growth behaviour was impacted by stress circumstances, indicating a potential function for stress response in *M. fortuitum* pathogenesis [70]

2.12. Role of *hadC* gene

2.12.1 *Mycobacterium species*

The *hadC* gene encodes for 3-hydroxyacyl-CoA dehydratase, which is a crucial enzyme involved in the β-oxidation pathway of fatty acids. β-oxidation is a fundamental metabolic process that breaks down fatty acids to generate acetyl-CoA, which feeds into the tricarboxylic acid (TCA) cycle for energy production. [71],[72]. The *hadC* gene product specifically catalyzes the dehydration step in this pathway, converting 3-hydroxyacyl-CoA to trans-2-enoyl-CoA [73]. The production of mycolic acids is essential to the survival of *M. tuberculosis*, the causative agent of tuberculosis (TB), within the host. Mycobacterial cell walls' structural integrity and resistance to a variety of external stresses, including host immune responses and antibiotics, depend on long-chain fatty acids known as mycolic acids [74].

Mycolic acid biosynthesis is a complicated process involving many different enzyme stages. One important enzyme in this route is 3-hydroxyacyl-CoA dehydratase, which is encoded by the *hadC* gene. In particular, *hadC* catalyzes the maturation and elongation of mycolic acids by dehydrating 3-hydroxyacyl-CoA molecules [75]. The conversion of complicated lipids into simpler compounds that may be used for energy generation and cell wall construction depends on this route. The *hadC* gene helps NTM species, such *M. fortuitum* and *M. abscessus*, adapt to a variety of environmental niches, providing them with resistance to antimicrobial treatments and the ability to survive inside host cells.

Research has indicated the role that the *hadC* gene plays in the etiology of NTM infections [76]. The deletion of the *hadC* gene in *M. avium*, for example, resulted in altered cell wall composition and decreased intracellular survival in macrophages, suggesting a critical involvement in virulence. Like this, mutations in the *hadC* gene in *M. abscessus* have been

linked to modifications in lipid metabolism and heightened vulnerability to antibiotics like macrolides, which are frequently employed in NTM treatment plans.

Apart from its function in lipid metabolism and antibiotic resistance, the *hadC* gene has promise as a target for both therapeutic and diagnostic approaches [77], [78]. The 3-hydroxyacyl-CoA dehydratase enzyme is a desirable option for the creation of molecular diagnostic tests that are exclusive to NTM species due to its distinct biochemical characteristics [79].

2.13 Gene knockdown

The process of gene knockdown includes reducing the expression of one or more genes in an organism. Reverse genetics is the name of the methodology, which aims to determine a gene's function by analyzing its sequence. Knockdown strains are created using an antisense oligo, which is a short DNA molecule with a sequence corresponding to a gene. When the antisense oligos attach to the gene, transcription is blocked. Gene knockout is a procedure that involves causing perturbations to a cell's or model organism's genomic DNA in order to permanently stop a particular gene from being expressed. Target genes are harmed and rendered non-functional by gene knockdown techniques like homologous recombination and site-specific nuclease-mediated knockout.

The knocked-out gene's functional product can never be expressed by the cells or model organisms that survive the knockout event. A frameshift mutation that results in the introduction of a stop codon close to the transcription start point (5' end of the gene) is all that is needed for the occurrence rather than the actual removal of the gene from the genome. As a result, the corresponding gene is deleted from the genome and transcription stops downstream of the stop codon.

2.14 Other bacteria

The *hadC* gene plays a major role in cellular metabolism and survival in non-mycobacterial bacteria, including *E. coli* and *P. aeruginosa*. The well-studied model organism *E. coli* uses the beta-oxidation pathway to convert fatty acids into acetyl-CoA units, which are then used by the tricarboxylic acid cycle (TCA) to provide energy. [80]. By transforming 3-hydroxyacyl-CoA molecules into trans-2-enoyl-CoA and so accelerating the release of energy and metabolic intermediates, the *hadC* gene, which codes for 3-hydroxyacyl-CoA dehydratase, catalyzes an

essential step in this route. The significance of the *hadC* gene in lipid metabolism and membrane synthesis has been emphasized by studies conducted on *E. coli* [81].

The *hadC* gene can be mutated or disrupted to reduce the breakdown of fatty acids, which can accumulate harmful intermediates and disturb cellular homeostasis. Research has demonstrated that the *hadC* gene is crucial for sustaining bacterial fitness and adaptability since its deletion in *E. coli* can change lipid profiles, membrane fluidity, and sensitivity to environmental stresses. For example, the *hadC* gene-mediated beta-oxidation pathway aids in the metabolism of fatty acids during growth and differentiation in *B. subtilis*, a gram-positive bacteria recognized for its strong sporulation and stress tolerance. Research has linked the *hadC* gene to the regulation of membrane integrity and cellular lipid composition, both of which are essential for B. subtilis's ability to adapt to changing environmental circumstances [82][83].

Similar to this, the *hadC* gene plays a role in virulence and lipid metabolism in the adaptable opportunistic bacterium *P. aeruginosa*. Fatty acids are used by *P. aeruginosa* as carbon sources when they infect and colonize host tissues [84]. The *hadC* gene's 3-hydroxyacyl-CoA dehydratase activity, which is part of the beta-oxidation pathway, is essential for the use of fatty acids under a variety of infection-related environmental circumstances. Research has indicated that mutations impacting lipid metabolism, such as those pertaining to the *hadC* gene, might affect *P. aeruginosa's* virulence and resistance to antibiotics. Furthermore, the *hadC* gene is linked to virulence and antibiotic resistance in harmful bacteria including *S. pneumoniae* and *S. aureus* [85].

Due to their need on the metabolism of fatty acids for both energy production and membrane formation, these bacteria may become pathogenic if lipid metabolism pathways, such as beta-oxidation, are disrupted. Studies have shown that focusing on enzymes such as 3-hydroxyacyl-CoA dehydratase, which is encoded by the *hadC* gene, may be a viable approach to creating innovative antimicrobial treatments for these infections [86][87].

CHAPTER – 3

MATERIAL & METHODS

3 .1 Material Methods

3.1.1. Bacterial & Fungal strains

Table 2: List microbial strains used.

Strains	Sources
E. coli DH5α	Institute of Microbial Technology (IMTECH), Chandigarh, India
Bacillus subtilis	Institute of Microbial Technology (IMTECH), Chandigarh, India
Mycobacterium smegmatis MC^2 *155*	Central Drug Research Institute (CDRI), Lucknow, India
M. fortuitum ATCC 6841	Central Drug Research Institute (CDRI), Lucknow, India
Aspergillus flavus 9367	Institute of Microbial Technology (IMTECH), Chandigarh, India

3.1.2 Instruments used.

Table 3: List of instruments used.

Laminar Air Flow	Rescholar
Weighing Balance	Citizon
Incubator Shaker	Macflow Enginerring
PH Meter	Eutech
Centrifuge	Eppendorf
PCR	Thermo Fisher Scientific
Water bath	NSW India
Autoclave	REFLITECH
Incubator	Thermo Scientific
Light Microscope	OLYMPUS
Gel Doc	BioRad
Vortex	REMI
4°C Storage	Ailled Frost
-20°C Storage	Blue star

3.1.3 Media and chemicals

Table 4: List of chemicals

Nutrient Broth	HIMEDIA
Agar	HIMEDIA
Luria Broth	HIMEDIA
Potato Dextrose Agar	HIMEDIA
Crystal Voilet	Loba Chemie
Grams Iodine	HIMEDIA
95% Alcohol	Loba Chemie
Safranin	HIMEDIA
Basic fuschin	HIMEDIA
Lacto Phenol Cotton Blue	Loba Chemie
Methylene Blue	Fisher scientific
Tween - 80	SRL labs
Glycerol	Merck
Sodium Chloride	Merck
Agarose	HIMEDIA
Antibiotic (Powder) Ampicillin	HIMEDIA
Saturated Phenol	SRL
Chloroform	SRL
Isoamyl	SRL
SDS	Bio Rad

3.2 Methods

3.2.1 Basic Microbiological Techniques

3.2.1.1 Simple Streaking

1. The bottom of the petri plate was labelled with the name of the microorganism, and date.
2. The inoculation loop was sterilized by holding it on the flame until it became red hot.
3. The inoculation loop was allowed to cool.
4. With the help of an inoculation loop a single colony was picked from another cultured plate.
5. The loop was dragged gently on the fresh nutrient agar plate in a zig-zag pattern.
6. The loop was again sterilized until it became red hot.
7. For 24 hours, the plates were incubated at 37°C in an incubator.

3.2.1.2 Quadrant Streaking

1. The bottom of the petri plate was labelled with the name of the microorganism, and date.
2. The inoculation loop was sterilized by holding it on the flame until it became red hot.
3. The inoculation loop was allowed to cool.
4. With the help of an inoculation loop a single colony was picked.
5. A smear was prepared at one extreme end and the loop was dragged back and forth across the agar surface.
6. The loop was sterilized again.
7. After turning the plate 90 degrees, new streaks were created starting from the end of the preceding one.
8. The same process was repeated three more times.
9. For 24 hours, the plates were incubated at 37°C in an incubator.

3.2.1.3 Gram Staining

1. Clean grease free glass slide was taken,
2. A few drops of normal saline were added.
3. A single culture colony was picked using a sterile loop, and a smear was made.
4. The slide was kept for air drying, and heat fixed.
5. Crystal violet was added and kept for 30 to 60 sec.

6. A few drops of grams iodine (mordant) were added for 1 min and again washed using distilled water.
7. Then the slide was washed with 95% alcohol for about 10 to 20 seconds and again washed with distilled water.
8. A few drops of safranin (counter stain) were added for 35 to 45 sec and again washed with distilled water.
9. The slide was air dried and observed under microscope.

3.2.1.4 Fungal Staining

1. A clean grease free slide was taken.
2. On the centre of the glass slide, one drop of Lactophenol Cotton Blue was applied.
3. With the help of an inoculating loop, the fungus was taken from the cultured plate.
4. The slide was covered with cover slip.
5. After that slide was observed under the light microscope.

3.2.1.5 Ziehl – Nelson Staining

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

3.2.1.6 Isolation of microorganisms from soil.

1. Sample soil was taken in sterilized falcon tube.
2. 1g of soil was weighed and dissolved in 10 ml autoclaved distilled water.
3. Different Eppendorf tubes was taken and filled with 900µl of Luria broth in each tube.

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

3.2.2 Basic Molecular techniques

3.2.2.1 Isolation of Genomic DNA

1. *E. coli* sample was inoculated in Luria broth and kept for incubation for 24 hours at 37°C.
2. After 24 hours the *E. coli* culture was transferred to sterilized Eppendorf tubes and centrifuged at 9500 x g for 5 mins.
3. Supernatant was discarded and pellet was kept in tubes.
4. 400µl of SDS lysis buffer was added to the pellet and mixed the pellet by pipetting.
5. After mixing the tubes were incubated at 65°C for 30 minutes in water bath.
6. After incubation 400µl of Phenol: Chloroform (1:1) was added into the tubes.
7. Tubes were centrifuged at 16060 x g for 10 minutes at Room temperature.
8. The upper layer (Aqueous layer) was transferred to the fresh tubes.
9. For the DNA precipitation, an equivalent amount of cold isopropanol was added to the tubes.
10. Tubes were kept at -20°C for 18 hours.
11. Tubes were again centrifuged at 13684 x g for 15 minutes at 4°C.
12. After discarding the supernatant, 500µl 70% ethanol was used to wash the DNA pellet.
13. Tubes were again centrifuged at 9500 x g for 5 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 provided to gel for running.
21. The DNA bands were visualized by keeping the gel in Gel Doc system.

3.2.2.2 Plasmid isolation

1. *E. coli* containing pUC 19 culture was grown overnight.
2. Overnight grown culture was poured to 1.5ml Eppendorf tubes.
3. Then tubes were centrifuged at 9500 x g for 5 min.
4. The supernatant was discarded.
5. Alkaline lysis solution 1 was added (300µl) and mixed properly by tapping.
6. Alkaline lysis solution II was added (600µl) and mixed carefully by slowly tilting the tubes.
7. Eppendorf tubes were incubated at 37°C for 5 mins.
8. Alkaline lysis soultion III was added (450µl) was added and tubes were incubated in ice for 45 mins.
9. Then Eppendorf tubes were centrifuged at 4656 x g for 15 mins and supernatant was transferred to fresh Eppendorf tube.
10. An equal amount of chilled isopropanol was added to the supernatant.
11. Eppendorf tubes were kept at -20°C for overnight.
12. Eppendorf tubes were centrifuged at 13684 x g for 15 min.
13. Supernatant was discarded and 70% ethanol was added, centrifuged at 9500 x g for 10 mins and pellet was stored.
14. Again 96% ethanol was added to the pellet and centrifuged at 9500 x g for 10 mins.
15. Supernatant was discarded and pellet was air dried to remove the ethanol.
16. Nuclease free water was added to the pellet.
17. After that these tubes were kept in ice for 1 hr.
18. 1% Agarose was prepared for gel electrophoresis.
19. In agarose 3µl of Etbr (ethidium bromide) was added.
20. The gel was placed in electrophoresis chamber and 1X TAE was added.
21. Sample was loaded in the well with loading dye and 100 v of current was set for running of gel.
22. The DNA bands were visualized in Gel doc system.

3.2.2.3 Restriction digestion of pUC 19

Fig 2: Restriction profile of pUC19.

1. PCR vials were autoclaved.
2. Isolated plasmid, water, buffer and restriction enzymes were added in vials.
3. The vials were incubated at 37°C for 1 hr.
4. 1% Agarose was prepared and homogenized for gel electrophoresis.
5. In agarose 3µl of Etbr (ethidium bromide) was added.
6. The gel was placed in electrophoresis chamber and 1X TAE was added.
7. Sample was loaded in the well with loading dye and 100 v of current was set for running of gel.
8. The DNA bands were visualized in Gel doc system.

Table 5: Reaction mixture for restriction digestion.

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				

3.2.2.4 *Mycobacterium* CFU calculation

1. *Mycobacterium smegmatis* was grown in LBGT (Luria broth with 2% glycerol and 0.5% Tween 80) media for 3 days at 37°C under shaking conditions at 200 rpm.
2. Sterilized nine Eppendorf vials were taken and labelled.
3. 1 ml of bacterial culture was taken from the culture grown overnight and labelled as neat.
4. Add 900µl of Tween Normal Saline (TNS) in 8 Eppendorf.
5. Add 100µl from neat and transferred to vial containing Tween Normal Saline (TNS) and all vials were serially diluted as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, $10^{-8.}$
6. 100µl of sample was taken from each t Add 100µl from neat and transferred to vial containing Tween Normal Saline (TNS) and all vials were serially diluted as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, $10^{-8.}$
7. 100µl of sample was taken from each tube and spread on NAT (Nutrient agar with 0.05% Tween 80) plates and incubation at 37°C for 3 days.
8. After 3 days the colonies were counted and cfu was calculated.

3.2.2.5 *M. fortuitum* CFU calculation

1. *M. fortuitum* was grown in LBGT (Luria broth with 2% glvcerol and 0.5% Tween 80) media for 3 days at 37°C under shaking conditions at 200 rpm.
2. Sterilized nine Eppendorf vials were taken and labelled.
3. 1 ml of bacterial culture was taken from the culture grown overnight and labelled as neat.
4. Add 900µl of Tween Normal Saline (TNS) in 8 Eppendorf.
5. Add 100µl from neat and transferred to vial containing Tween Normal Saline (TNS) and all vials were serially diluted as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, $10^{-8.}$
6. 100µl of sample was taken from each tube and spread on NAT (Nutrient agar with 0.05% Tween 80) plates and incubation at 37°C for 3 days.
7. After 3 days the colonies were counted and cfu was calculated.

3.2.2.6 Isolation of Genomic DNA from *M. fortuitum*

1. *M. fortuitum* culture was grown in LBGT media at 37•C in incubator shaker.
2. Two 50 ml Falcon tubes were filled with bacterial culture, and the tubes were centrifuged for five minutes at 9500 x g.
3. The pellet was stored and supernatant was discarded.

4. Then add 6 ml of TES (Tris EDTA Saline) buffer was added and mix properly with pellet.
5. The falcon tubes were placed in a water bath and incubated for one hour at 80°C.
6. After the incubation 2mg/ml of lysozyme was added and incubated at 37°C for 1 hr.
7. Then 1.5 % Sodium Dodecyl Sulphate and 100µg/ml Proteinase K was added and incubated at 50°C for 1 hr.
8. After the incubation saturated phenol (½ the volume of TES buffer) was added and centrifuged at 9500 x g for 20 mins.
9. The aqueous layer was transferred to sterilized Eppendorf vials.
10. Chloroform and isoamyl (24:1) were in the vials containing aqueous layer and centrifuged at 9500 x g for 20 mins.
11. After moving the aqueous layer to a new tube, adding chloroform and isoamyl, and centrifuging for 20 minutes at 9500 x g.
12. Again, the aqueous layer (upper layer) was transferred into fresh vial and equal volume of chilled isopropanol was added into the vial.
13. The Eppendorf vial was incubated overnight at -20°C.
14. Following incubation, the Eppendorf was centrifuged for 20 minutes at 9500 x g, and the supernatant was disposed of.
15. Then 70% ethanol was added into the Eppendorf for washing of pellet and it was centrifuged at 9500 x g for 15 mins.
16. The Supernatant was discarded, and the pellet was air dried to remove the ethanol.
17. Autoclaved distilled water was added to the pellet.
18. After that these tubes were kept in ice for 1 hr.
19. 1% Agarose was prepared for gel electrophoresis.
20. In agarose 3µl of Etbr (ethidium bromide) was added.
21. The gel was placed in electrophoresis chamber and 1X TAE was added.
22. Sample was loaded in the well with loading dye and 100 v of current was set for running of gel.
23. The DNA bands were visualized in Gel doc system.

3.2.2.7 PCR: Amplification of *hadC* gene in *M. fortuitum*

Table 6: Primer sequence for amplification of *hadC* gene in *M. fortuitum*.

Table 6: list of Primer used.

MF*hadC*_Fwd	5'-CCGGAATTCCGGATGGCACTCAAGGCTGACATTCG-3'
MF*hadC*_Rev	5'-CCGGAATTCCGGTTACTCTCCGGCCGCCTTC-3'

1. Sterilized PCR vials were taken.
2. DNA Template, Forward and reverse primer, Master mix and Nuclease free water were added to the PCR vials.

Table 7: PCR mixture.

DNA template	12 µl
Forward Primer	3 µl
Reverse Primer	3 µl
Master mix	36 µl
Nuclease free water	6 µl
Total = 60 µl	

Table 8: PCR Conditions.

Conditions	Temperature	Time
Initial denaturation	95°C	5 minutes
Cyclic Denaturation	95°C	1 minutes
Annealing	63°C	1 minutes
Cyclic Extension	72°C	45 sec
Final Extension	72°C	5 minutes
Hold	4°C	∞

30

3. 2% Agarose was prepared and homogenized for gel electrophoresis.
4. In agarose 3µl of Etbr (ethidium bromide) was added.
5. The gel was placed in electrophoresis chamber and 1X TAE was added.
6. Sample was loaded in the well with loading dye and 100 v of current was set for running of gel and the DNA bands were visualized in Gel doc system.

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

3.3.1.2 Hypoxic Stress

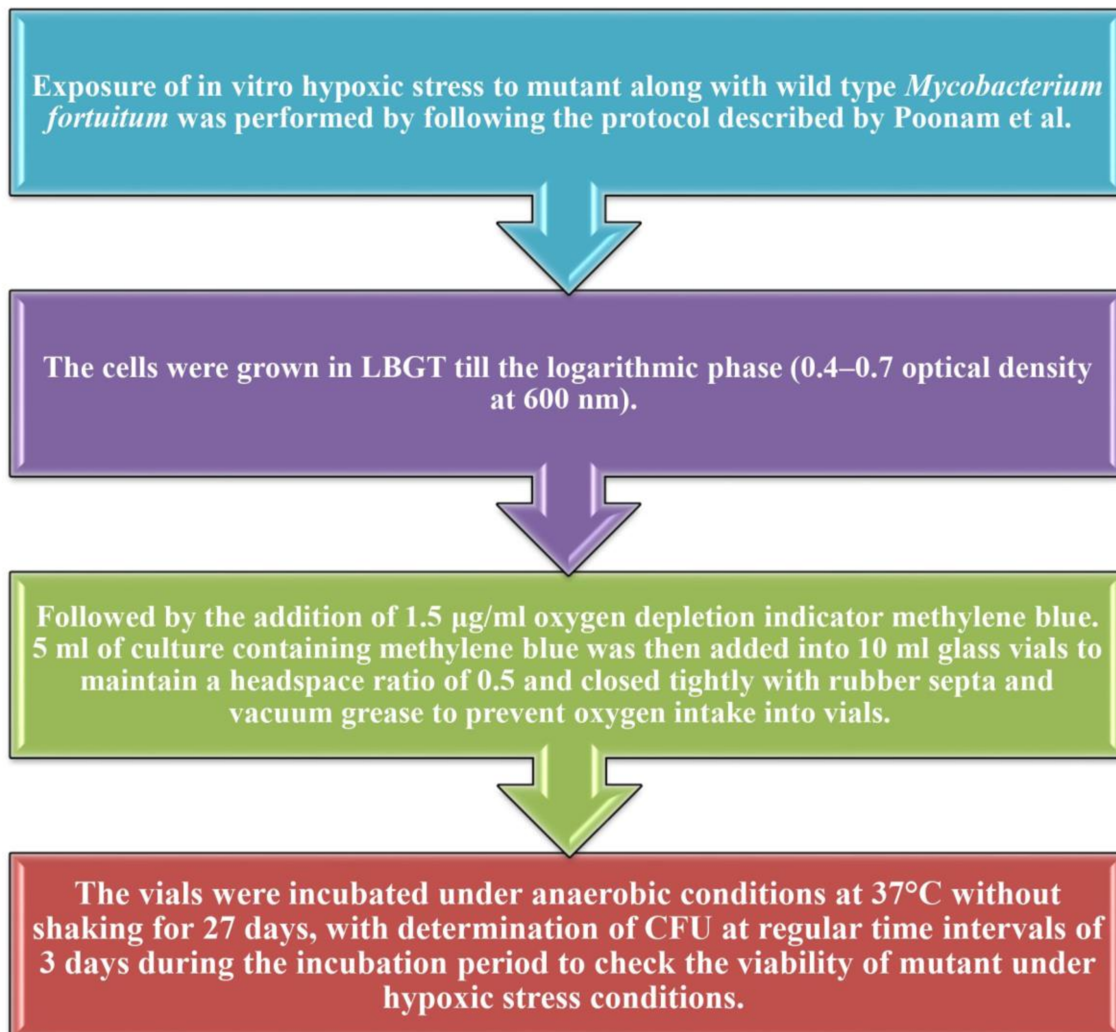


Fig 3: Flow chart showing the detailed protocol employed for study of survival and growth of *M. fortuitum* under hypoxic stress conditions [Protocol adapted from Poonam et. al [67] with minor modification].

3.3.1.2 Acidic stress

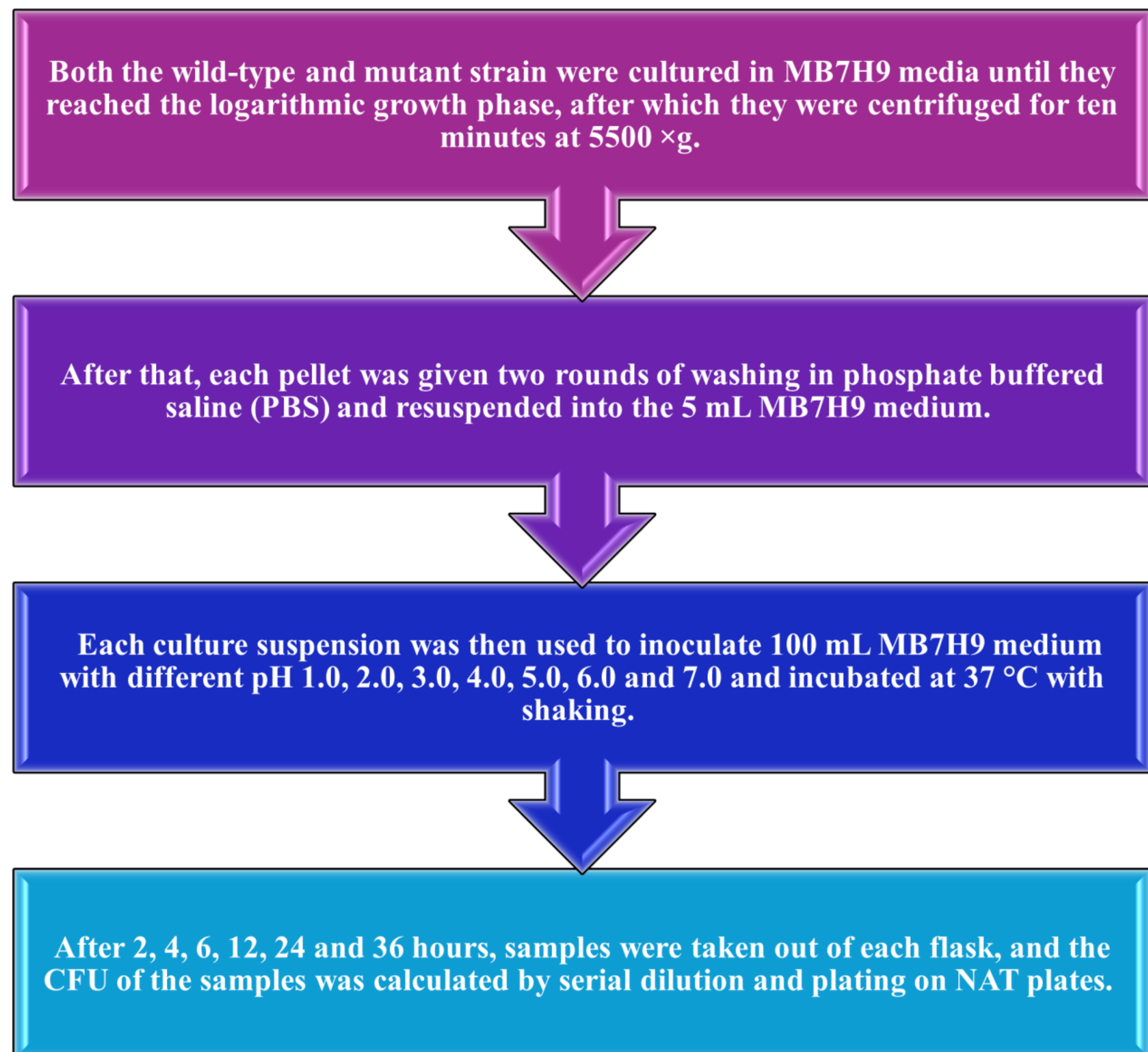


Fig 4: Flow chart showing the detailed protocol employed for study of survival and growth of *M. fortuitum* under acidic stress conditions [Protocol adapted from Poonam et. al [67] with minor modification].

3.3.1.3 Nutrient Starvation

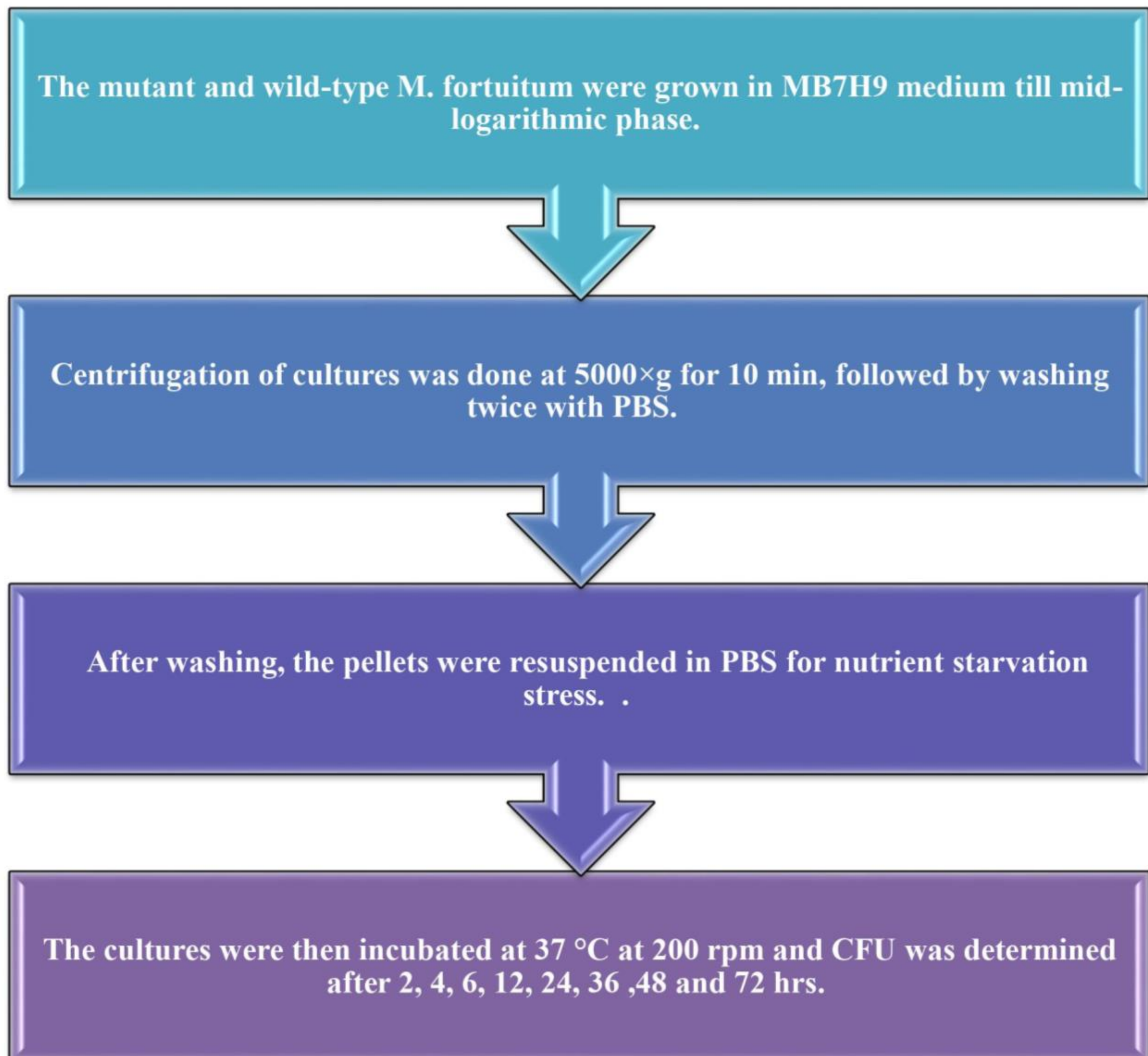


Fig 5: Flow chart showing the detailed protocol employed for study of survival and growth of *M. fortuitum* under nutrient starvation [Protocol adapted from Poonam et. al [67] with minor modification].

3.3.1.3 Oxidative stress

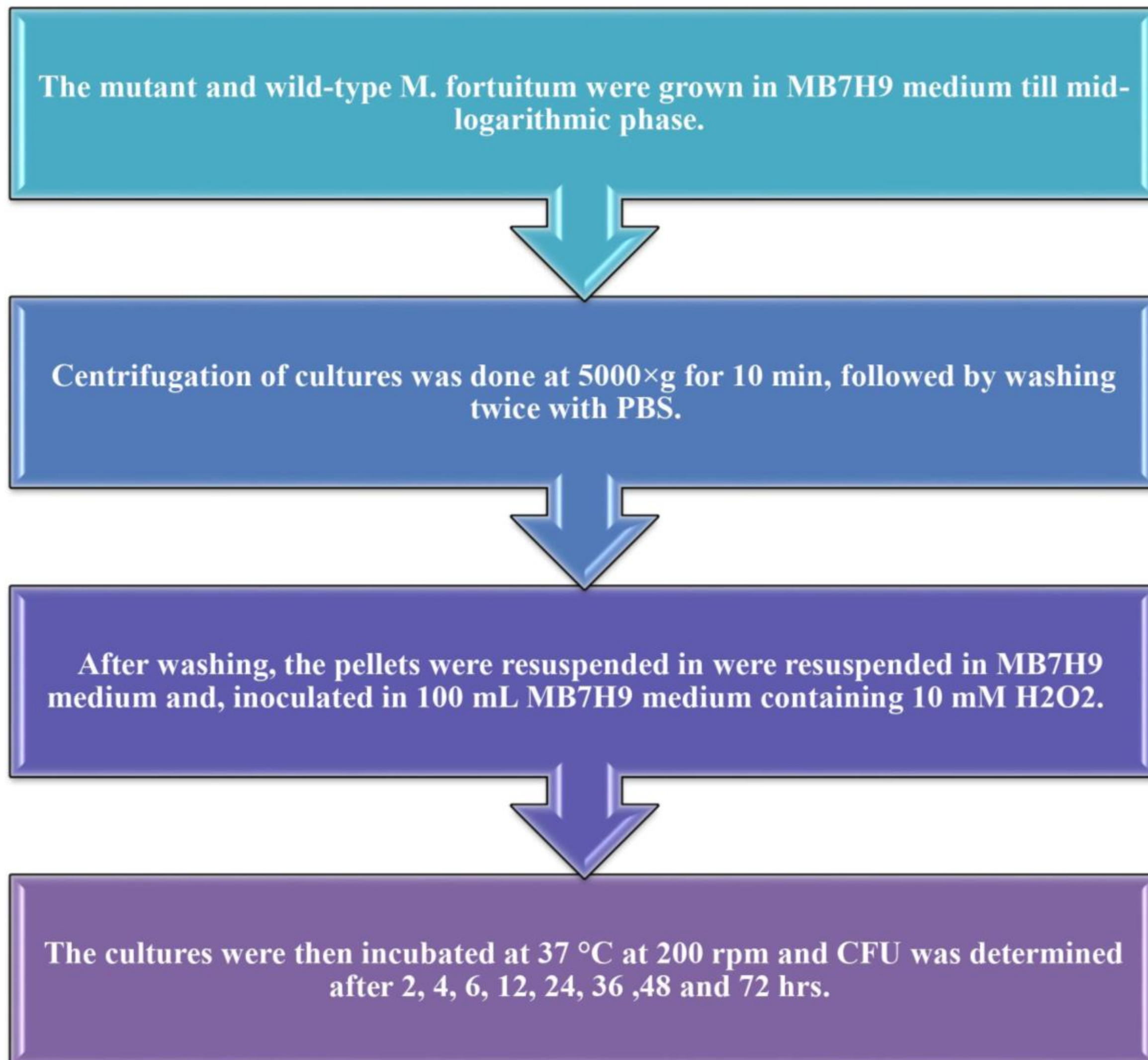


Fig 6: Flow chart shows the detailed protocol employed for study of survival and growth of *M. fortuitum* under oxidative conditions [Protocol adapted from Poonam et. al [67] with minor modification].

3.3.1.4 Detergent stress

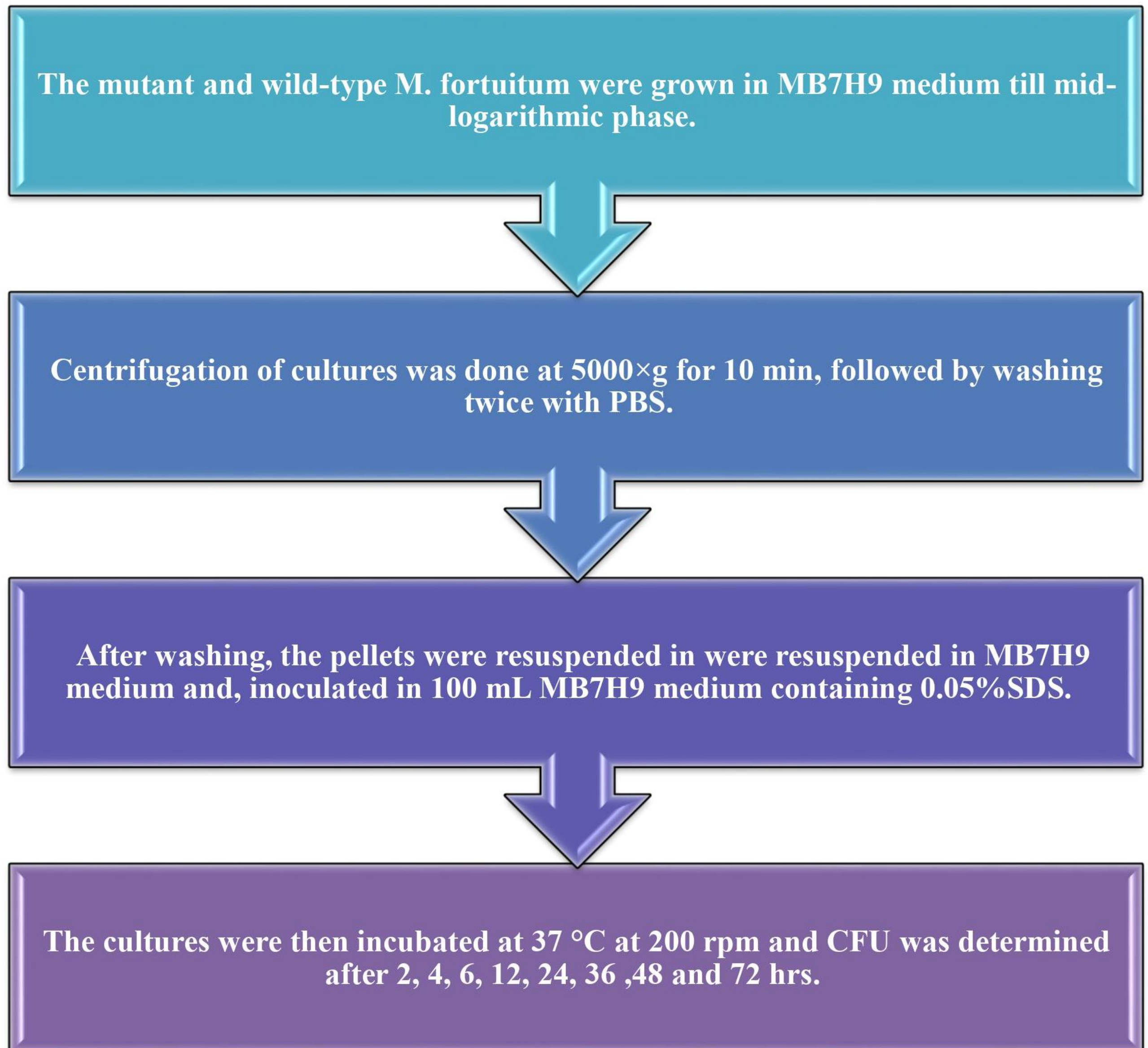


Fig 7: Flow chart shows the detailed protocol employed for study of survival and growth of *M. fortuitum* under detergent stress conditions [Protocol adapted from Poonam et. al [67] with minor modification].

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.

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

Formula for calculating Standard Error:

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

Here: $\bar{x}$ = Mean of sample

N = Size of sample

MS Excel was used to evaluate Statistical significance.

CHAPTER – 4

RESULTS

4. Result

4.1 Basic Microbiological Techniques

4.1.1 Simple Streaking

Cultures of *E. coli, B. subtilis*, *and M. smegmatis* were streak on NA (Nutrient agar) and NAT (Nutrient agar with 0.05% Tween 80) media to increase the amount of inoculum for short term preservation of culture. Representative images of culture plates [Fig:8] are shown below**.**

(a)

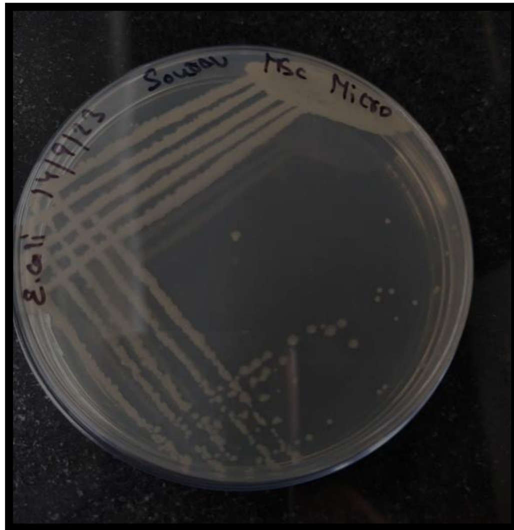
(b)

(c)

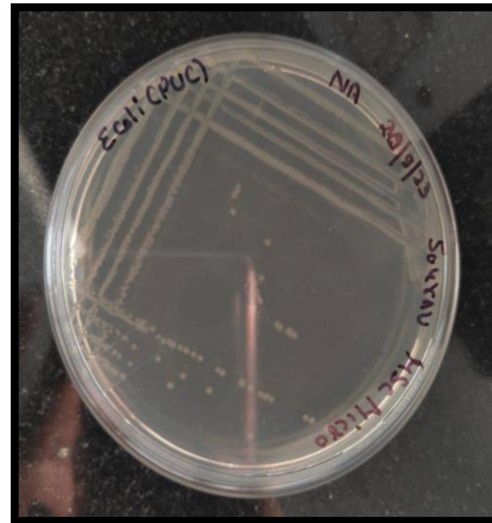
Fig 8: (a)Simple streaking of *Bacillus subtilis*, (b) *Simple streaking of E. coli & (c)* Simple streaking of *M. smegmatis*

4.1.2 Quadrant Streaking

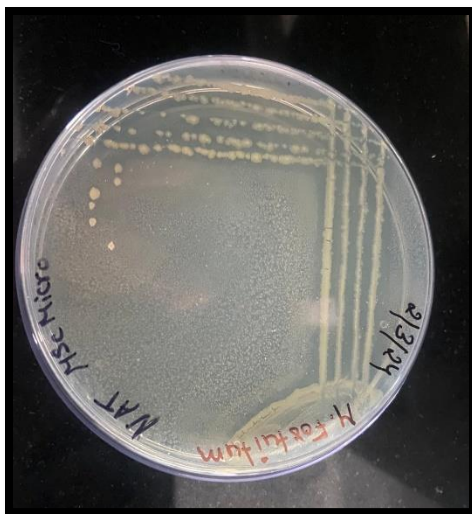
To obtain isolated colonies quadrant streaking of *E. coli, M. fortuitum and M. smegmatis* was done. And these strains were used in further experiment.



(a)



(b)



(c)

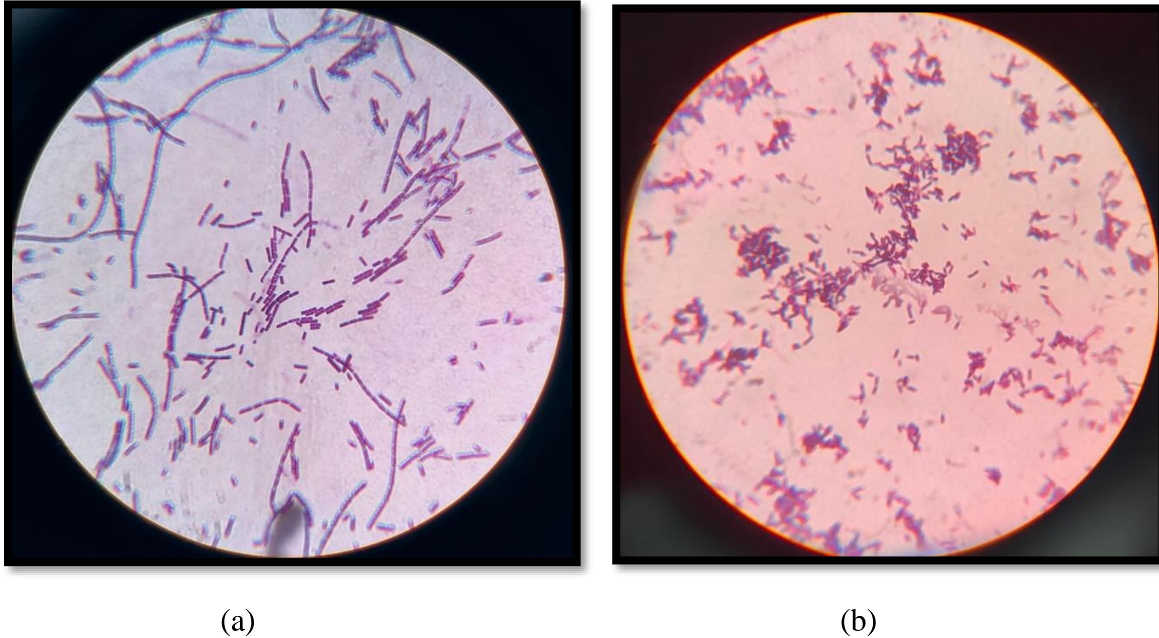
(d)

Fig 9: (a)Quadrant streaking of *E. coli,* (c) *Quadrant Streaking M. fortuitum,* (d) Quadrant Streaking of *M. smegmatis.*

4.1.3 Staining

Gram Staining

Gram staining of *E. coli and B. subtilis* was done to check the weather these microbial cultures are pure.



(a) (b)

Fig 10: (a) Gram of *bacillus subtilis.* (b) Gram staining of *E. coli.*

4.1.4 Fungal Staining

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

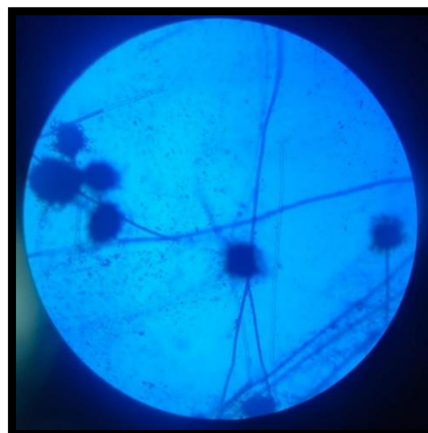
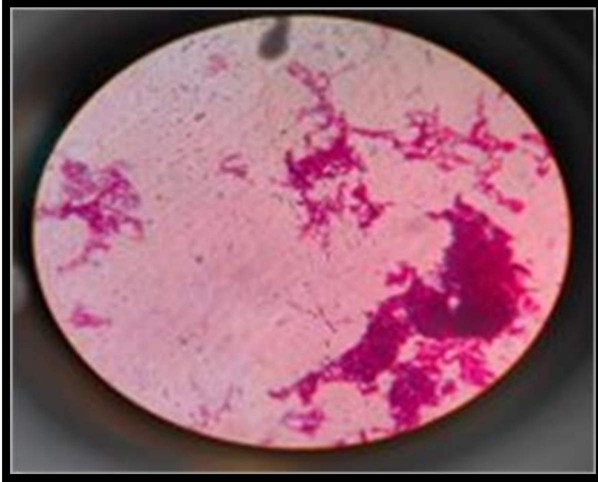


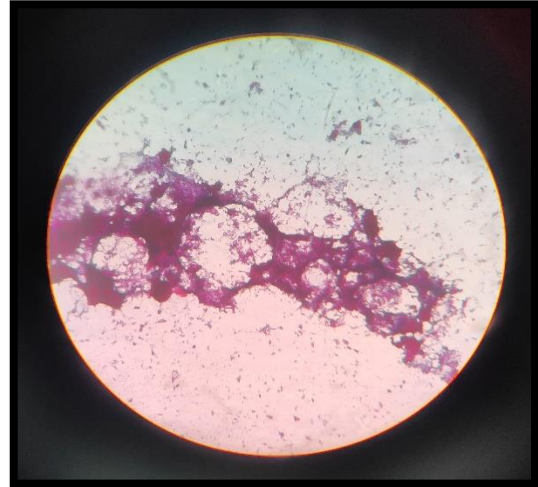
Fig 11: Fungal staining of *A. flavus.*

4.1.5 Ziehl Neelsen Staining

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



(a)



(b)

Fig 12: (a) Ziehl Neelsen Staining of *M. smegmatis and* (b) Ziehl Neelsen staining of *M. fortuitum.*

4.1.6 Isolation of Microorganism from soil sample

Soil sample was taken from the JUIT campus and CFU count of soil sample was done to determine number of cells were present in the soil. Serial dilution of samples was made and 100 µL of each dilution was plated in duplicate, Fig: 13 shows representative plates obtained after incubation of plates. No. of colonies obtained in the study has been shown in Table:9.

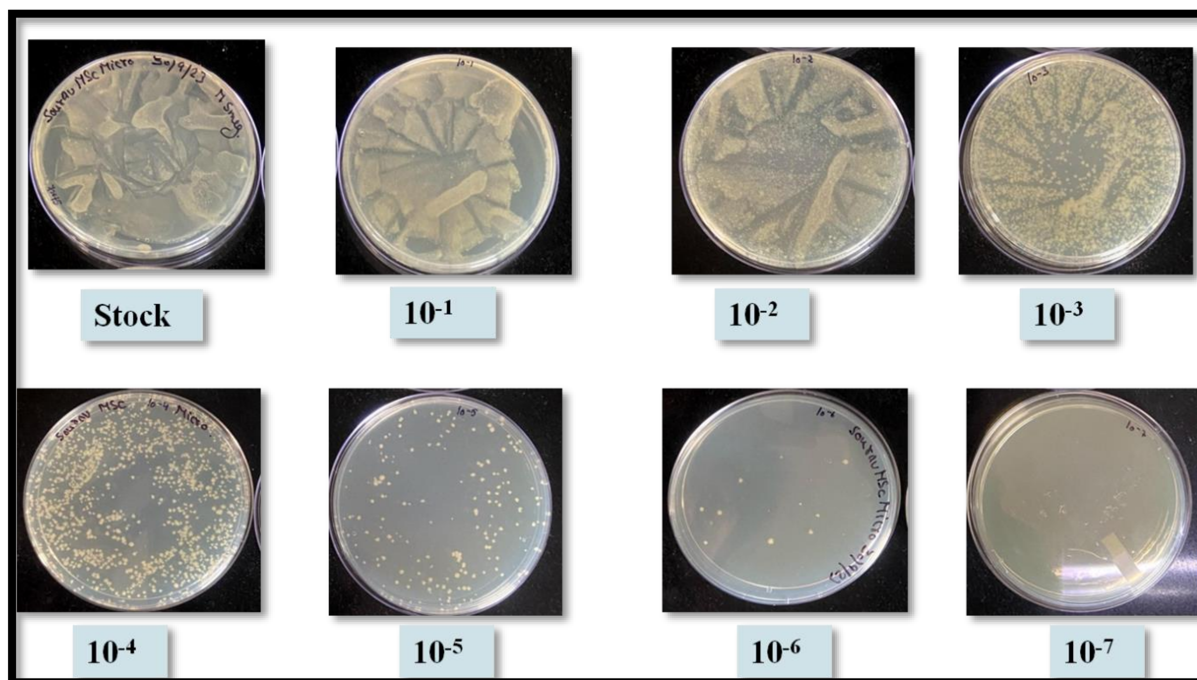


Fig 13: Petri plates showing colonies obtained on spreading of different dilution of water sample.

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}	110
10^{-6}	10
10^{-7}	0

$$\text{CFU/ml} = \frac{\text{Number of colonies x Dilution Factor}}{\text{Volume plated (ml)}}$$

$$\text{CFU/ml} = \frac{110 \text{ x } 10^5}{0.1}$$

$= 1.1 \text{ x } 10^8$ CFU/ml

$$\text{CFU/ml} = \frac{10 \text{ x } 10^6}{0.1}$$

$= 1 \text{ x } 10^8$ CFU/ml

Average CFU = $1.04 \text{ x } 10^8$ CFU/ml

4.1.7 *Mycobacterium smegmatis* CFU calculation

CFU count of *M. smegmatis* was done to know that how many colonies are present in 1 ml of culture. Broth cultures were serially diluted till 10-7 dilutions and 100 µL of each dilution was plated in duplicate, Fig: 14 shows representative plates obtained after incubation of plates. No. of colonies obtained in the study has been shown in Table:10.

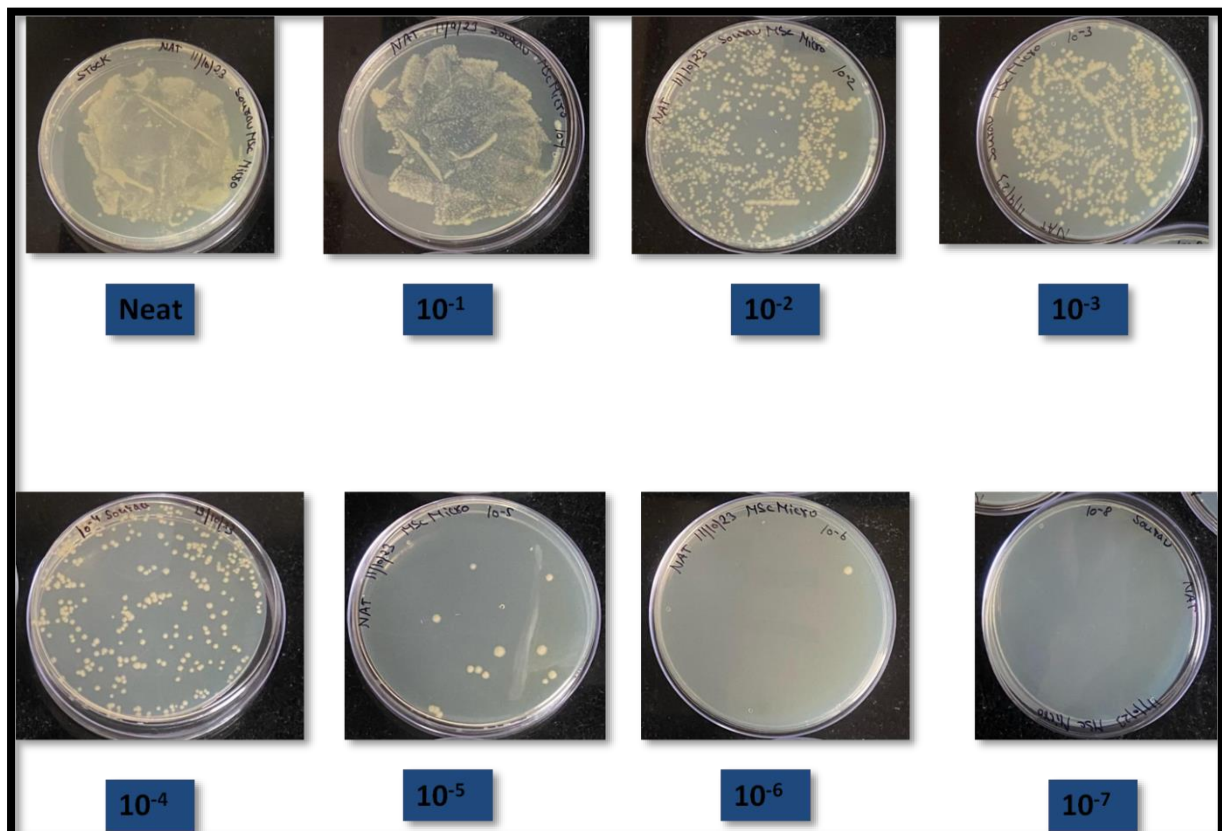


Fig 14: Petri plates showing colonies obtained on spreading of different dilution of water sample.

Table 10: 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}	116
10^{-5}	15
10^{-6}	1
10^{-7}	0

.

CFU= $\frac{\text{Number of colonies x Dilution Factor}}{\text{Volume plated (ml)}}$

CFU/ml = $\frac{116 \times 10^4}{0.1}$

= 1.16×10^7 CFU/ml.

CFU/ml = $\frac{15 \times 10^5}{0.1}$

= 1.5×10^7

Average = 1.33×10^7

4.2 Basic Molecular Techniques

4.2.1 Isolation of Genomic DNA from *E. coli*

Overnight-grown *E. coli* culture was used to isolate its genomic DNA. The genomic DNA was run on 0.8% of agarose gel [Fig:15].

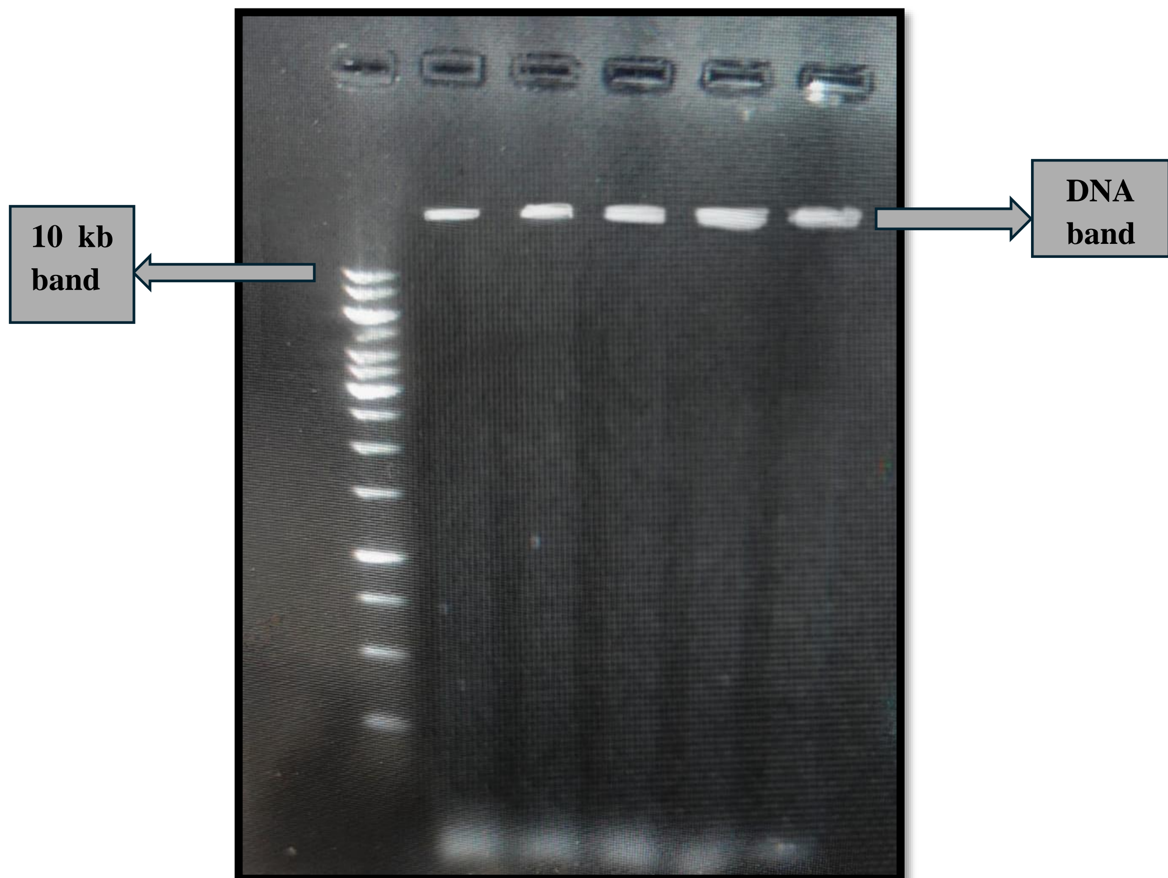


Fig 15: Agarose Gel electrophoresis of genomic DNA of *E. coli*. In Lane 1: 1 kb DNA ladder (GeneRuler) (size of fragments in base pairs) (from bottom to top: 250, 500,1000,1500,2000,2500,3000,3500, 4000,5000,6000,8000,10000), Lane 2,3,4,5: DNA isolated from *E. coli.*

4.2.2 Isolation of plasmid

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.

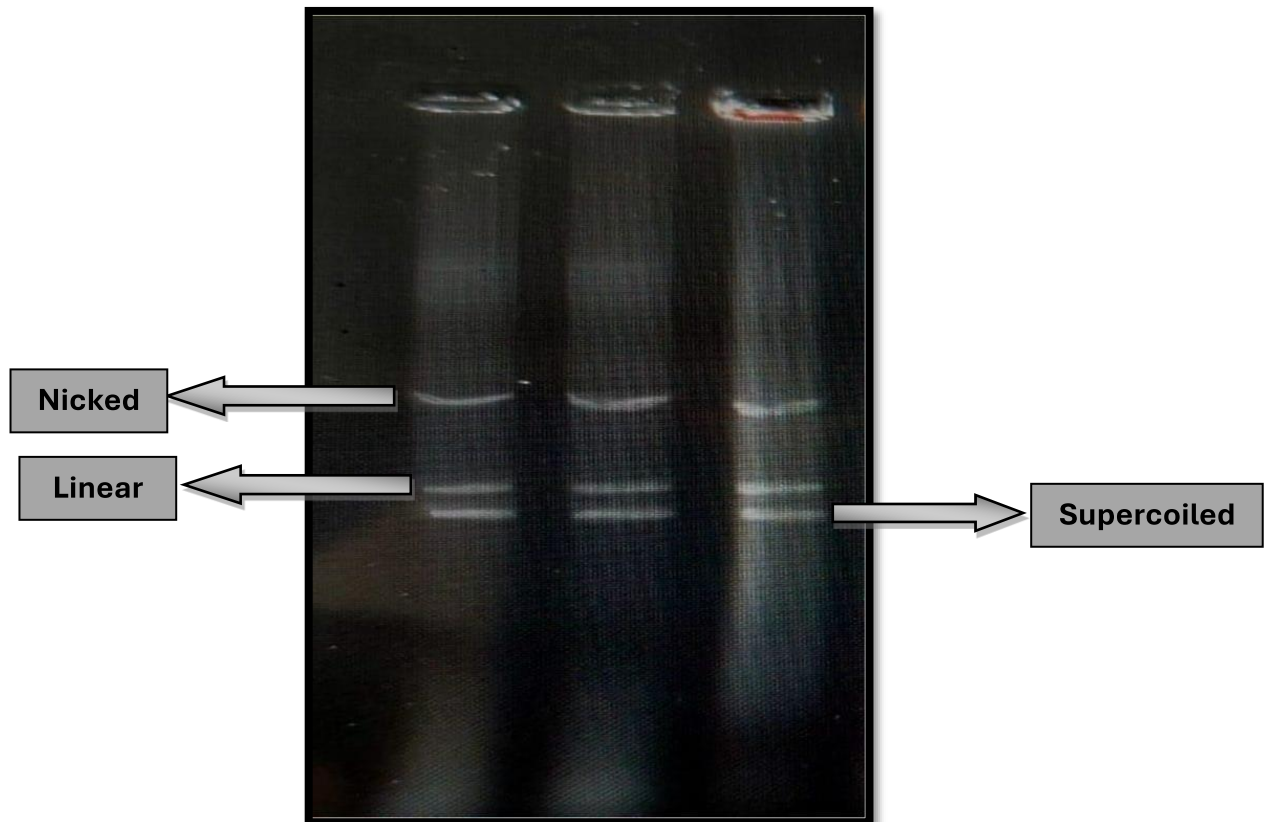


Fig 16: Agarose gel electrophoresis of isolated plasmid. Lane 1, 2,3: Plasmid DNA isolated from *E. coli* containing pUC 19. Different shapes show supercoiled, linear and nicked structure of plasmid.

4.2.3 Restriction digestion of plasmid

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:17.

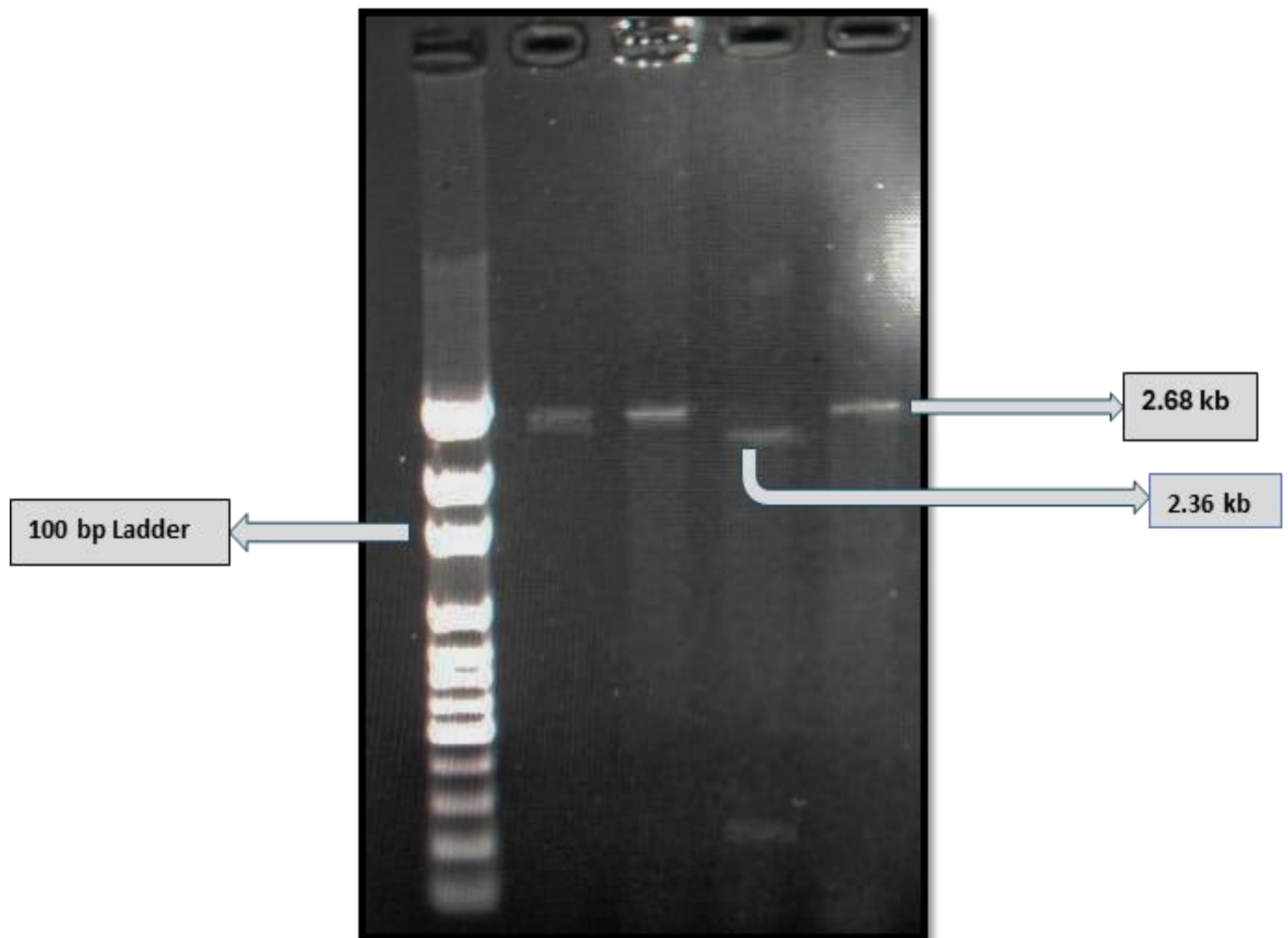


Fig 17: Agarose gel electrophoresis of digestion of pUC 19 with **different restr**iction enzymes. In lane 1: 100bp ladder (GeneRuler) (Size of fragments in bp) (from bottom to top; 100, 200, 300, 400 , 500, 600, 700, 800, 900, 1000, 1200 , 1500, 2000, 3000), Lane 2: digested with HindIII (2.68kb), Lane 3: digested with EcoRI (2.68kb), Lane 4: digested with pvuII (2.36kb) and in Lane 5: double digestion with HindIII and EcoRI(2.6kb and 52bp).

4.2.3 *M. fortuitum* CFU count

CFU count of *M. fortuitum* was done to know that how many colonies are present in 1 ml of culture. Broth cultures 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:11.

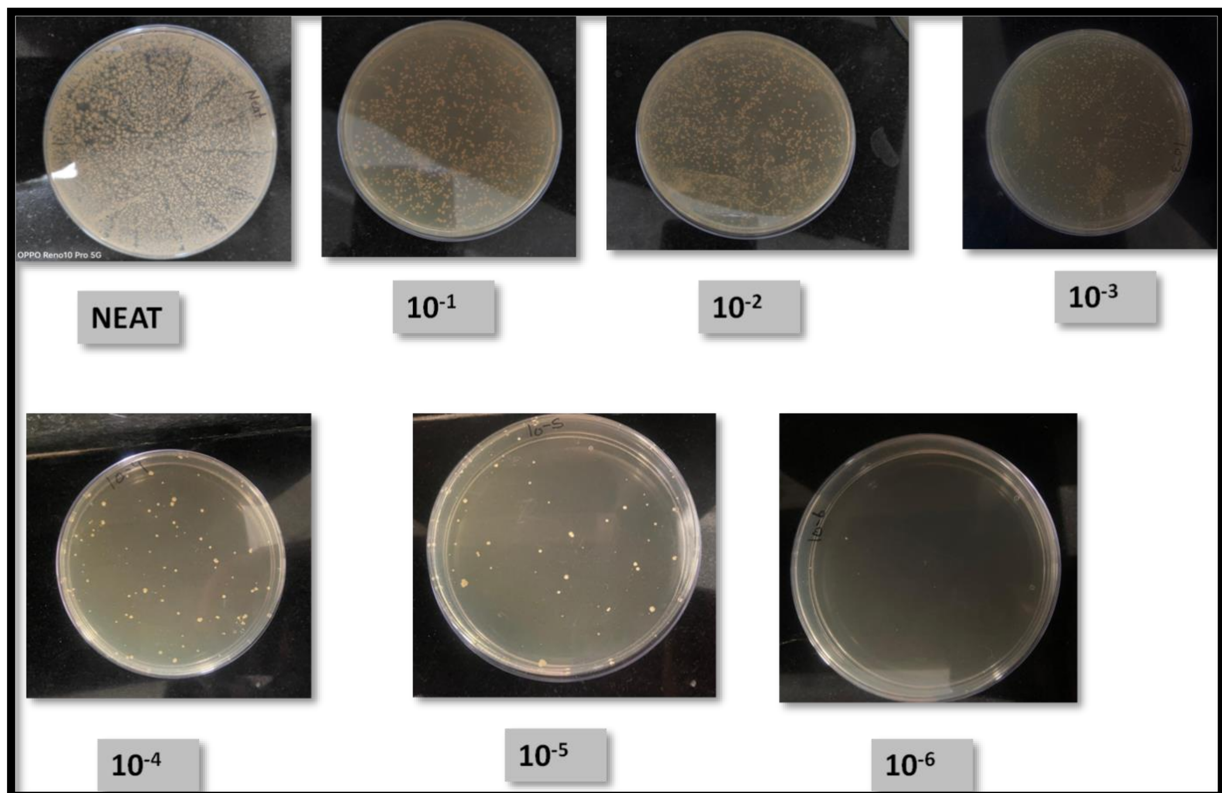


Fig 18: Petri plates showing colonies obtained on spreading of different dilution of water sample.

Table 11: 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}	78
10^{-5}	26
10^{-6}	0

$$\textbf{CFU} = \frac{\text{Number of colonies x Dilution Factor}}{\text{Volume plated (ml)}}$$

$$\text{CFU/ml} = \frac{78 \times 10^4}{0.1}$$

$= 7.8 \times 10^6$ CFU/ml

$$\text{CFU/ml} = \frac{26 \times 10^5}{0.1}$$

$= 2.6 \times 10^7$

4.2.4 Isolation of *M. fortuitum* genomic DNA

Isolation of DNA from *M. fortuitum* was done and genomic DNA was run on 0.8% agarose gel [Fig:19]. Genomic DNA was employed for amplification of the gene *(hadC)***.**

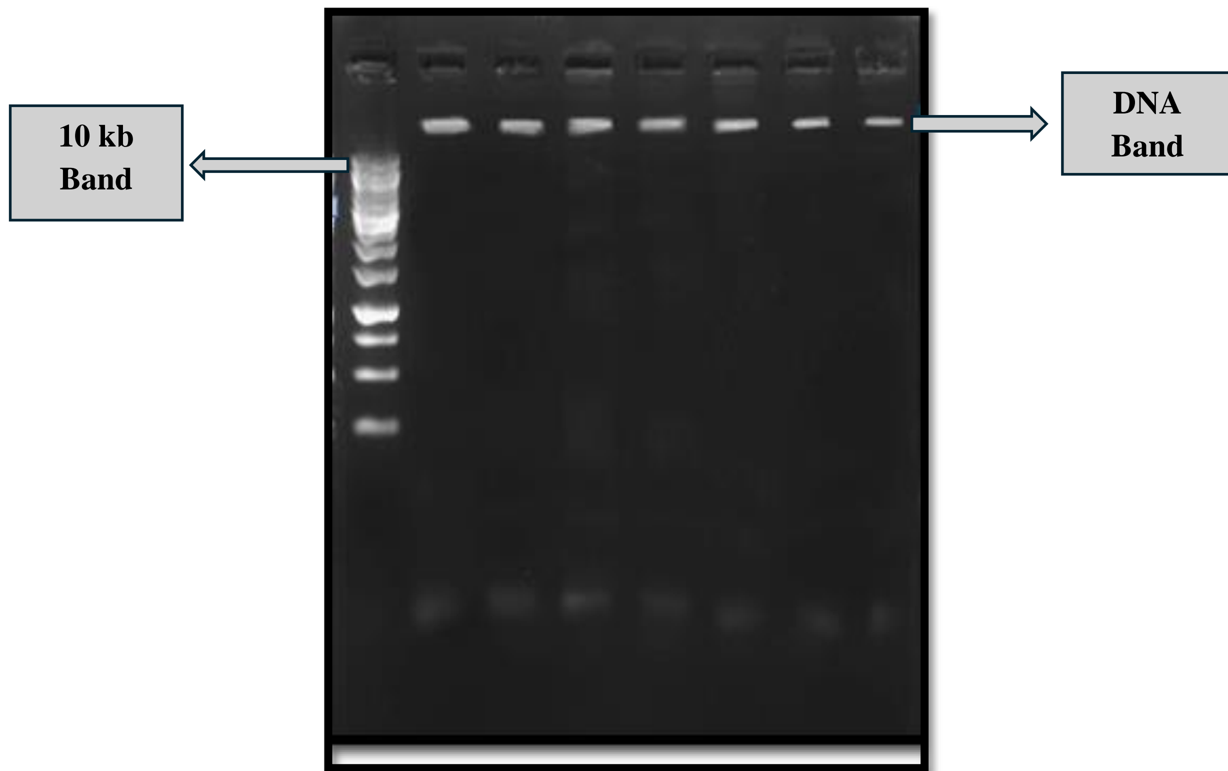


Fig 19: Agarose gel electrophoresis of genomic DNA of *M. fortuitum*. Lane 1: 1 kb DNA ladder (GeneRuler) (size of fragments in base pairs) (from bottom to top: 250, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 5000, 6000, 8000, 10000), Lane 2,3,4,5: DNA isolated from *M. fortuitum.*

4.2.5 Amplification of *hadC* gene

After the isolation of genomic DNA from *M. fortuitum* the gene (*hadC*) was amplified by PCR and the amplicon was run on agarose gel. Results of the amplification are shown in Fig: 20.

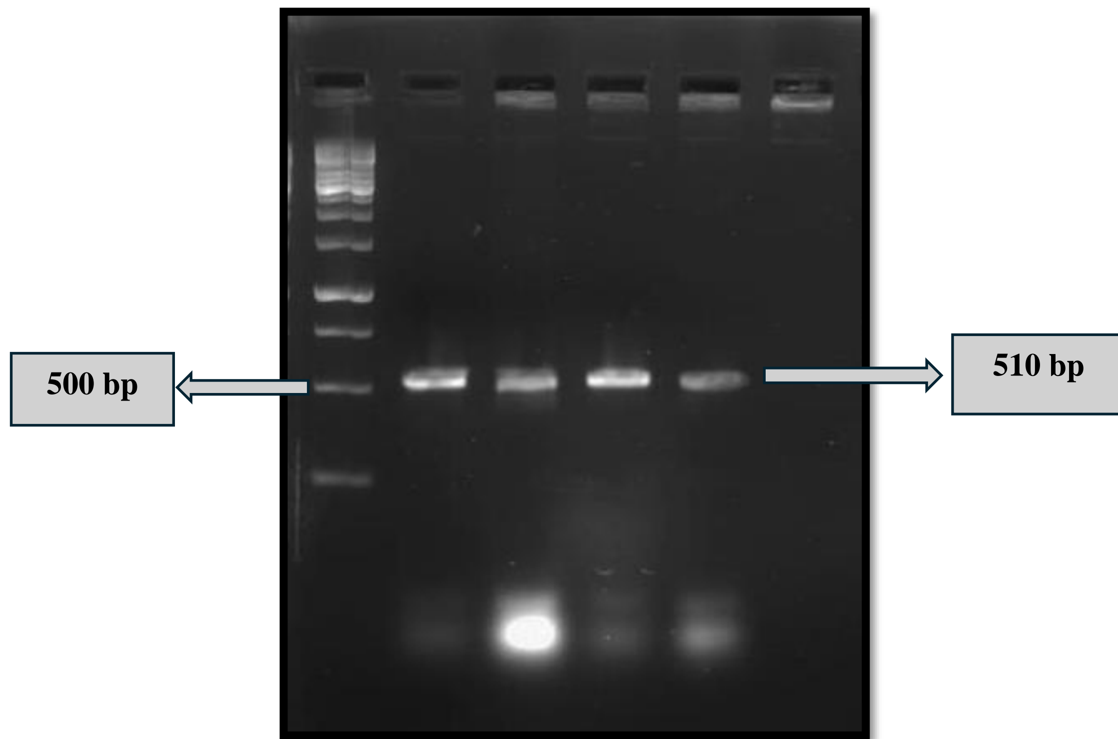


Fig 20: Agarose gel electrophoresis of amplification of *haC* gene from *M. fortuitum* DNA. Lane 1: 1 kb DNA ladder (GeneRuler) (size of fragments in base pairs) (from bottom to top: 250, 500,1000,1500,2000,2500,3000,3500, 4000,5000,6000,8000,10000), Lane 2,3,4,5: *hadC* gene (510 bp).

4.3 Study of wild type *M. fortuitum* and mutant *M. fortuitum hadC* knockdown strain under different stress conditions.

4.3.1 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°C without shaking. After 6 days the colour changes from blue to colourless 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.

Wild Type – 0 day

Mutant – 0 day

Wild Type – 6 days

Mutant – 6 days

Wild Type – 12 days

Mutant – 12 days

Wild Type – 18 days

Mutant – 18 days

Wild Type – 24 days

Mutant – 24 days

Wild Type – 30

Mutant – 30 days

Fig 21: Hypoxic stress. Image shows that Methylene Blue was added to the vials as oxygen indicator. After 6 days it became colourless due to 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 after 15 days remains constant till 30 days. Whereas in case of wild type it shows a constant growth after 12 days. However, after 33 days no survival was seen in case of mutant strain as shown in Fig:22.

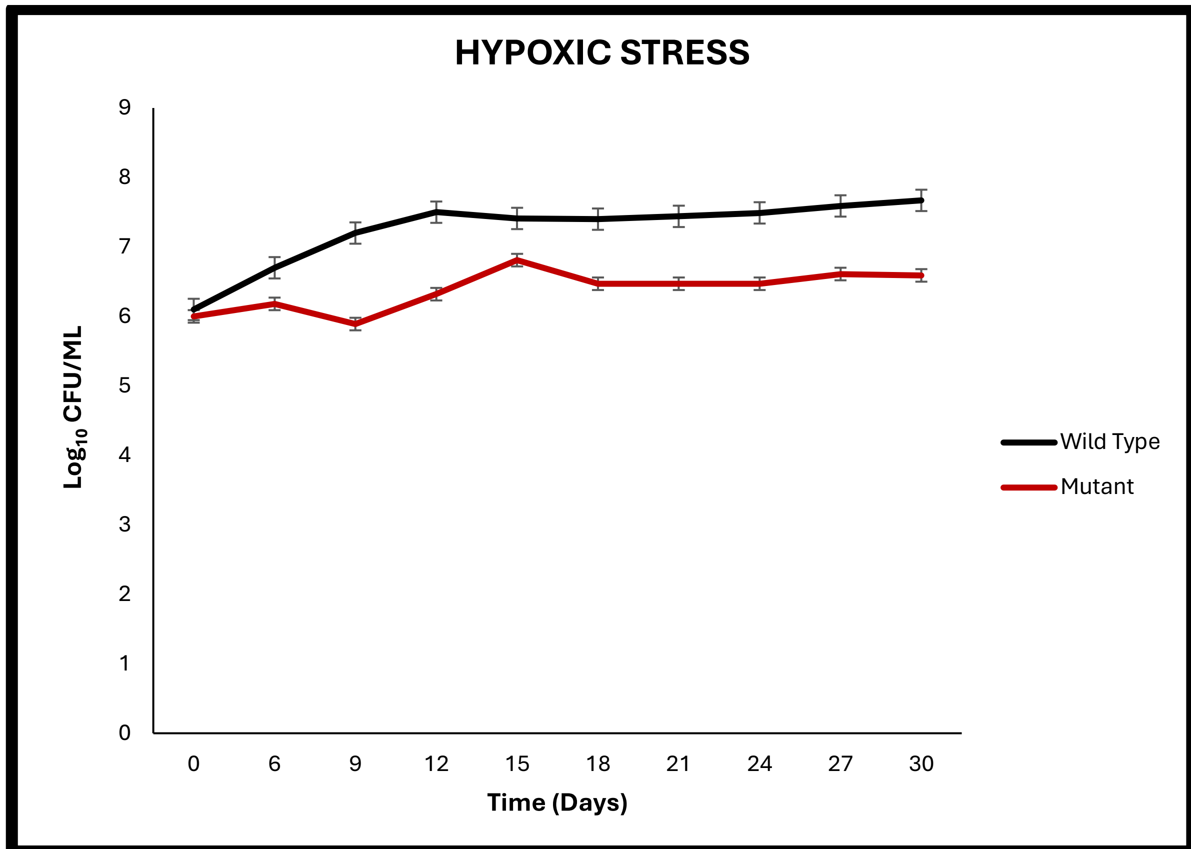
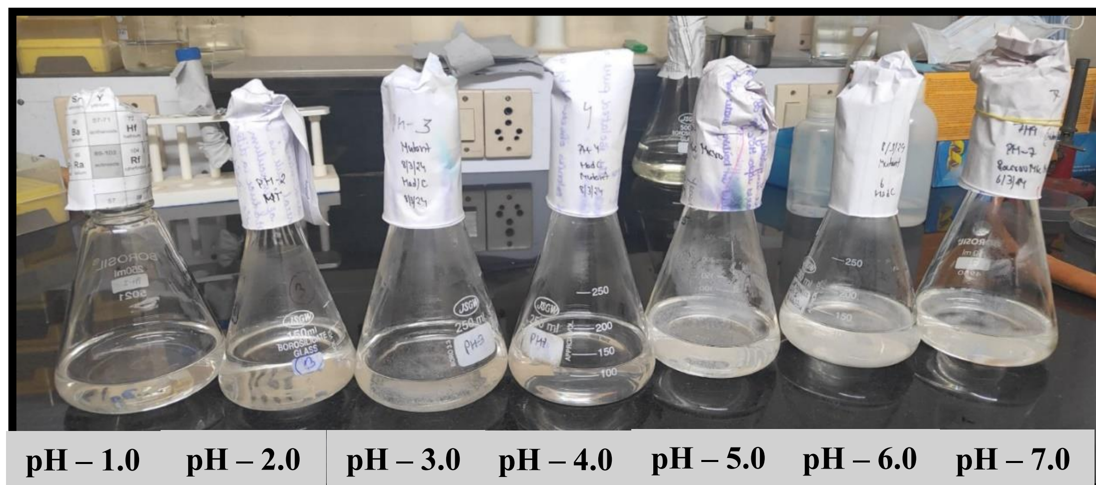


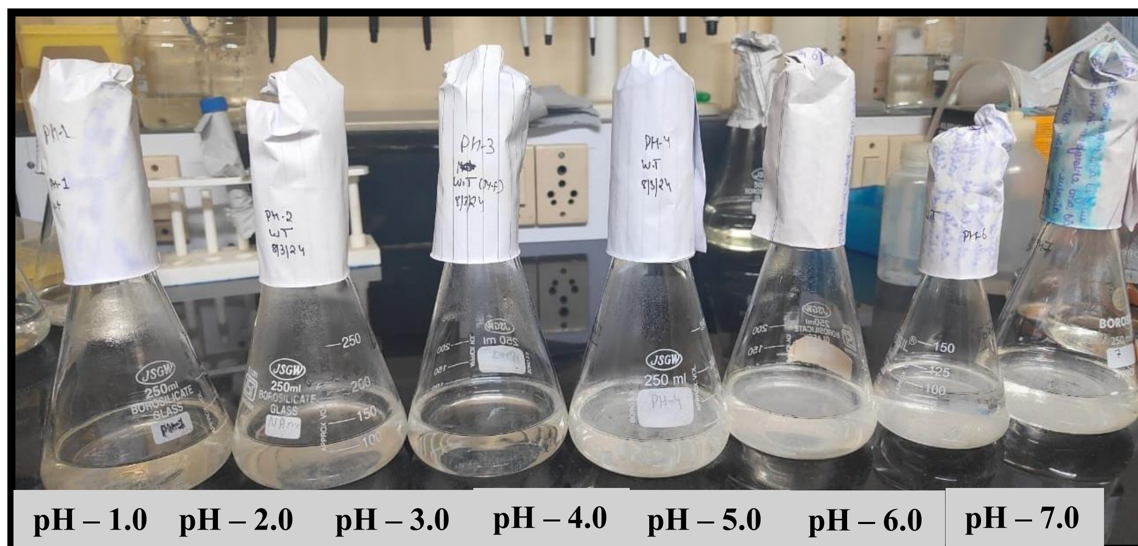
Fig 22: Survival and growth of *M. fortuitum hadC* gene mutant knockdown strain under hypoxic conditions. Graph shows that both wild type and mutant *hadC* showing equal growth and survival at hypoxic conditions.

4.3.2 Acidic stress

Mutant (*fabG4*) strain and Wild type strain of *M. fortuitum* were inoculated in different pH media as shown in Fig:23 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 23:(a), (b) Survival and growth of *M. fortuitum hadC* gene mutant knockdown strain under acidic stress at different pH from 1.0 to 7.0 after 48 hrs. In 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 declined after 12 hours. However, in case of mutant a steep decline was observed. After 24 hours no survival was seen as shown in Fig:24.

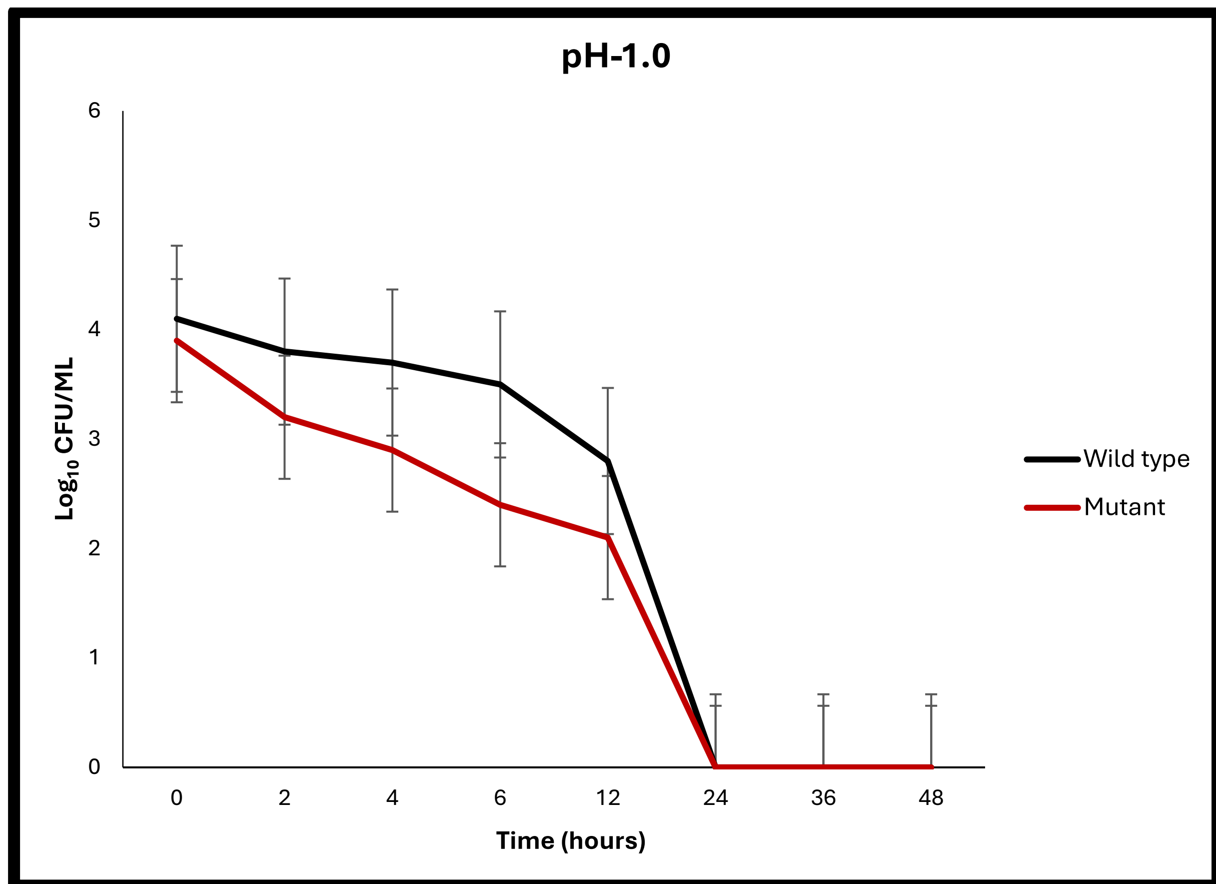


Fig 24: Survival and growth of *M. fortuitum hadC* gene mutant knockdown strain at pH- 1.0. Figure shows that at pH-1.0 growth of WT declined after 12 hours, however mutant growth is also declined but at faster rate as compared to WT. Growth of both WT and mutant strain show constant decline in growth 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 declined after 12 hours. However, in case of mutant a steep decline was observed. After 36 hours no survival was seen in both mutant and wild type as shown in Fig: 25.

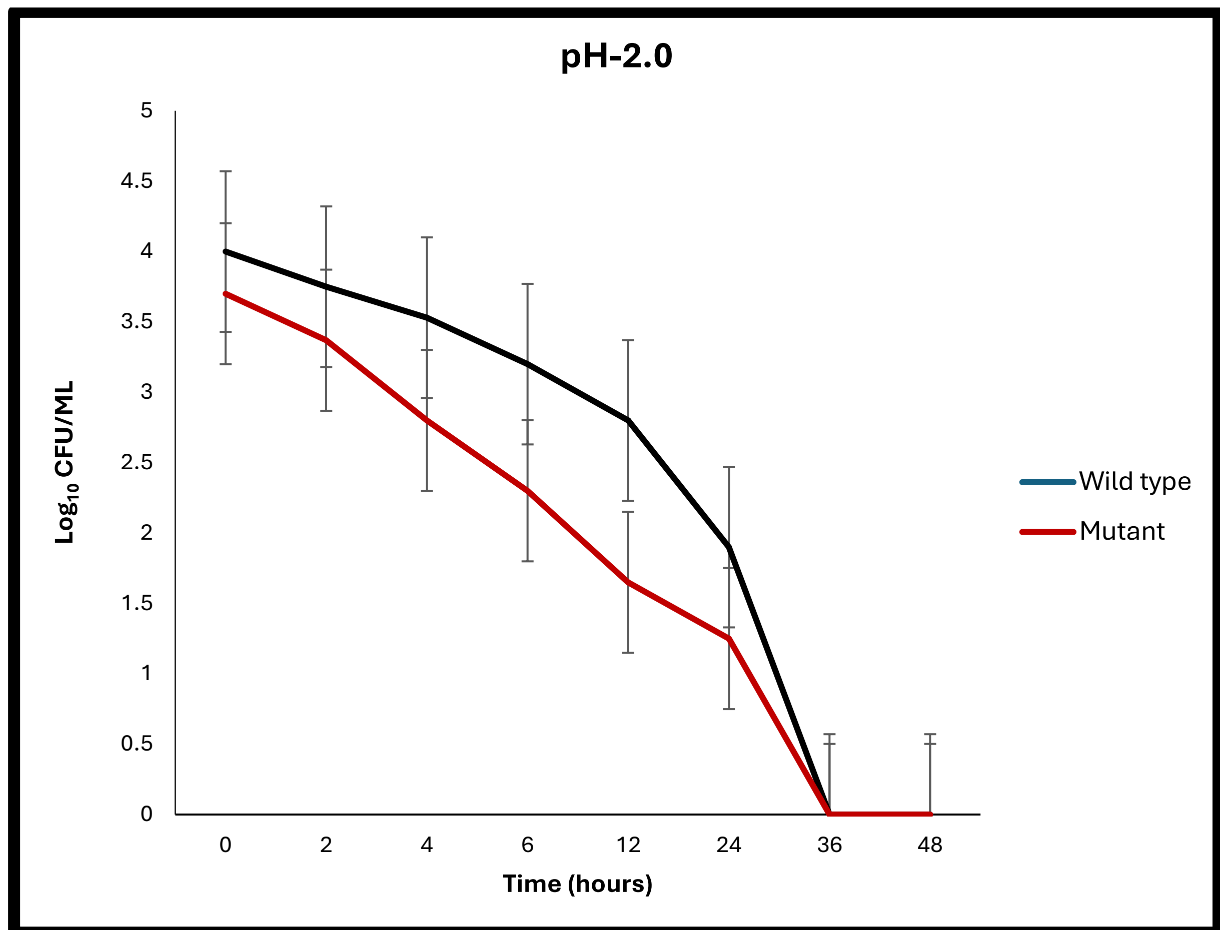


Fig 25: Survival and growth of *M. fortuitum hadC* gene mutant knockdown strain at pH- 2.0. It shows that the at pH-2 growth of mutant and wild type decline after 12 hours. After 36 hours no survival was observed in both mutant and wild type.

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 declined after 12 hours. However, in case of mutant a steep decline was observed. After 36 hours no survival was seen in both mutant and wild type as shown in Fig: 26.

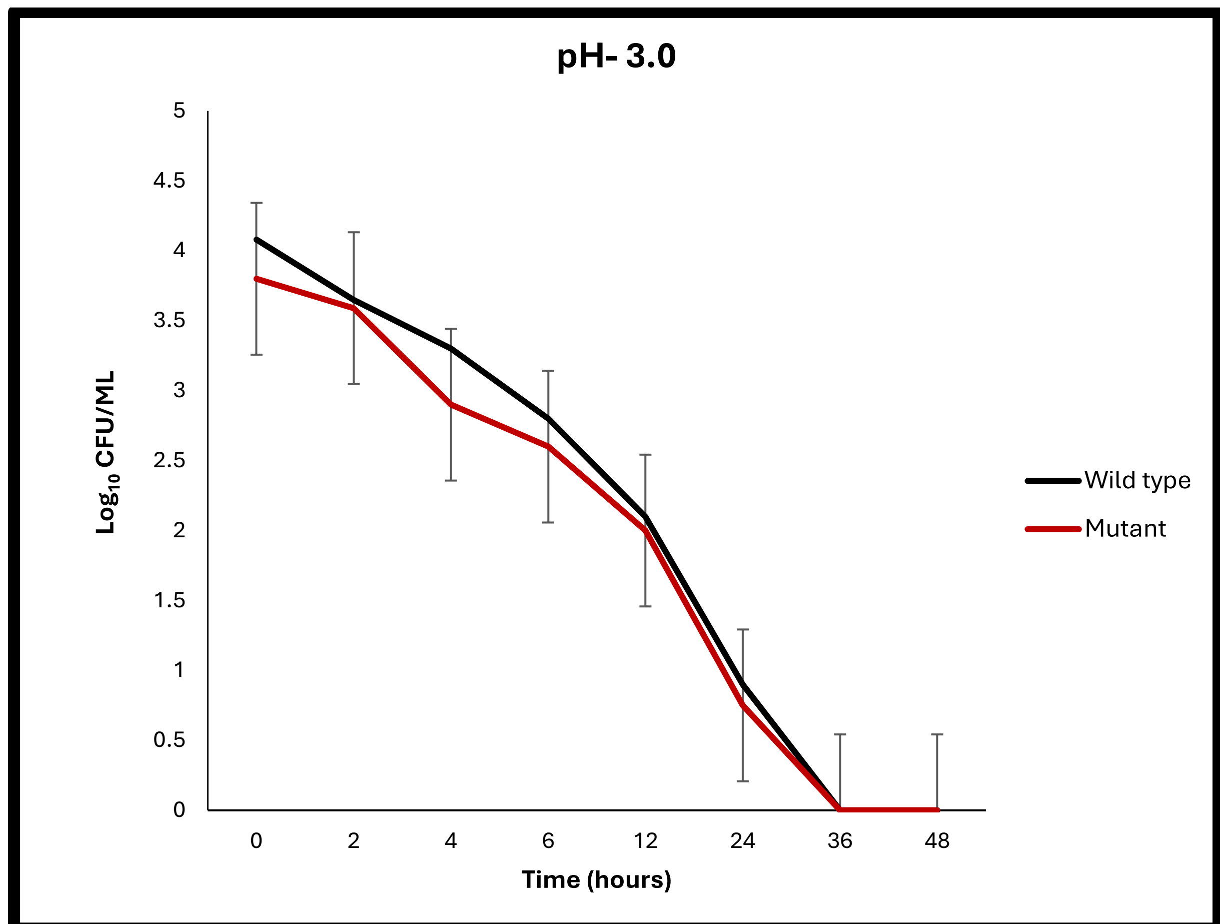


Fig 26: Survival and growth of *M. fortuitum hadC* gene mutant knockdown strain at pH- 3.0. It shows that the at pH-3 growth of mutant and wild type decline after 12 hours. After 36 hours no survival of both mutant and wild type was observed.

pH – 4

Mutant and wild type strain of *M. fortuitum* were inoculated in MB7H9 medium of pH-4.0. The growth of both wild type and mutant strain constant declined after 6 hours. However, in case of mutant a decline was observed after 12 hours as shown in Fig: 27.

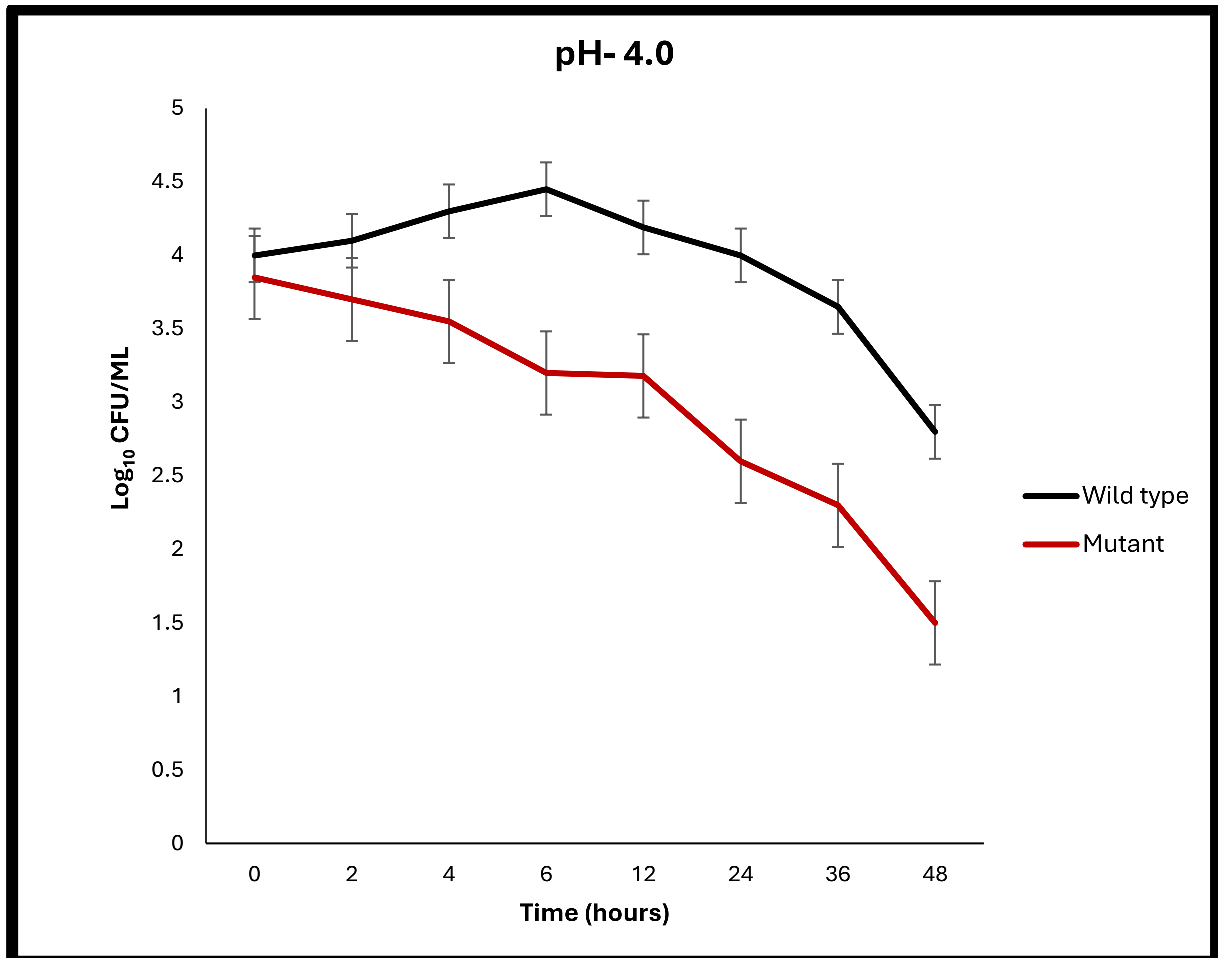


Fig 27: Survival and growth of *M. fortuitum hadC* gene mutant knockdown strain at pH- 4.0 It shows that at pH-4, Growth of the mutant and wild type both declines after 6 hours. After 36 hours both mutant and wild type was able to survive.

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: 28.

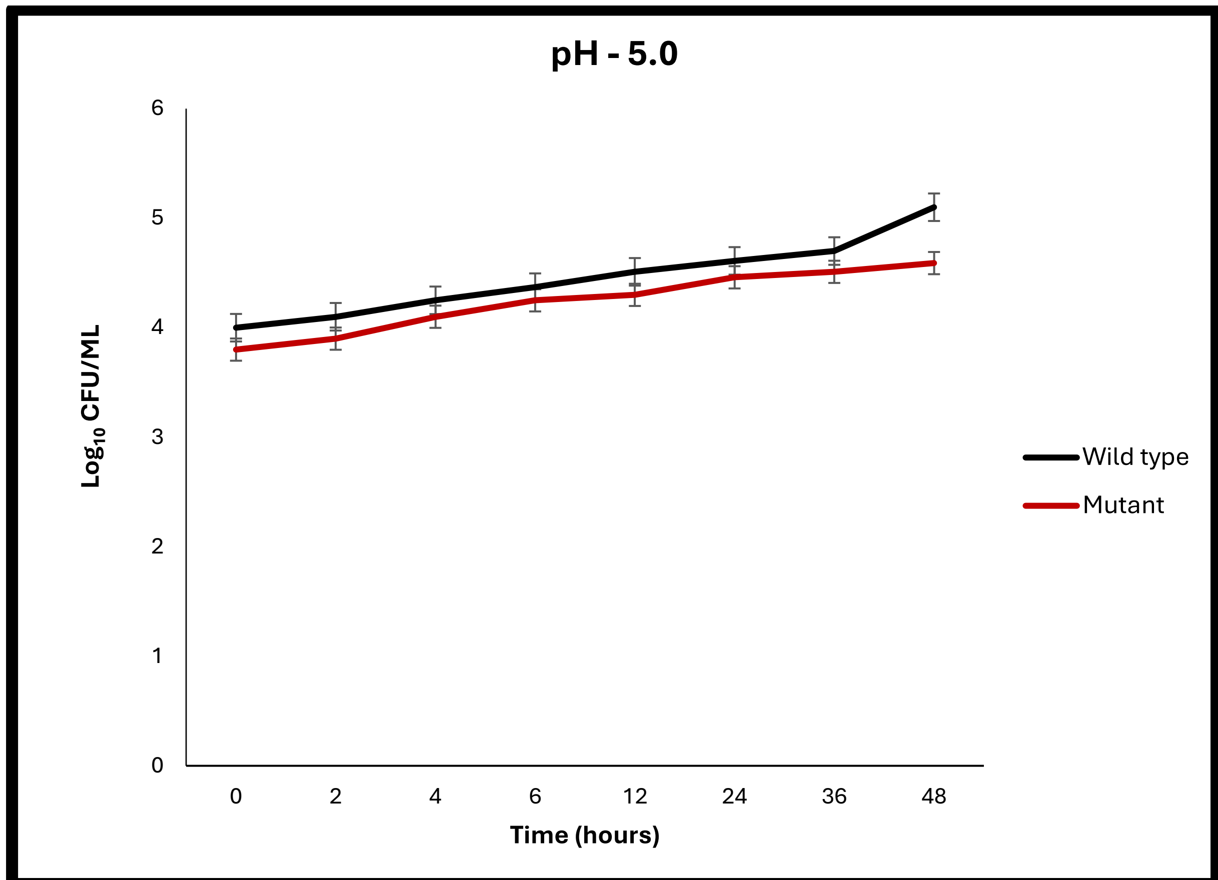


Fig 28: Survival and growth of *M. fortuitum hadC* gene mutant knockdown strain at pH- 5.0. Graph shows that at pH -5 the growth of both mutant and wild type increases simultaneously. After 36 hours both mutant and wild type was able to survive.

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: 29.

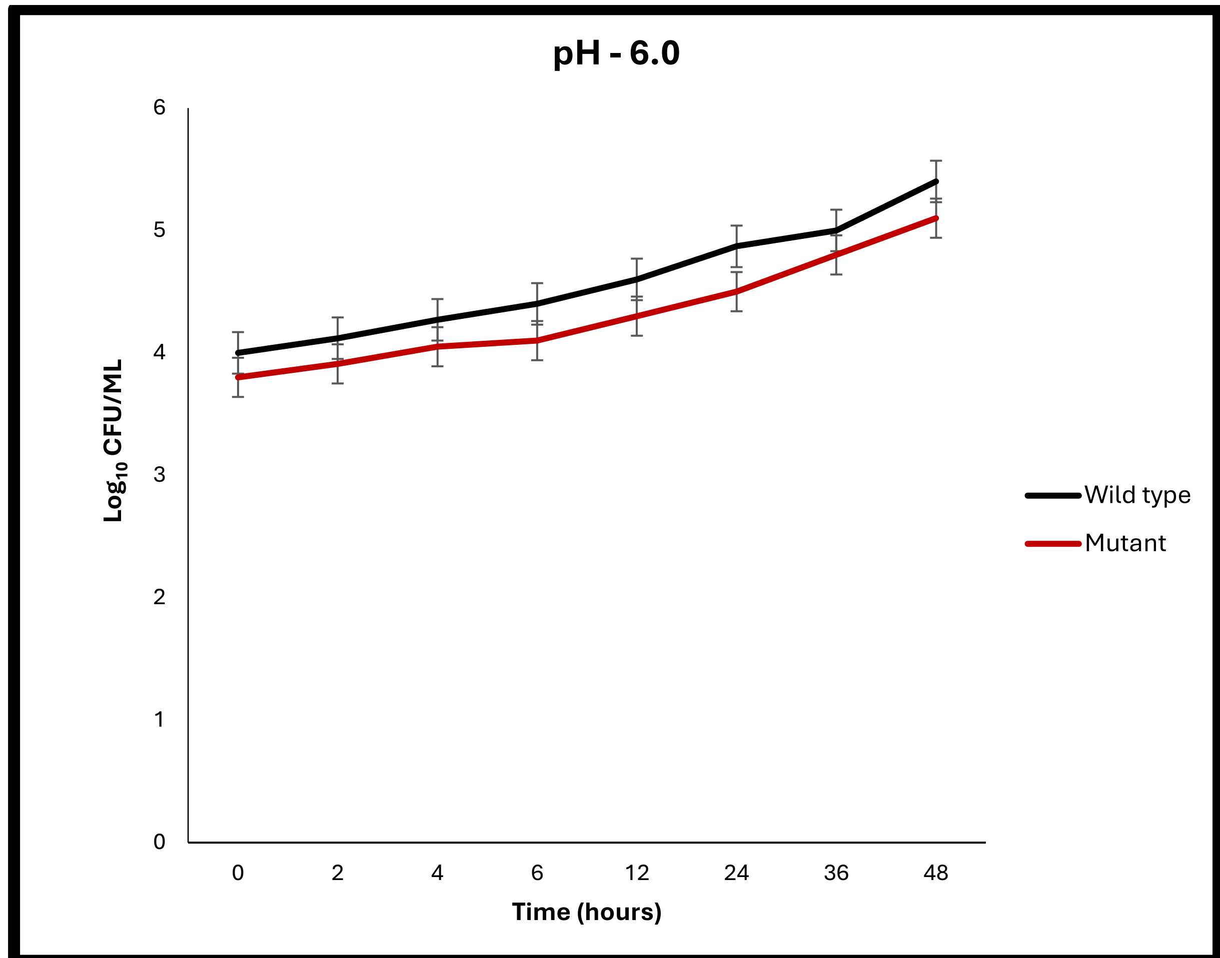


Fig 29: Survival and growth of *M. fortuitum hadC* gene mutant knockdown strain at pH- 6.0. Graph shows that at pH-6 the growth of both mutant and wild type increases simultaneously. After 36 hours both mutant and wild type was able to survive.

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: 30.

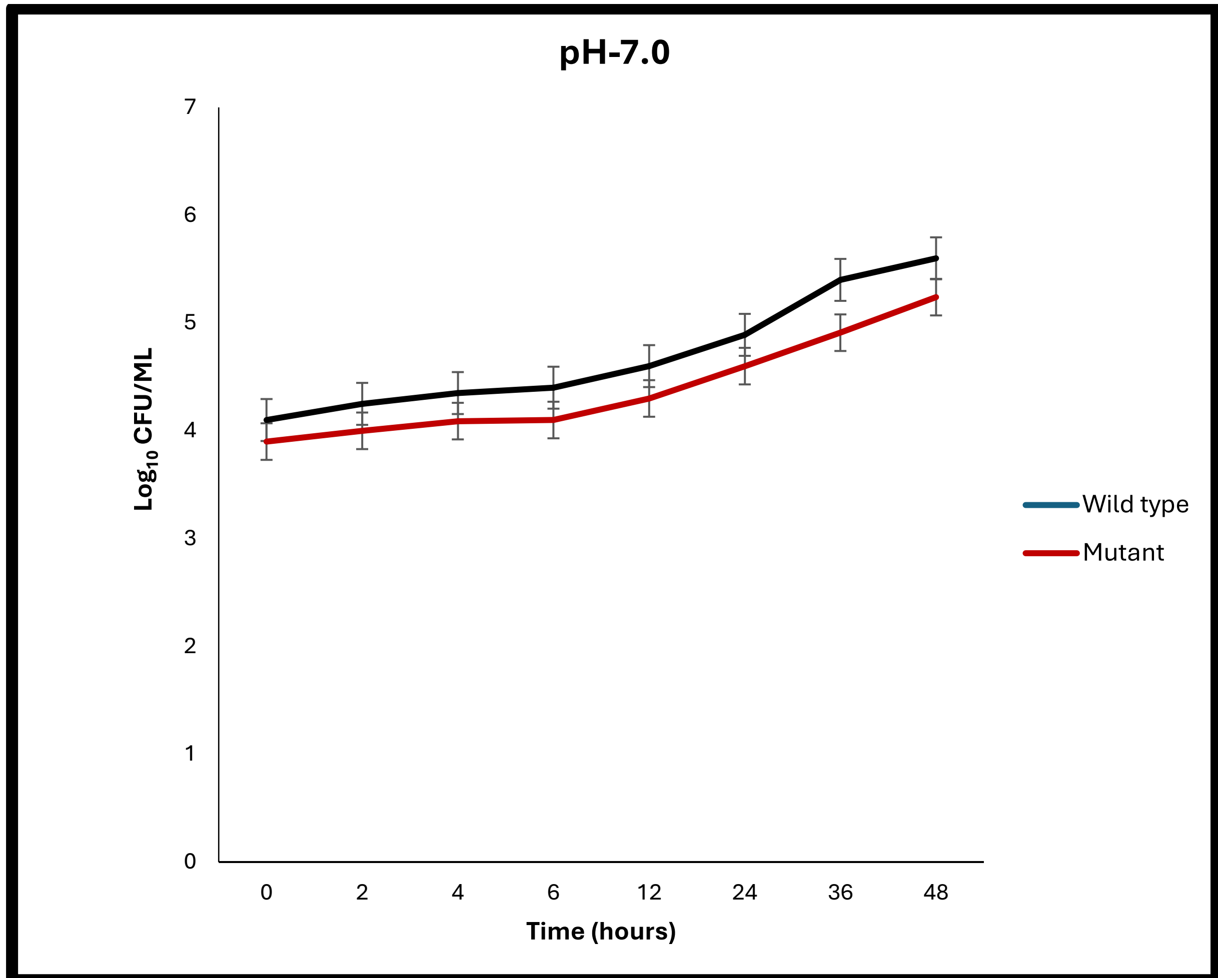


Fig 30: Survival and growth of *M. fortuitum hadC* gene mutant knockdown strain at pH- 7.0. Graph shows that at pH 7 the growth of both mutant and wild increases simultaneously. After 36 hours both mutant and wild type was able to survive.

4.3.3 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.

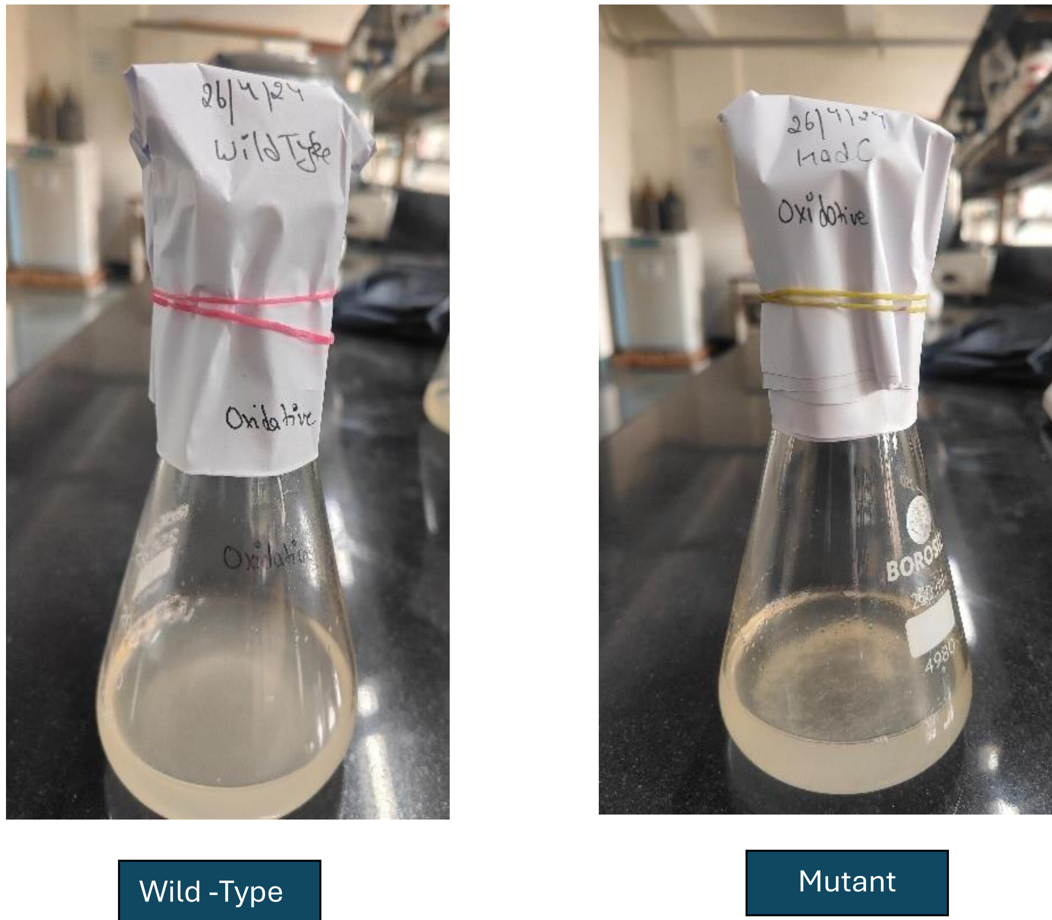


Fig 31: Mutant (*hadC*) and wild type (*M. fortuitum*) were inoculated in conical flasks containing MB7H9 medium and and 10mM H_2O_2. In image we can observe the turbidity after 72 hours.

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 48 hours the growth of mutant declined from log 3.8 to log 3.2 as shown in Fig: 37.

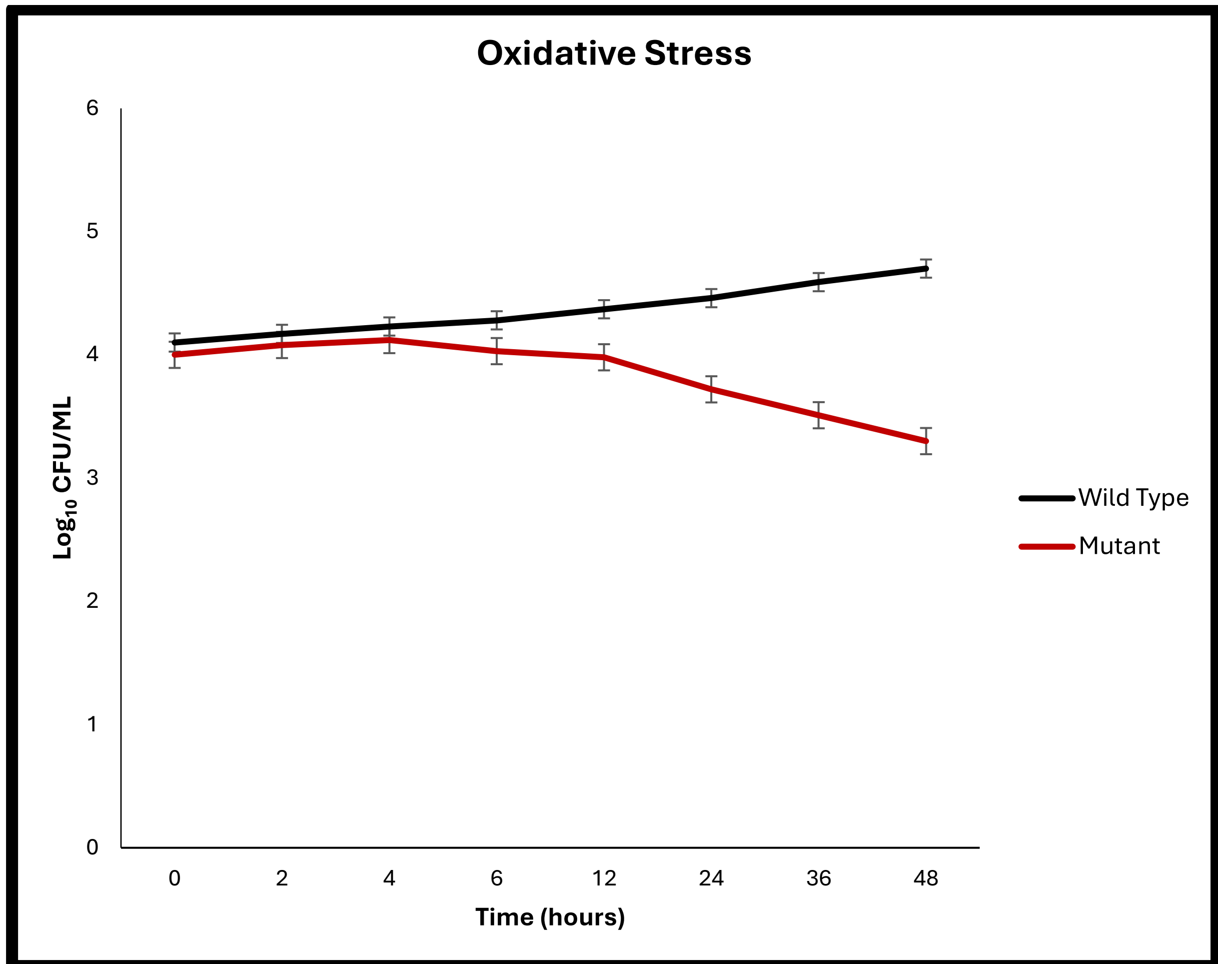


Fig 32: Survival and growth of *M. fortuitum hadC* gene mutant knockdown strain under oxidative stress. Graph shows that After 12 hours, mutant growth starts to slow down, whereas wild type growth remains constant. Whereas in case of wild type there was increase in growth after 4 hours.

4.3.4 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°C under shaking conditions at 180 rpm as shown in Fig:33 and samples were collected at different time points and plated on NAT plates to determine CFU.

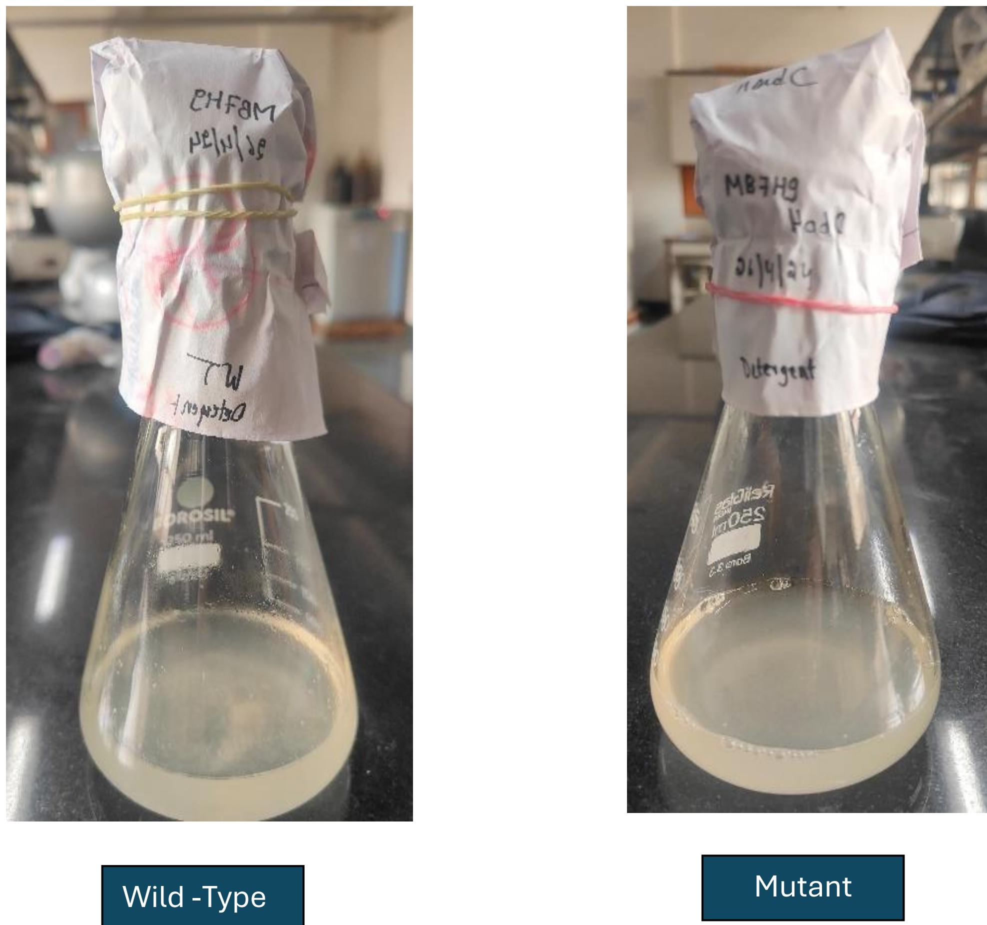


Fig 33: Mutant (*hadC*) and wild type (*M. fortuitum*) were inoculated in conical flasks containing MB7H9 medium and 0.05% SDS. In this image we can observe the turbidity after 72 hours.

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.

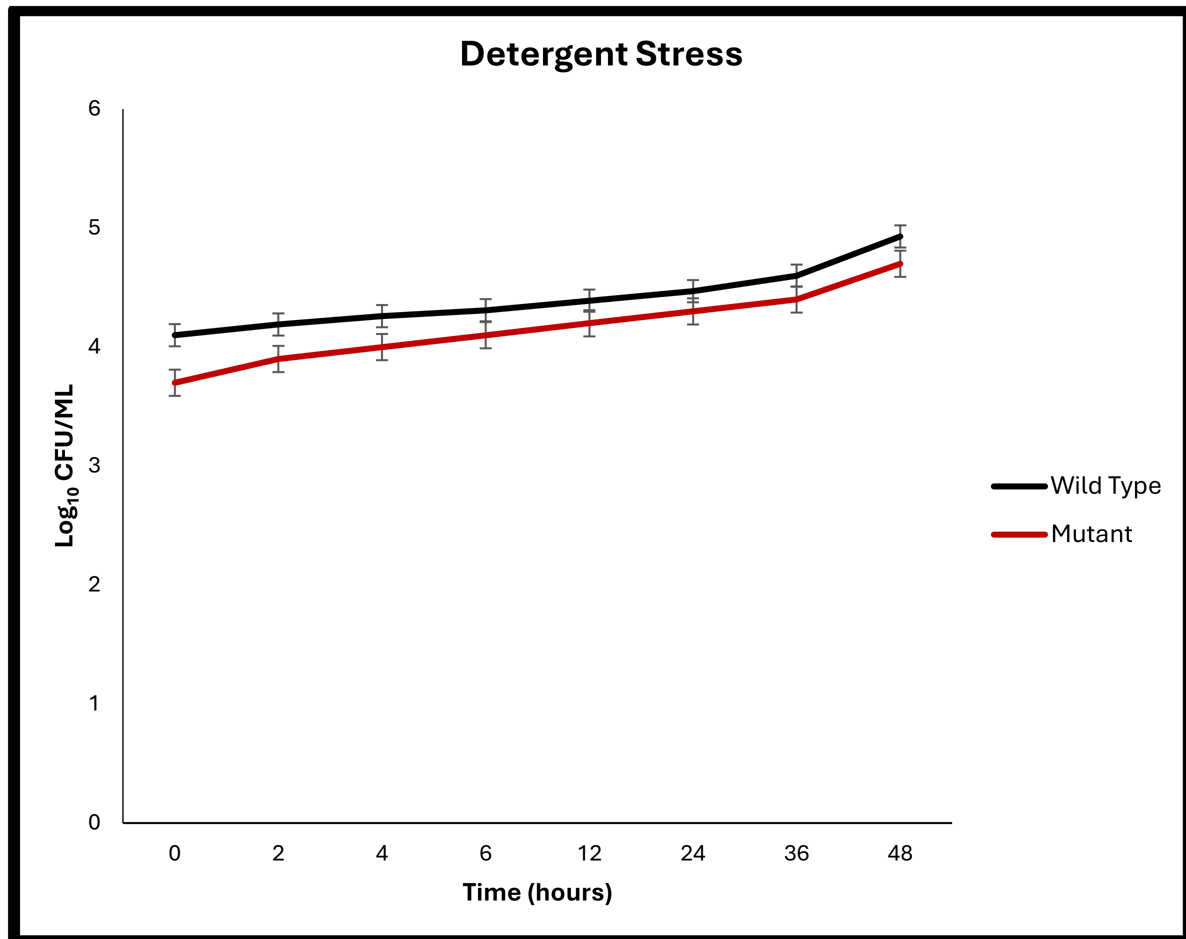
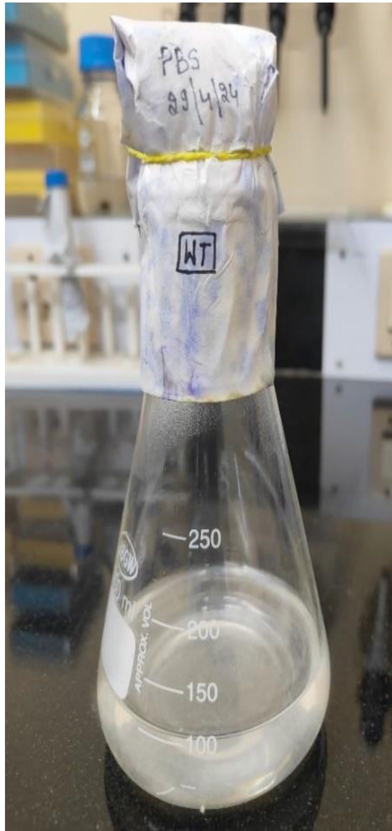


Fig 34: Survival and growth of *M. fortuitum hadC* gene mutant knockdown strain under detergent stress. Figure shows that over the course of the measurement period, both strains are growing at same rate.

4.3.4 Nutrient starvation stress

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°C under shaking conditions at 180 rpm as shown in Fig: 35 and samples were collected at different time points and plated on NAT plates to determine CFU.



Wild Type **Mutant**

Fig 35 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 image we can observe the turbidity after72 hours.

Mutant and wild type strain were grown in 1X PBS buffer. After 12 hours both mutant and wild strain decline. However, after 36 hours the growth of mutant and wild type remains constant as shown in Fig: 36.

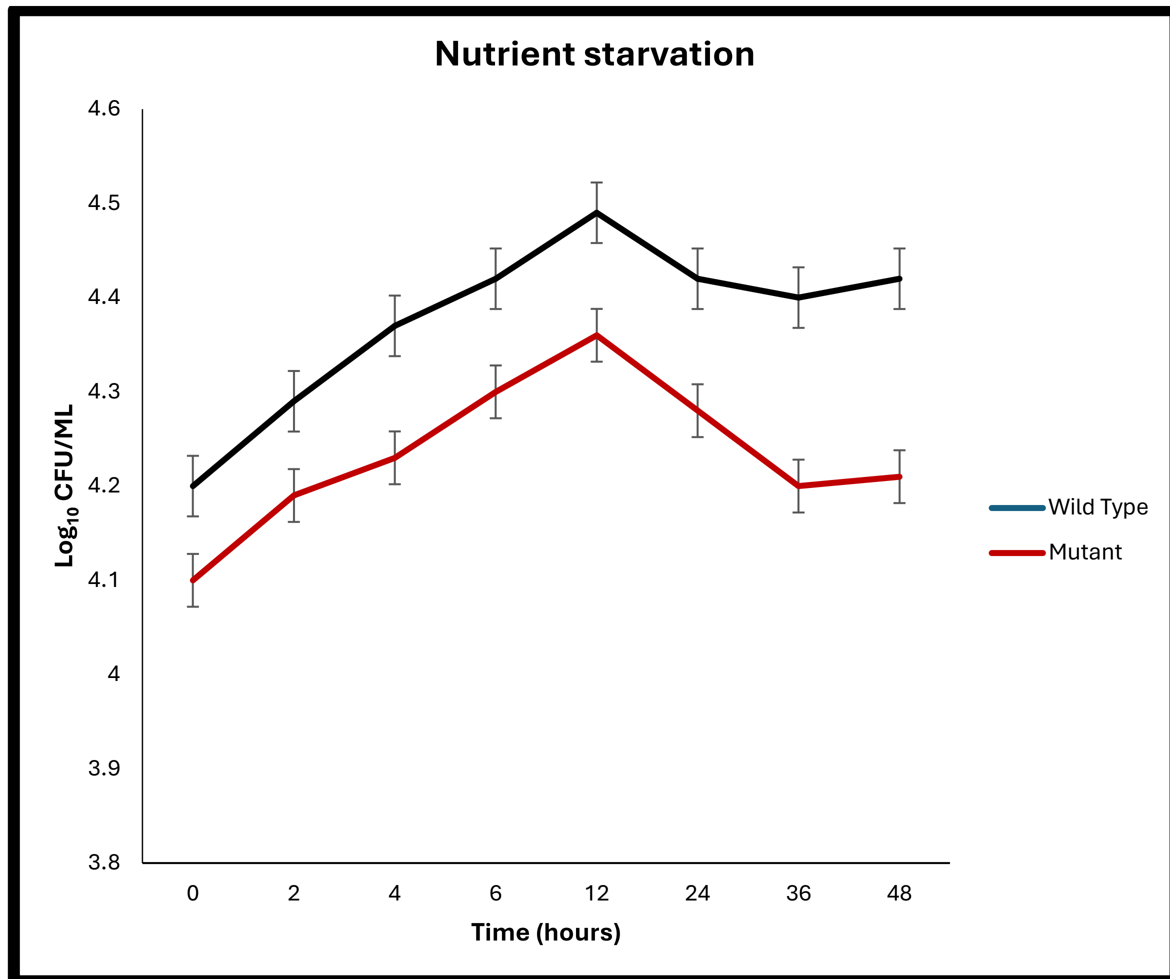


Fig 36: Survival and growth of *M. fortuitum and hadC* gene mutant knockdown strain under detergent stress. Figure shows that mutant growth decline after 12 hr. The wild type is also decline after 12 hrs but after that both are growing at constant rate. After 36 hours the growth of mutant and wild type remains constant.

CHAPTER – 5

DISCUSSION & CONCLUSION

5. Discussion & Conclusion

Species that do not cause TB are known as non-tuberculous mycobacteria (NTM), which are distinct from the *M. tuberculosis* complex [13]. Non-Tuberculosis Mycobacterium are essentially free-living, naturally occurring creatures. As of now, around 140 NTM species have been discovered. Pulmonary infections are the most common (65–90%) of the several mycobacterial illnesses they may cause. Human disorders such skin infections, implant-associated infections, disseminated diseases in immunocompromised people, and superficial lymphadenitis are mostly caused by NTM.

M. fortuitum is a fast-growing non-tuberculous mycobacterium (NTM) that has drawn more attention from the scientific and medical communities since it may infect people with a range of illnesses, regardless of their immune system status. Because NTM prevalence varies geographically and reporting methods and diagnostic criteria have changed over time, it is challenging to pinpoint the exact number of NTM cases worldwide. *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.

This study examined the function of the *hadC* gene in *M. fortuitum*, a common bacterium renowned for its environmental resistance and opportunistic pathogenicity. By contrasting the growth patterns of a *hadC* mutant strain with the wild-type strain under a variety of stress scenarios, including as acidic stress, hypoxic stress, food starvation, detergent treatment, and oxidative stress, we were able to investigate the functional importance of *hadC*. Our research sheds new light on *hadC*'s role in *M. fortuitum*'s stress tolerance processes. The *hadC* mutant and wild-type strain responded to acidic conditions quite differently, as we were able to detect.

When the pH was adjusted to 1, the mutant showed a noticeable reduction in growth in just two hours, but the natural type continued to develop for a longer period of time, showing a dip only after six hours. When opposed to the wild type's delayed reduction at the same time point, the mutant showed a decrease after 24 hours, continuing this trend at pH 2. Within 24 hours, there was a reduction in both strains at pH 3. Remarkably, at pH 4, the growth trend of the mutant and wild type was identical, with a drop occurring only after 36 hours. Remarkably, both strains showed increased growth at pH values of 5, 6, and 7, indicating a possible adaption mechanism for the bacterium in these nearly neutral conditions.

These results imply that *hadC* is essential for *M. fortuitum* to be able to tolerate acidic stress, especially at lower pH values. The creation of acid-resistant systems, pH homeostasis, and cellular integrity may all be impacted by the *hadC* gene product. The mutant and wild type showed a clear difference when exposed to hypoxic circumstances, which are low oxygen availability. Within 24 hours, the *hadC* mutant showed a notable reduction in growth, whereas the wild type exhibited a longer survival time. The results indicate that *hadC* plays a crucial role in *M. fortuitum*'s ability to adapt to low oxygen conditions.

Other respiratory routes or the synthesis of oxygen-scavenging enzymes, which are essential for life in hypoxic environments, may be mediated by the *hadC* gene product.Following a 12-hour period of stagnation during which growth remained mostly steady, both the mutant and wild type showed a small drop in growth when subjected to nutritional restriction. This shows that both strains have strategies for energy conservation and nutrient-limited survival. *HadC* may not have a substantial role in *M. fortuitum*'s reaction to food shortage, though, as there is no discernible difference between the mutant and wild type.

It's interesting to note that under detergent stress, the wild type and *hadC* mutant showed comparable growth trends. This implies that *hadC* may not play a major role in the bacterium's resistance to the disruption that detergents cause to cell membranes. The strains might be distinguished from one another by their reactions to reactive oxygen species-induced oxidative stress. The *hadC* mutant showed a marked reduction in growth after just 12 hours, but the wild type continued to develop for up to 48 hours. According to this research, *hadC* is essential for shielding *M. fortuitum* from oxidative damage.

The *hadC* gene product may be involved in the synthesis of antioxidant enzymes or in the restoration of cellular components that have been oxidatively damaged. *M. fortuitum* is a fast-growing non-tuberculous *Mycobacterium* (NTM) that has drawn more attention from the scientific and medical communities since it may infect people with a range of illnesses, regardless of their immune system status. Because NTM prevalence varies geographically and reporting methods and diagnostic criteria have changed over time, it is challenging to pinpoint the exact number of NTM cases worldwide.

The role of the *hadC* gene in certain stress responses was demonstrated by studying *M. fortuitum* under a variety of stress scenarios. Although the mutant and wild type were able to withstand acidic settings with pH values ranging from 5.0 to 7.0, the mutant was able to survive in acidic situations with pH range of 1.0 to 4.0 like the wild type. Remarkably, both strains

fared just as well in settings with little oxygen and nutrients. Both strains were not considerably affected by detergent stress, however the mutant was at a growth disadvantage due to oxidative damage. Based on these results, it appears that *hadC* is probably essential for *M. fortuitum*'s ability to withstand oxidative stress. This implies that *hadC* might not play a major role in the stress response mechanisms of *M. fortuitum*. Homologues of *MFhadC* is not used as drug target for related pathogenic mycobacteria.

CHAPTER – 6

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CHAPTER – 7

APPENDIX

APPENDIX

8.1. 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].

8.1.1 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

8.1.2 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)

8.1.3 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	1.1 % (v/v)

8.1.4 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.

8.1.5 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)

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

8.2.1 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

8.2.2 Acid alcohol (decolourizer)

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

8.2.3 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.

1.3 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

1.4 Reagents and Buffers

8.4.1 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)

8.4.2 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.

8.4.3 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.

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

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

NaCl	9g

Tween 80	0.1 (v/v)

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

8.5.1 Tris-EDTA saline (TES) buffer

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

8.5.2 Lysozyme [Storage at -20°C]

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

8.5.3 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).

8.5.4 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.

8.5.5 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.

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

6.6.1 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.

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

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

8.6.3 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

8.6 Agarose gel Electrophoresis

8.6.1 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

8.6.2 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

PUBLICATIONS

1. Oral Microbiome as Tool for Diagnosis and Prognosis of Diseases.
   <u>Sourav Kumar</u>, Diksha Suman, 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. Role of Oral Microbiome in Prognosis and Diagnosis**.**
   <u>Sourav Kumar,</u> Diksha Suman, Rahul Shrivastava*.
   Book chapter submitted and accepted for Book entitled "Oral Microbiome: Hygiene and Health".

3. Biogenic Silica in Ocean and Terrestrial Environment.
   Diksha Suman, **<u>Sourav Kumar,</u>** Ashok Kumar Nadda, Rahul Shrivastava*.
   Book chapter submitted and accepted for publications to Royal Society of Chemistry for Book entitled "Biogenic Silica".

4. Study role of *fabG4* and *hadC* genes in *M. fortuitum* under stress conditions.
   Diksha Suman, **<u>Sourav Kumar</u>**, 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.