BIOCHEMICAL STUDIES OF NITRITE REDUCTASE IN *MYCOBACTERIUM SP.*

Thesis submitted to University of Pune

For the degree of

DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY

By

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DEDICATION

I dedicate this thesis to my parents, father Md. Salahuddin and my mother Asgari Begum without their support I had never completed my PhD work. I want to sincerely thanks them for their patience and believe in me throughout the life and especially during the PhD period when I required it the most. Their faith always gave confidence to deliver each and every time when I fail or become week. My elder brother, Nasim Akhtar is another very important person in my life, who always supports me without knowing the fate of the path that I have chosen. He is and will always be the true inspiration of my life and I truly believe that by following his way of choosing his carrier path will definitely guide me throughout my life. Lastly my Bhabhi Mrs. Farha Akhtar who supported me mainly during the last stage of my thesis submission has helped me in completion of this goal.

"Many a man never fails because he never tries"

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CERTIFICATE

This is to certify that the work incorporated in the thesis entitled "**Biochemical studies of nitrite reductase in *Mycobacterium sp.***" submitted by **Mr. Shamim Akhtar** was carried out under my supervision at Combi Chem. Bioresource Center, Organic Chemistry Division, National Chemical Laboratory, Pune – 411008, Maharashtra, India.

Dr. Dhiman Sarkar

(Research Guide)

DECLARATION BY RESEARCH SCHOLAR

I hereby declare that the thesis entitled "**Biochemical studies of nitrite reductase in *Mycobacterium sp.***", submitted for the Degree of ***Doctor of Philosophy*** to the University of Pune, has been carried out by me at Combi Chem. Bioresource Center, Organic Chemistry Division, National Chemical Laboratory, Pune-411 008, Maharashtra, India, under the supervision of Dr. Dhiman Sarkar (Research supervisor). The work is original and has not been submitted in part or full by me for any other degree or diploma to any other University.

Mr. Shamim Akhtar

(Research Scholar)

ABSTRACT

Mycobacterium tuberculosis (MTb), the disease causing organism for tuberculosis (TB) is responsible for 2 million deaths annually worldwide. The bottle neck of complete eradication of this disease is survival of Mtb in latent form which can reactivate anytime when the immune system of host is compromised. The current scenario of TB has become worse than expected by the outcome of XDR (Extremely drug resistance) strain and the rate of death caused by TB in HIV positive patients. The present strategies are insufficient to control the pathogen; henceforth there is urgent requirement of screening of new genes and its product, proteins which can be potential future drug targets.

MTb is active reducer of nitrate as nitrogen source during latency. It is well known that during latency, nitrogen metabolism play important role for bacterial survival because of requirement of certain proteins in large amount to support its survival during latency. Hence most of the genes involved in protein metabolism are reported as potential drug target including nitrate reductase (nr) and glutamine synthatase (gs). Unfortunately the connecting link between nitrate reductase and glutamine synthatase which is nitrite reductase (nir) is missing. So far, there is no report about the nir from mycobacterium about its functional active role. This is an important enzyme of nitrogen metabolic pathway which helps in the survival of any organism including mycobacteria. Here in our thesis we tried to evaluate the biochemical role of nitrite reductase in *mycobacterium sp.* during its survival in aerobic and latent form.

Here we first report about the presence of functional nitrite reductase (nirB) from MTb as well as *M. smegmatis* during both actively aerobic growing as well as hypoxia induced dormant form of bacilli. We observed its induced expression at gene as well as protein activity level in presence

of nitrite as sole nitrogen source. Further, we saw that the increased expression of nirB gene during hypoxia induced dormancy as well as within THP-1 macrophage infection model. The inhibitory effect of nitrite reductase inhibitor on growth of the bacilli under both in vitro and ex-vivo conditions suggested its important role during the survival of mycobacteria during dormant stage.

During the course of this study, we observed that high concentration of nitrite in growth medium has bacteriostatic effect mainly through the production of nitric oxide. This NO induces dormancy in bacteria which was characterized by the increased expression of dos-SR regulon and change in cells morphology which is hall-mark of dormant cells along with inhibition in biofilm formation as well as staining properties.

Lastly, NirB protein from *M. smegmatis* was cloned and expressed in *E. coli* system. Characterization of certain biochemical properties was also done using this partially purified NiRB in order to better understand the mechanism of the enzyme as well as to use the enzyme for development of enzyme based screening assay to search nitrite reductase specific inhibitors.

TABLE OF CONTENTS

Abbreviations

ACR:	Alpha Crystalline Protein
AFB:	Acid Fast Bacilli
ATCC:	American Type Culture Collection
ATP:	Adenosine triphosphate
AO:	Aldehyde Oxidase
BCG:	Bacillus Calmette Guerin
BLAST:	Basic Local Alignment Search Tool
BSA:	Bovine Serum Albumin
p-CA:	para Caumaric Acid
CTAB:	Cetyl Trimethyl Ammonium Bromide
CFU:	Colony Forming Unit
CP:	Cytochrome P-450)
DAFDA:	Dichloro Acetate Fluorescein Di Acetate
DAPI:	Di Amidino-2- Phenyl Indole
DEPC:	Di Ethyl Pyro Carbonate
DETA-NO:	Di Ethyl Tri Amine
DOOR:	Database of prOkaryotic OpeRon
DOTS:	Directly observed therapy shortcourse
DOS:	Dormancy Oxygen Sensor
E. coli:	*Escherichia coli*
EDTA:	Ethylenediaminetetraacetic acid
EMB:	Ethambutol
EPR:	Electron paramagnetic Resonance
ETH:	Ethionamide
FBS:	Fetal Bovine serum
FAD:	Flavin adenine dinucleotide
FDA:	Food and Drug Administration
GAN:	Gene Accession Number
glnA:	Glutamine synthtase gene
GS:	Glutamine Synthetase
HIV:	Human Immunodeficiency Virus
HSP:	Heat shock protein
HSR:	Head Space Ratio
HTS:	High throughput screening
IFN-γ:	Interferon-γ
INH:	Isoniazid
IPTG:	Isopropyl β-D-1-thiogalactopyranoside
KEGG:	Kyoto Encyclopedia of Genes and Genomes
LAM:	Lipoarabinomannan

M. bovis:	*Mycobacterium bovis*
MDR:	Multidrug resistance
$MgCl_2$:	Magnesium Chloride
$MgSO_4$:	Magnesium sulfate
MOPS:	3-(N-morpholino) propanesulfonic acid
M. avium:	*Mycobacterium avium*
M. lepre:	*Mycobacterium lepre*
M. smegmatis:	*Mycobacterium smegmatis*
M. tuberculosis:	*Mycobacterium tuberculosis*
MOI:	Multiplicity of Infection
MW:	Molecular Weight
NADH:	Nicotinamide Adenine Dinucleotide Reduced
NCBI:	National Center for Biotechnology Infromation
NEDD:	*N*-(1-naphthyl) ethyldiamine hydrochloride
NH4:	Ammonia
Nir:	Nitrite Reductase
NO_3:	Nitrate
NO_2:	Nitrite
NO:	Nitric oxide
NOS:	Nitric Oxide Synthase
NR:	Nitrate Reductase
NRP:	Non Replicating Persistence
OD:	Optical Density
ORF:	Open Reading Frame
PAS:	Para Amino Salicylic Acid
PBS:	Phosphate Buffer Saline
PCR:	Polymerase Chain Reaction
PPD:	Purified Protein Derivative
PI:	Post Infection
PZA:	Pyrazinamide
RIF:	Rifampicin
RH:	Routin Hydrate
RNI:	Reactive Nitrogen Intermediates
ROS:	Reactive Oxygen Species
rRNA:	ribosomal Ribonucleic Acid
SCID:	Severe Combined Immune Deficiency
SDS-PAGE:	Sodium Dodecyl Sulphate- Poly Acryl amide Gel Electrophoresis
SEM:	Scanning Electron Microscopy
STM:	Streptomycin
TB:	Tuberculosis
THP-1:	Human acute monocytic leukemia cell line
TNF-α:	Tumor Necrosis Factor α
WHO:	World Health Organization
XDR:	Extremely Drug resistant

PUBLICATIONS FROM THESIS

[1] **Akhtar S.,** Sarkar S., Mishra A., Sarkar D., 2011. A method to extract intact and pure RNA from mycobocateria. *Analytical Biochemistry* 417(2) 268-288. (Re-print in Global Medical Discovery Magazine, http://globalmedicaldiscovery.com/key-scientific-articles/a-method-to-extract-intact-and-pure-rna-from-mycobacteria/)

[2] **Akhtar S.,** Sarkar S., Mishra A., Sarkar D., 2011. A method to extract intact and pure RNA from mycobocateria, *Analytical Biochemistry.* 417(2) 268-288.

[3] **Akhtar S**., Sarkar D. Presence of functional nitrite reductase in mycobacterium sp. (Manuscript under preparation)

[4] **Akhtar S**., Sarkar D., Nitrite dependent nitric oxide production induces dormancy in *Mycobacterium smegmatis* under aerobic condition. (Manuscript under preparation)

PUBLICATIONS OUTSIDE OF THESIS

[1] Singh U., **Akhtar S**., Mishra A., Sarkar D., 2011. A novel screening method based on menadione mediated rapid reduction of tetrazolium salt for testing of anti-mycobacterial agents. *J Microbiolgical Methods* 84(2) 202-207.

[2] Khan A., **Akhtar S**., Ahmad JN., Sarkar D.,2008. Presence of a functional nitrate assimilation pathway in *Mycobacterium smegmatis*. *Microbial Pathogenesis* 2008 44(1) 71-77.

[3] Sarkar S., **Akhtar S.**, Sarkar D., Depletion of Intracellular ATP due to Acute Hypoxia Triggers Necrosis of Mycobacterium tuberculosis Infected Human Macrophages. (Under Review)

[4] Mishra A., Shurpali K., **Akhtar S**., Sarkar D., Essential role of endogenous superoxide in regulation of growth and dormancy in *Mycobacterium smegmatis* (Under Review)

CHAPTER ONE

Introduction:

"Overview of tuberculosis and its causative agent"

1.1 A brief review of Tuberculosis

Tuberculosis, **MTB**, or **TB** (short for *tubercle bacillus*) is a common, and in many cases lethal, infectious disease caused by various strains of Mycobacteria, usually *Mycobacterium tuberculosis* in human being. It has been recorded in history since the Greco-Roman and Egyptian civilizations, with evidence of spinal tuberculosis being recorded as long ago as 3400 BC (Fig 1.1 C). Evidence for the infection in the form of a gibbus as well as spinal deformaties, typical of tuberculous infection of the vertebrae (also known as Potts disease or tuberculous spondylitis), was observed dating as far back as pre-dynastic Egyptian mummies some 3000BC (Fig. 1.1 A) [1]. The first written record of a tuberculous-type disease was formulated by a Babylonian monarch who documented a chronic lung disease in cuneiform script (the oldest known writing system) on a stone pillar approximately 2000 years before Christ. At the time of the famous Greek physician, Hippocrates (460-370BC), scholars described the most common condition of its day as 'phthisis' which is derived from the Greek for "wasting away". The disease was later colloquially known as 'consumption' or 'white plague' due to the same observed effects. Although Hippocrates identified the first tubercules in animal tissues, they were at the time not connected to pulmonary phthisis and it was believed that the disease was hereditary in nature. At about the same time, Aristotle identified 'scrofula' (now known as tuberculous cervical lymphadenitis, Fig. 1.1 B).

Ancient Indian scriptures also mention this disease [2], with the first known description of tuberculous spondylitis being written in Sanskrit sometime between 1500 and 700 BC. However, the modern name of the disease has been attributed to Laennec in the 1800s [3]. It has been postulated that *M. tuberculosis* existed as an unimportant pathogen to man until the coming of the industrial revolution [4].

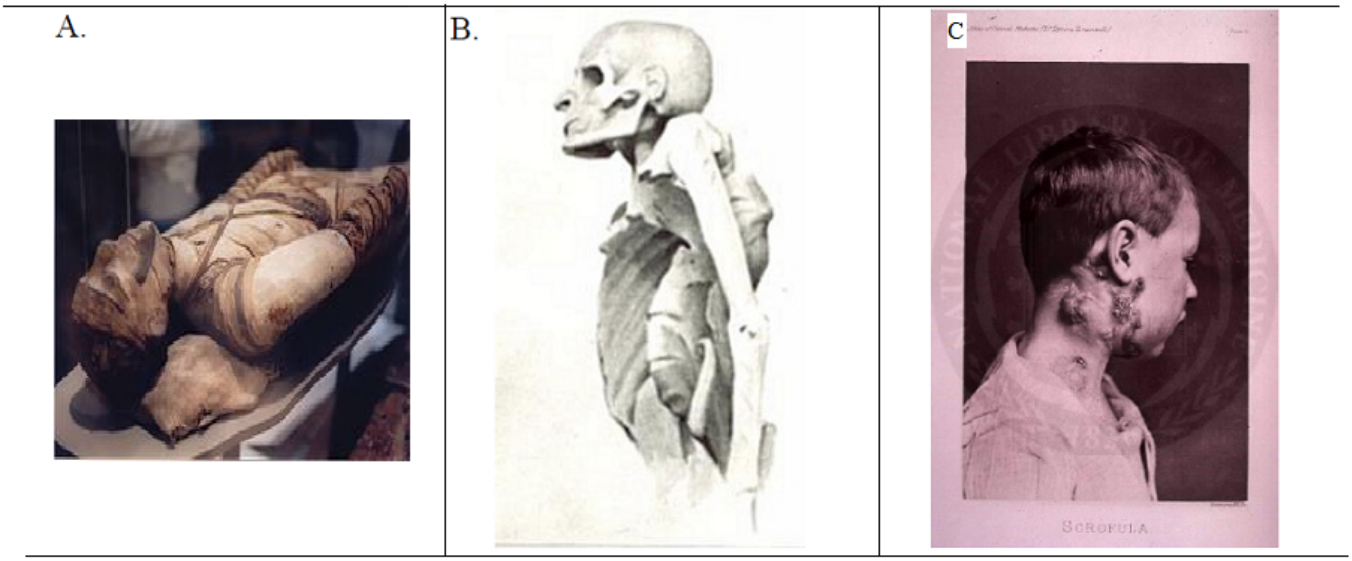


Figure 1 .1*: (A, C) Evidence for Pott's disease (tuberculosis of the spine) in an Egyptian mummy dated approximately 1000BC. B) Photograph of the condition historically known as 'scrofula'. The swelling of the lymph nodes in the neck is attributed to Mycobacterium tuberculosis infection (Source: http://en.wikipedia.org).*

With resulting urbanization and propinquity of living, a new epidemic, described as 'a great white plague', evolved. In the newly industrialized countries, the incidence of tuberculosis probably increased sharply from the mid 1700s with subsequent pandemic spread throughout Western Europe over the next century and a peak incidence around 1800 [5]. Migration probably resulted in spread to the United States, central Africa and also to South and South-east Asia. As recently as 1950, tuberculosis has affected previously completely uninfected and, therefore, non-immune populations, such as the Inuit Eskimos of Northern Canada and the natives of the highlands of Papua New Guinea [6, 7]. It has been stated that as tuberculosis moves through a non-immune population, natural selection would eventually resulted in a resistant population and subsequent gradually decline of the disease pandemic. In most persons, infection with *M.*

tuberculosis is initially contained by host defences, and the infection remains latent. However, latent tuberculous infection has the potential to develop into tuberculosis at any time, and persons with active tuberculosis become sources of new infections [8]. Today, tuberculosis remains endemic in most of the developing countries. In common with many other developed countries, Belgium faces a resurgence of tuberculosis. After declining for more than a century, notification rates began to increase in the mid 1980s and the long-term downward trend in mortality also shows signs of leveling off [9]. Several factors may have contributed to these trends, including immigration from countries with a high prevalence and the epidemic of HIV and AIDS. In addition, other underlying diseases (diabetes mellitus, chronic renal failure, chronic obstructive pulmonary disease, liver cirrhosis, leukemias and lymphomas)

and numerous sociological factors contributed to the re-emergence of tuberculosis: a growing elderly population; overcrowded prisons; poor living facilities; poor nutrition status, alcohol and drug abuse, persons in long-term care-facilities and homelessness [10, 11]. Among health care workers, the risk of occupational tuberculosis varies among and within institutions, but workers involved in autopsies and cough-inducing procedures seem to be at higher risk [12]. Finally, it is known that immigrants visiting their country of origin can "bring back" tuberculosis on their return [13]. Tuberculosis may arise in two different ways: either from a recent infection with *M. tuberculosis* or from the reactivation of dormant tubercle bacilli years or decades after initial infection resulting in tuberculous disease. As a consequence, the present level of tuberculosis comprises both individuals with "new" exogenous infections and those with a reactivation of "old" endogenous disease. The annual risk of developing pulmonary tuberculosis following a recent primary infection is estimated to be 300 times greater than the risk of disease from endogenous reactivation [14]. However, older people having lived through a period of high

tuberculosis incidence are very likely to have been infected with *M. tuberculosis* and now comprise a growing population group. In contrast, younger people who have acquired primary infections have done so during a period of much lower incidence and consequently comprise a smaller subgroup. Therefore, it has been stated that disease in the elderly largely consists of endogenous reactivation whilst most tuberculosis in younger people is the result of new exogenous infection [4].

The tuberculosis epidemic reached a peak during the 19th century when it became the leading cause of death in the western world [15]. The overcrowded unsanitary cities of the industrial revolution were devastated, especially within the poorer sections of the cities with malnourished populations. By this time the disease was pervasive throughout society and was no longer a disregarded scourge of the poor. In artistic circles, tuberculosis became a romantically tragic disease, and it was the subject of works of art, literature and music. By the end of the 19th century the incidence of tuberculosis was steadily declining [16]. Many reasons are cited, including the Pasteur's discovery of microbes as a cause of disease, but perhaps most important was an improvement in the standard of living in many countries [17].

The global distribution of TB cases at present is skewed heavily towards low-income and emerging economies (Fig.1.2). The highest prevalence of cases is in Asia, where China, India, Bangladesh, Indonesia, and Pakistan collectively make up over 50% of the global burden. Africa, and more specifically sub-Saharan Africa, has the highest incidence rate of TB, with approximately 83 and 290 per 100,000, respectively. TB cases occur predominantly (approximately 6 million of the 8 million) in the economically most productive 15- to 49-year-old age group [18].

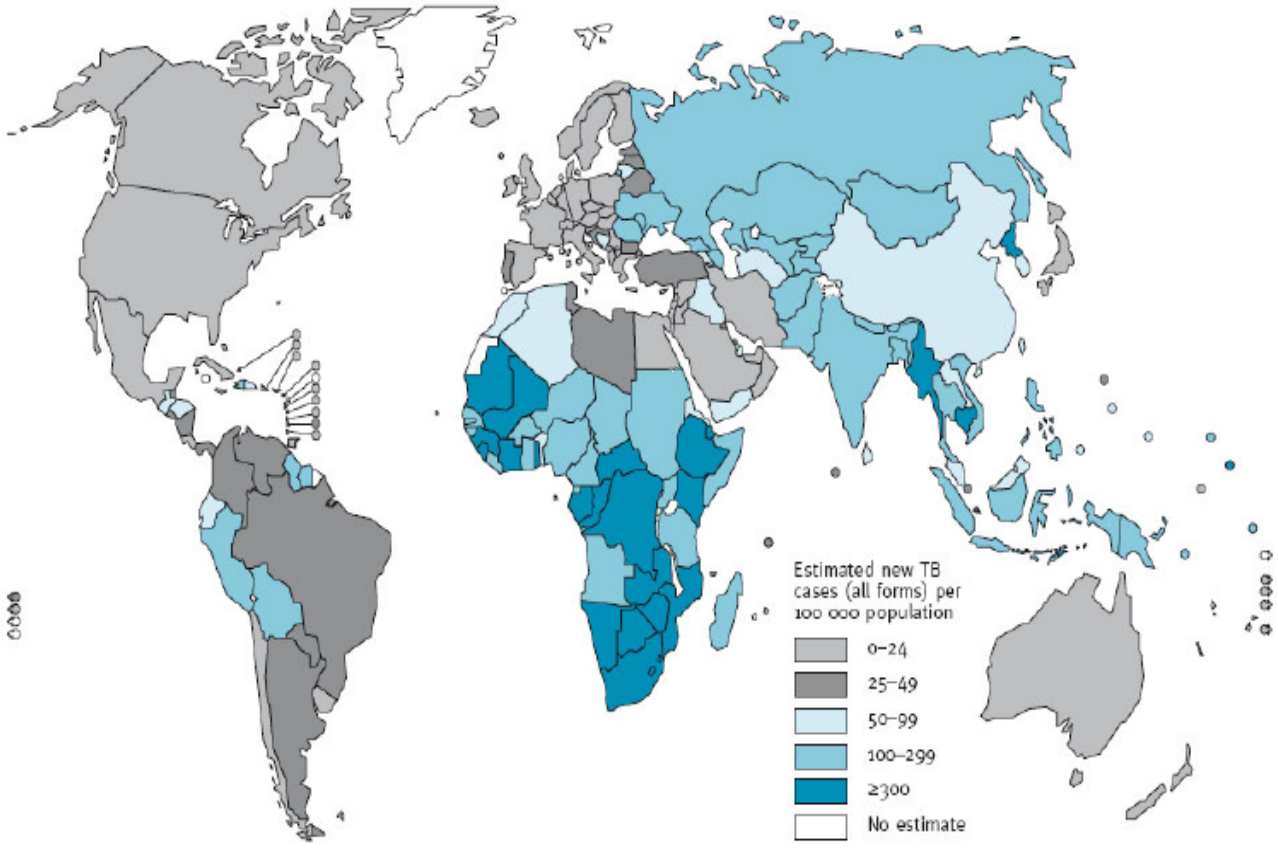


***Figure 1.2**: Estimated number of TB cases by countries 2009 (Extracted from WHO report 2010, Global Tuberculosis Control: Surveillance, Planning and Financing)*

Control of TB infection has been further complicated by the worldwide increase in the incidence of drug resistance *M. tuberculosis* strains. The high morbidity and mortality due to multi drug resistance TB (MDR-TB) has caused major concerns regarding the clinical management and prevention of the dissemination of the disease [19]. According to WHO estimates, in 2003 there were 8.73 million new cases of TB worldwide, of which 3.83 million were positive [20]. Globally, 22 countries (TB 80 group) carry 80% of the estimated new TB cases (Table 1).

TABLE 1. Estimated incidence of TB: high-burden countries*

COUNTRY	POPULATION 1000s	ALL Cases 1000s	RATE PER 100 000	RANK
India	1065 462	1 788	168	1
China	1304 196	1 334	102	2
Indonesia	219 883	627	285	3
Nigeria	124 009	364	293	4
Bangladesh	146 736	361	246	5
Pakistan	153 578	278	181	6
Ethiopia	70 678	252	356	7
South Africa	45 026	242	538	8
Philippines	79 999	237	296	9
Kenya	31 987	195	610	10
DR Congo	52 771	195	369	11
Russian Federation	43 246	161	112	12
Viet Nam	81 377	145	178	13
UR Tanzania	36 977	137	371	14
Brazil	178 470	110	62	15
Uganda	25 827	106	411	16
Thailand	62 833	89	142	17
Mozambique	18 863	86	457	18
Zimbabwe	12 891	85	659	19
Myanmar	49 485	85	171	20
Afghanistan	23 897	80	333	21
Cambodia	14 144	72	508	22
Total, high-burden countries	3 942 338	7 027	178	

* 22 countries, Global Tuberculosis Control; surveillance, planning, financing. WHO report 2005. Geneva, world health organization (WHO/HTM/TB/2005.349).

It is well established that the impairment of the immune system as a result of human immunodeficiency virus (HIV) infection predisposes to the development of tuberculosis and the disease is now regarded as a "sentinel" manifestation of the progression from HIV to AIDS [21-23]. The specific targeting of the CD4 helper cells by the HIV carries a greater risk of endogenous reactivation of any latent tuberculous infection. However, in patients infected with HIV, opportunistic infection with *M. tuberculosis* most commonly occurs as a result of exogenous infection [22]. The risk of developing progressive primary tuberculosis within the first year in HIV-infected persons is almost 30% in contrast with the 3% risk of the non-HIV-infected persons [24]. Infection with *M. tuberculosis* has been reported as one of the most pathogenic of the HIV/AIDS opportunistic infections [22]. Foley *et al*., described an increase in the proportion of tuberculous patients infected with HIV whilst the total number of TB notifications remains largely unchanged and suggested a direction of causality from the wider population to the AIDS group [25]. Tuberculosis as the primary cause of death has also been reported in patients suffering from AIDS and tuberculosis [26, 27].

1.2 The causative agent of Tuberculosis: *Mycobacterium tuberculosis*

Robert Koch famously identified *Mycobacterium tuberculosis (M. tb)* as the organism that causes tuberculosis in 1882 (Fig.1.3). He received the Nobel Prize in physiology or medicine in 1905 for this discovery. ***Mycobacterium tuberculosis*** (MTB) is a pathogenic bacterial species in the genus *Mycobacterium* and the causative agent of most cases of tuberculosis (TB).

Classification of *Mycobacterium tuberculosis*

Kingdom: Bacteria

Phylum: Actinobacteria

Class: Actinobacteridae

Order: Actinomycetales

Suborder: Corynebacterineae

Family: Mycobacteriaceae

Genus: *Mycobacterium*

Species: *Mycobacterium tuberculosis*

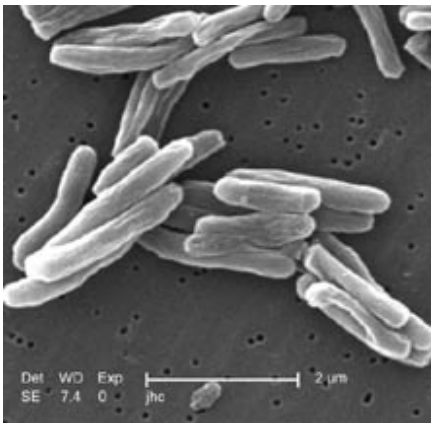


***Fig1.3**: SEM Image of M. Tb*

The mycobacteria are nonmotile, rod shaped, aerobic bacillus which are characterized by the presence of mycolic acids in their cell walls, being acid-fast and rich in G+C content of their genomes (61-71%) [28]. The structure of mycobacterial cell wall is very unique which is located outside the cytoplasmic membrane yet contains large amounts of lipids, many of them with unusual structure (Fig 1.4). The major fraction of the cell wall is occupied by unusually long-chain fatty acids containing 70-90 carbons, the mycolic acids [29]

The peptidoglycan, which contains N-glycolylmuramic acid instead of the usual N acetylmuramic acid, is linked to arabinogalactan via a phosphodiester bridge. About 10% of the arabinose residues of arabinogalactan are in turn substituted by mycolic acids, producing the covalently connected structure of the cell wall [30]. The cell wall also contains several types of "extractable lipids" that are not covalently linked to this basal skeleton; these include trehalose-containing glycolipids, phenol- phthiocerol glycosides, and glycopeptidolipids [31].

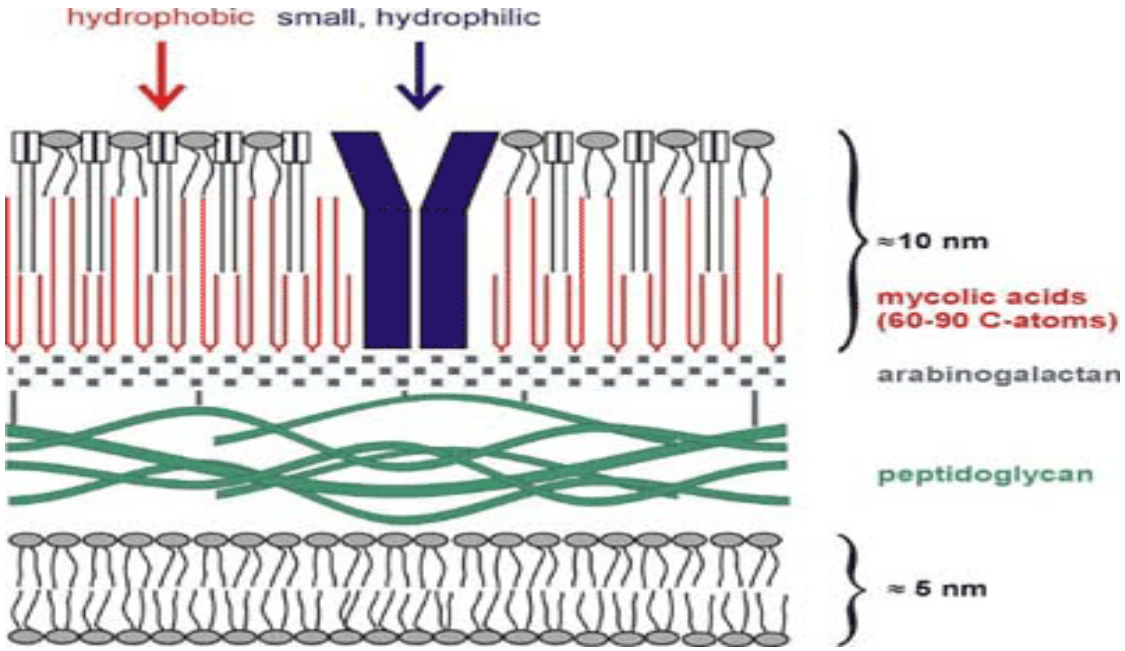


Figure 1.4: *Schematic of the bacterial cell wall. Credit: Michael Niederweis, Universität Erlangen, Germany*

Since mycobacteria have very prominent outer cell wall, it is kept under gram (+) bacteria although it does not stain with crystal violet due to unique kind of lipid present in its cell wall [32]. However it stains with acid fast stain hence also called acid fast bacteria. Acid-fast organisms are difficult to characterize using standard microbiological techniques, though they can be stained using concentrated dyes, particularly when the staining process is combined with heat [33]. Once stained, these organisms resist the dilute acid and/or ethanol-based de-colorization procedures common in many staining protocols—hence the name *acid-fast* (Fig1.5). The most common staining technique used to identify acid-fast bacteria is the Ziehl-Neelsen stain, in which the acid fast bacilli are stained bright red and stand out clearly against a blue background (Fig. 1.6) [34].

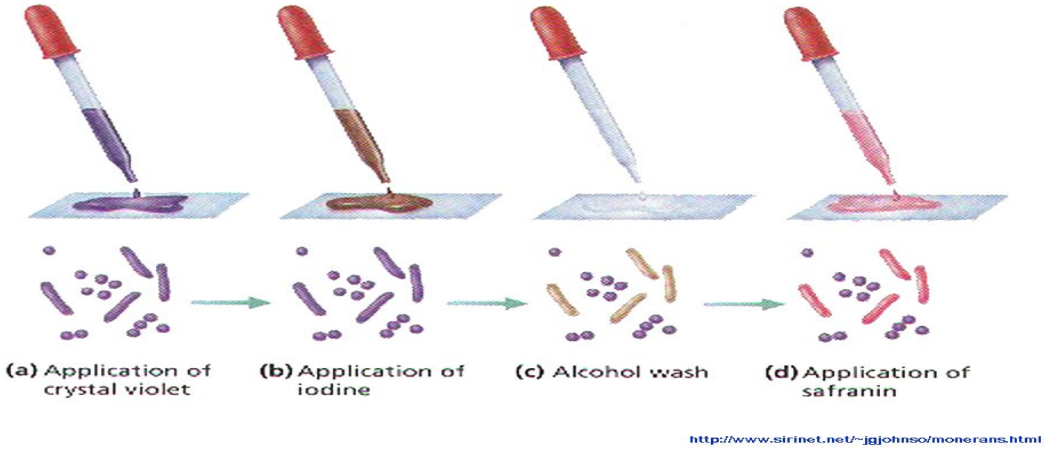


***Figure 1.5**: Staining procedure of mycobacteria using acid fast staining*

Another method is the Kinyoun method, in which the bacteria are stained bright red and stand out clearly against a green background. Acid-fast bacteria can also be visualized by fluorescence microscopy using specific fluorescent dyes (auramine-rhodamine stain) [35].

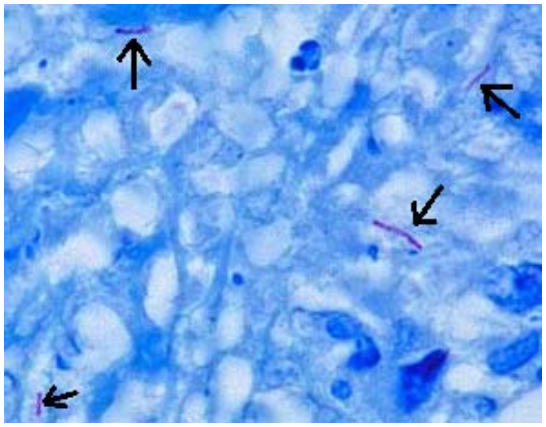


***Figure 1.6**: Bacterial staining by acid fast stain. The bacterial are pink (arrow) color in blue background*

Another very unique character of mycobacterium tuberculosis is that, it is very slow growing pathogen. It divides every 16 to 20 hours, which is an extremely slow rate compared with other

bacteria. Additionally, the 16S rRNA studies reveal an extended helix at position 451-482 for most slow-growers with the exception of *M. genavense, M. intermedium, M. interjectum, M. simiae* and *M. triviale* [36]. In evolutionary terms, phylogenetic analysis seems to suggest that the rapidly growing organisms are older than their slow growing relatives. Several causes have been postulated to explain the growth rate differences. These include differences in the number of rRNA (*rrn*) operons as well as the orientation of genes with respect to the direction of replication. Ribosomes are thought to function at a constant maximum efficiency. Therefore a faster growth rate is equated with a higher ribosome concentration; the number of ribosomes present within a cell is based on the production of rRNA, which, in turn depends on the number of *rrn* operons. Likewise, genes transcribed in the same direction as the replication fork are considered to be expressed more efficiently. Slow growing mycobacteria such as *M. leprae* and *M. tuberculosis* have a single *rrn* operon while rapid growers such as *M. phlei* and *M. smegmatis* have two *rrn* operons [36, 37]. The amount contrasts to several *rrn* operons observed in species such as *Escherichia coli* (7 *rrn* t_d <30 min) [38] or the extremely rapidly growing *Vibrio natriegens* (~13 *rrn* t_d <10 min) [39]. Investigations into the macromolecular compositions of *M. bovis* BCG (Bacillus Calmette Guerin) using a chemostat model for mycobacterial growth determined an 82% decrease in the number of ribosomes per cell between *M. bovis* BCG with a t_d of 23 h versus *M. bovis* BCG with a t_d of 69 h supporting a connection between the number of ribosomes and growth rate [40]. The ribosomes present in slow growing mycobacteria also appear to function at only 12% of maximal activity and may involve additional regulatory factors. Matsumoto and colleagues discovered a novel mycobacterial DNA binding protein, 'MDPI' localized to the nucleoid, 50S ribosomal subunit and cell surfaces, that was capable of transforming rapidly growing bacteria to slow growing [41]. Cell proliferation also requires the

uptake of essential nutrients and energy consumption [42]. Another possible explanation for the differences in growth rates could be differences in energy metabolism or transport process for oxygen and/or essential nutrients across the cellular membrane. Recently, Mailaender and coworkers demonstrated that the uptake of nutrients such as glucose in mycobacteria is 1430-fold slower than other fast-growing bacteria [43]. In their work they expressed MspA, the main porin of *M. smegmatis*, in *M. bovis* BCG that led to an increased uptake of nutrients and accelerated the growth rate by 7%.

The slow-growers tend to be pathogenic and those that cause tuberculosis disease in mammals are grouped within the *M. tuberculosis* complex (Table 2). Other animals such as birds (*M. avium*) frogs and turtles (*M. chelonae*) and reptiles (*M. fortuitum*) are known to be infected by mycobacterial species.

Mycobacterial species	**Host**
M. tuberculosis	Human
M. bovis	Cattle, deer, elk, bison, African buffalo, badger, opossum, human
M. caprae	Goat
M. africanum	Human
M. microti	Vole
M. canetti	Human
M. pinnipedii	Seal
Dassie bacillus	Dassie or hyrax

Table 2: *Members of the Mycobacterium tuberculosis complex are given with their respective mammalian host species*.

Other known pathogenic mycobacterium includes *Mycobacterium leprae*, *Mycobacterium avium*, and *M. kansasii*. The latter two species are classified as "nontuberculous mycobacteria" (NTM). NTMs cause neither TB nor leprosy, but they do cause pulmonary diseases that resemble TB.

1.3 Infection of tuberculosis

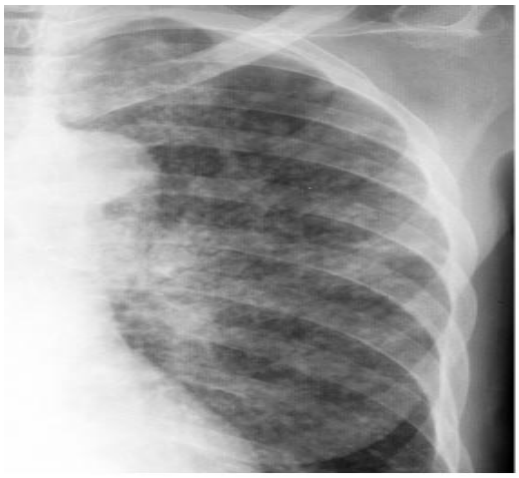
Inhalation is the predominant route of *M. tuberculosis* infection, making pulmonary tuberculosis the commonest form of tuberculous infection [44] *Mycobacterium tuberculosis* infection is in most cases due to the inhalation of an aerosolized droplet containing as few as one to three bacilli (Dharmadhikari and Nardell, 2009). The droplets are generally produced by respiratory movements such as coughing, sneezing, singing and speech. In rare cases, infection can occur via the gastro-intestinal tract when contaminated material such as milk is consumed. The organism gains access to the blood stream via the lymphohematogenous route and may then affect any organ. The incidence of extrapulmonary tuberculosis is increasing, especially because of HIV [45]. In patients infected with HIV, *M. tuberculosis* usually involves multiple extrapulmonary sites including the skeleton, abdominal organs, and central nervous system.

Tuberculosis may demonstrate a variety of clinical and radiological features depending on the organ site involved and as a consequence may mimic other pathologies. It is important to be familiar with the various radiological features of tuberculosis to obtain a presumptive diagnosis as early as possible [46].

1.3.1. Pulmonary tuberculosis

Pulmonary tuberculosis is classically divided into primary and postprimary (reactivation) tuberculosis. However, a considerable overlap in the radiological presentations of those entities may be seen. Although primary tuberculosis is the most common form of pulmonary tuberculosis

in infants and children, it has also been increasingly encountered in adult patients. Primary tuberculosis Primary disease accounts now for 23%-34% of all adult cases of tuberculosis [47]. Primary pulmonary infection occurs when an uninfected person inhales an infectious droplet, which successfully establishes infection in a terminal airway or alveolus [44]. The resultant primary parenchymal (Ghon) focus usually drains via local lymphatics to the regional lymph nodes. The combination of the Ghon focus, local lymphangitis and regional lymph node involvement is known as the Ranke complex. Sometimes, associated pleural reaction overlying a peripheral Ghon focus may be present. The formation of the Ghon complex is often subclinical and a random chest radiography following primary infection is often normal or reveals only a single component, mostly hilar adenopathy [44]. Disease progression may occur at the site of the Ghon focus, within the regional lymph nodes or as a result of lymphatic drainage with hematogenous dissemination or after local penetration across anatomical boundaries [48]. Penetration may occur into an adjacent anatomical space or structure, into an airway with additional intrabronchial spread or into a blood vessel with hematogenous dissemination. Two main types of hematogenous spread of *M. tuberculosis* are differentiated, but dissemination via the hematogenous route represents a condition of infinite gradation. Following dissemination, bacilli lodge in small capillaries where they may progress locally and give rise to further hematogenous spread. In the other type, disease progression may result in a caseous focus eroding into a blood or lymph vessel. Except for immunocompromised patients, the first type, contrary to the second one, rarely progresses to disseminated disease [49]. Primary tuberculosis typically manifests radiologically as parenchymal disease, lymphadenopathy, pleural effusion, miliary disease, or atelectasis, which may be either lobar or segmental (Fig 1.7). Parenchymal disease in primary tuberculosis affects the areas of greatest ventilation.



a.

Figure 1.7*: Miliary tuberculosis.* ***(a)*** *Radiograph of the left lung shows diffuse 2–3-mm nodules, findings that are typically seen in miliary tuberculosis*

Most commonly, the middle lobe, the lower lobes, and the anterior segment of the upper lobes are involved. Atelectasis is usually the consequence of bronchial obstruction by an enlarged hilar adenopathy.

1.3.2. Postprimary tuberculosis

Postprimary tuberculosis usually results from reactivation of a previously dormant primary infection in 90% of cases. In a minority of cases, it may result from the continuation of primary disease [50]. Reinfection is very rare. Reactivation of mycobacterial disease is almost exclusively seen in adolescence and adulthood. Reactivation occurs as the result from numerous causes such as poor nutrition status, neoplasia, infection or increasing age. Post-primary tuberculous lesions show a slow progressive course resulting in high morbidity and mortality if not adequately treated [51]. The radiologic features as seen in postprimary tuberculosis are the result from a continuous interaction between the individual patient, with his own immune status, and *M. tuberculosis* [52]. The radiologic features may be classified as parenchymal disease with

cavitation, airway involvement, and pleural extension. Parenchymal pulmonary disease may show caseous and liquefaction necrosis and communicate with the tracheobronchial tree to form cavities. A predilection for the apical or posterior segment of the upper lobes or the superior segment of the lower lobes has been reported [50]. Mostly, two or more segments are involved, and bilateral upper lobe involvement may also be noted [12]. Most commonly, cavities occurs within areas of consolidation, are multiple, and show thick irregular walls. However, thin and smooth cavity walls may also be seen. An air-fluid level within the cavity is an uncommon finding, and may reflect superimposed bacterial or fungal infection [53]. Bronchogenic spread is a common complication in postprimary tuberculosis and represents a chronic granulomatous infection in which active organisms spread via airways after caseous necrosis of bronchial walls. Endobronchogenic spread is characterized by multiple, ill-defined micronodules, distributed in a segmental or lobar distribution, distant from the site of the cavity formation and typically involving the lower lung zones [52]. If untreated, end stage disease may lead to lobar or complete lung opacification and destruction (Fig. 1.8).

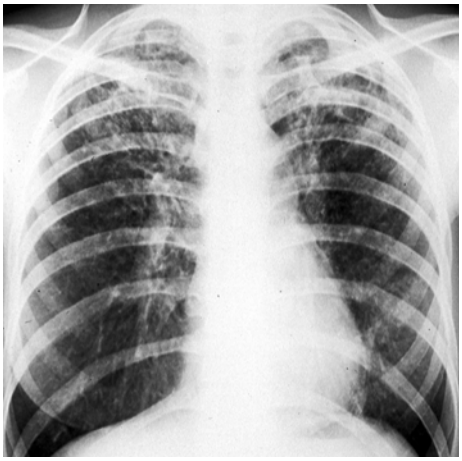


Figure 1.8*: Parenchymal postprimary tuberculosis. Chest radiograph demonstrates the characteristic bilateralupper lobe fibrosis associated with postprimary tuberculosis*

However, with chronic disease, fibroproliferative lesions composed of nodular opacities and clearly defined, medium to coarse reticular areas, may develop. Most often associated poorly marginated areas of increased density may be present. A marked fibrotic response is a common finding after postprimary tuberculosis and may result in atelectasis of the upper lobe, retraction of hilum, compensatory lower lobe hyperinflation, mediastinal shift towards the fibrotic lung and apical pleural thickening [52].

Central airway involvement in tuberculosis may be the result of direct extension from tuberculous lymph nodes, endobronchial spread of infection, or lymphatic dissemination to the airway [54]. Bronchialstenosis may result in segmental or lobar collapse, lobar hyperinflation, obstructive pneumonia, or mucoid impaction. A common complication of endobronchial tuberculosis consists of bronchiectasis resulting from pulmonary destruction and fibrosis, and central bronchostenosis. Pleural effusions in postprimary tuberculosis are usually small and associated with parenchymal disease. A loculated pleural fluid collection with parenchymal disease and cavitation may indicate tuberculous empyema and air-fluid levels in the pleural space indicate bronchopleural fistula. Occasionally, pericardial involvement may be seen with mediastinal and pulmonary tuberculosis and may cause calcific pericarditis [55].

1.4. Diagnosis of Tuberculosis

Tuberculosis is diagnosed by finding *Mycobacterium tuberculosis* bacteria in a clinical specimen taken from the patient, which may be difficult and give false result mainly due to its slow growing nature. Henceforth, complete medical evaluation for tuberculosis (TB) must include:-

a) Medical history
b) Chest X-ray
c) Microbiological examination (of sputum or some other appropriate sample). It may also include a tuberculin skin test, other scans and X-rays, surgical biopsy.

1.4.1. Medical History: The medical history includes the symptoms of pulmonary TB, Prolonged cough for 3-4 week or more, blood in cough and chest pain. Other symptoms include frequent low fever, chills, night sweats, appetite loss and weight loss [56]. Other medical history includes prior TB exposure, infection or disease; past TB treatment; demographic risk factors for TB; and medical conditions that increase risk for TB disease such as HIV infection. Tuberculosis should be suspected when a pneumonia-like illness has persisted longer than three weeks, or when a respiratory illness in an otherwise healthy individual does not respond to regular antibiotics.

1.4.2. Chest X-ray: Chest X-rays are effective in demonstrating airspace disease, the parenchymal nodule that represents the Ghon focus, diffuse interstitial disease and pleural effusions (Fig. 1.9). Revealing the presence of lymphadenopathy is an important diagnostic sign. However, lesions may appear anywhere in the lungs. In disseminated TB a pattern of many tiny nodules throughout the lung fields is common- the so called miliary TB. In HIV and other immunosuppressed persons, any abnormality may indicate TB or the chest X-ray may even appear entirely normal [57].

Abnormalities on chest radiographs may be suggestive of, but are never diagnostic of, TB. However, chest radiographs may be used to rule out the possibility of pulmonary TB in a person who has a positive reaction to the tuberculin skin test and no symptoms of disease.

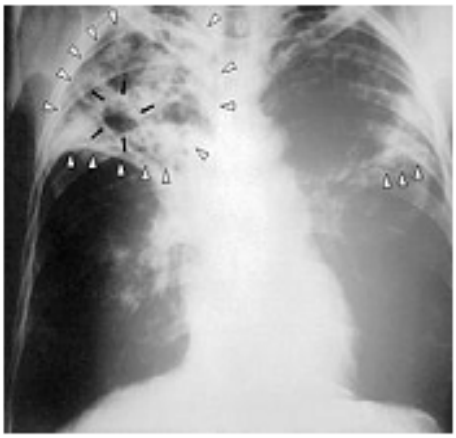


***Figure 1.9**: Chest X-ray of patient suffering from Pulmonary TB*

1.4.3. Microbiological examination: A definitive diagnosis of tuberculosis can only be made by culturing *Mycobacterium tuberculosis* organisms from a specimen taken from the patient (most often sputum, but may also include pus, CSF, biopsied tissue [58].

1.4.4. Sputum: Sputum smears and cultures should be done for acid-fast bacilli if the patient is producing sputum. The preferred method for this is fluorescence microscopy (auramine-rhodamine staining), which is more sensitive than conventional Ziehl-Neelsen staining [59]

The gold standard of diagnostics is confirmation with its growth in selective media [60]. This culturing is 1000 times more sensitive than microscopy, allows precise species identification, can be applied to drug susceptibility testing and may be useful to identify epidemiological links between patients or to detect laboratory cross contamination. In general, the sensitivity and specificity of culture method is 80-85% and 98% respectively [61]. However, their outcome is delayed by extremely low growth rate of mycobacteria. Contrary to a number of environmental mycobacteria that are rapid growers, yielding colonies in 7 days or less, *M. tuberculosis* exhibits a slow growth rate, requiring 14-21 days to generate visible colonies and does not produce any

pigment (Figure 1.10). With the advancement in culture system in 1980s BACTEC and biphasic culture methods were developed for faster recovery than traditional culture system [62].

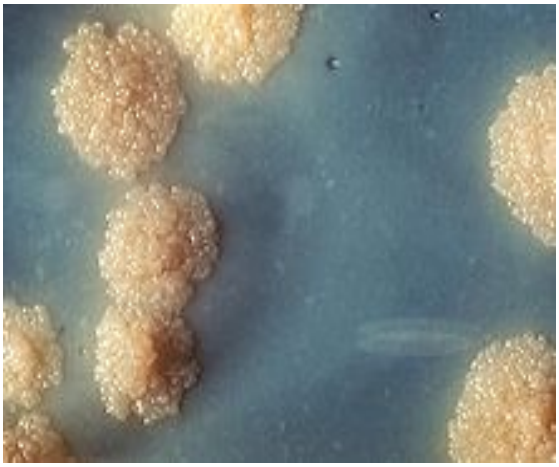


Figure 1.10: Appearance of colonies of M. tb on Dubos medium

1.4.5. PCR: Other mycobacteria are may also be acid-fast during the staining process. To overcome this false result and confirmation of *M. tuberculosis* in sample, PCR can be used as diagnostic method. Insertion element 6110 (IS6110) is a potential marker used to identify the *M. tb* in sample using specific primer in PCR reaction (Fig.1.11). Since this sequence is highly conserved and present only in MTB complex hence can be used for diagnostic purpose [63].

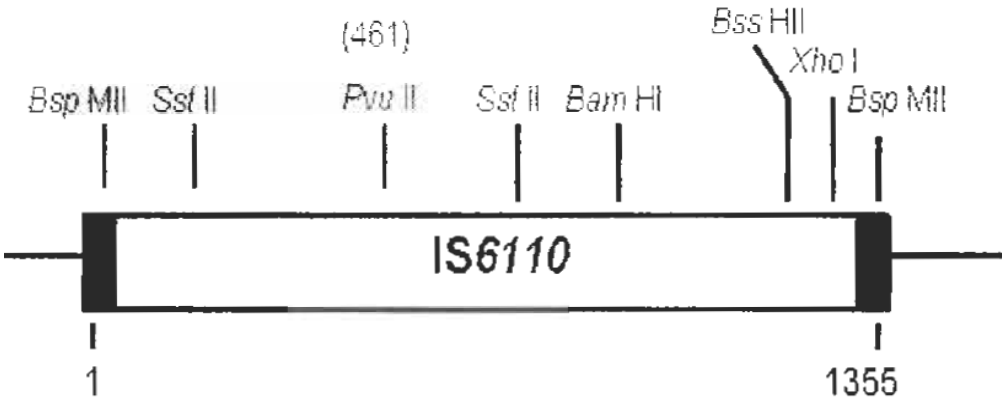


***Fig 1.11**: The gene sequence of IS6110 in M. tb*

1.4.6. Tuberculin Skin test: Tuberculin is a glycerol extract of the tubercle bacillus. Purified protein derivative (PPD) tuberculin is a precipitate of non-species-specific molecules obtained from filtrates of sterilized, concentrated cultures [64]. It was first described by Robert Koch in 1890 [65]. The test is named after Charles Mantoux, a French physician who built on the work of Koch and Clemens von Pirquet to create his test in 1907. Standard dose of 5 tuberculin units (0.1 mL) is injected intradermally (between the layers of dermis) and read 48 to72 hours later (Fig. 1.12) [66]. This intradermal injection is termed the mantoux technique. A person who has been exposed to the bacteria is expected to mount an immune response in the skin containing the bacterial proteins. The reaction is read by measuring the diameter of induration (palpable raised hardened area) across the forearm (perpendicular to the long axis) in millimeters. If there is no induration, the result should be recorded as "0 mm". Erythema (redness) should not be measured.

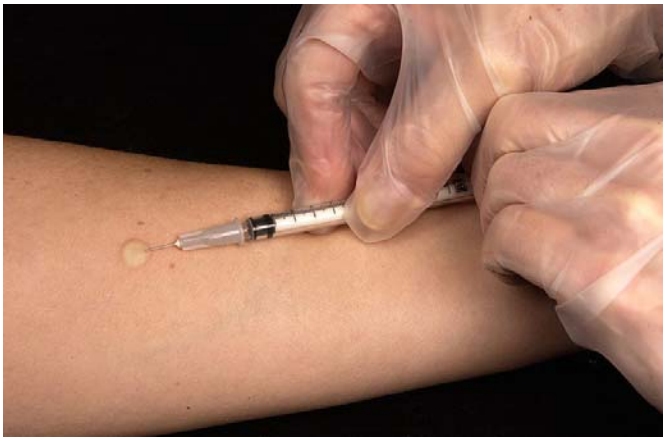


Fig 1.12: Tuberculin test performed using 2units of PPD

It can give false negative result due to the test's low specificity and immunologically compromised, especially those with HIV and low CD4 T cell counts patients, frequently show negative results from the PPD test [67]. A false positive result may be caused by nontuberculous mycobacteria or previous administration of BCG vaccine and prior vaccination with BCG may result in a false-positive result for many years afterwards [68].

1.4.7. Immunological assay: There is certain proteins (antigens) release in large quantity in extracellular medium specifically from mycobacterium tuberculosis in patients against which host releases interferon γ. Lymphocytes from the patient's blood are incubated with the antigens. If the patient has been exposed to tuberculosis before, T lymphocytes produce interferon γ in response. An ELISA format is used to detect the whole blood production of interferon γ with great sensitivity (89%). Hence forth, these proteins can be used for TB diagnostic purpose [69].

1.5. Treatment of tuberculosis

Prior to the 19th century and discovery of the *M. tuberculosis* bacillus by Robert Koch, aphthisitic diagnosis was associated with death. Ancient attempts to treat the disease included quarantine, burning of a consumptive's clothes and possessions, a healthy diet, fresh air, milk, sea voyages, regular bloodletting and exercise (such as horse-riding), amongst others. In Medieval Europe, it was believed that consumption could be cured by the touch of a king as they were considered to have magical or curative powers due to the position bestowed upon them by divine right. The idea that fresh air and a healthy diet could treat consumption persisted well into the 19th century with the consequent introduction of sanatoria. Herman Bremmer thought that it would be best to bring consumptive patients to an 'immune place' which he described as being free of known tuberculosis cases and with the emphasis on strict rest and healthy diet regimes, it was assumed that the disease would be cured. The first sanatorium was established by Bremmer in 1856 in the Sudeten Mountains of Silesia with numerous others observed in remote locations in 6 subsequent years. Although the condition of the patients improved under these conditions, it was later observed that the treatment was by no means curative and additional more invasive treatments were introduced. In the 1930's lung collapse therapy, artificial pneumothrax, phrenicectomy or thoracoplasty was applied to literally collapse and thereby immobilise the

diseased lung to provide localised rest for the infected areas [70]. These are rather extreme methods of treatment often caused severe side effects and were abandoned soon after the discovery of antituberculosis drugs.

Early in the 20th century, the French duo: Albert Calmette (1863–1933) and Camille Guérin (1872 – 1961) continued with attempts to develop a vaccine for tuberculosis. They were able to show that after more than 230 passages of *Mycobacterium bovis* on a medium consisting of potato slices and ox gall, that the bacterium was no longer able to create lesions in laboratory animals [71]. This attenuated strain was known as *M. bovis* BCG or bacilli Calmette-Guérin and was first used in Paris in 1921 to immunize children against *M. tuberculosis* with great success. The vaccine became popular in the rest of Europe; however, it suffered a setback in 1930 in the form of the 'Lübeck disaster' where babies were inadvertently administered with live bacilli causing 73 fatalities. Although the protection afforded by BCG vaccination against pulmonary tuberculosis in adults is variable [72], the efficacy of the vaccine in preventing disseminated forms of disease especially in children has resulted in an 80% global coverage today.

The middle of the Second World War saw the dawning of the anti-tubercular drug era with the discovery of streptomycin by Selman A. Waksman (1888-1973) and his student Albert Schatz [73, 74]. Although numerous other scientists had previously observed the inhibitory effect of metabolic products from organisms such as *Bacterium termo, Bacterium prodigiosum* and *Aspergillus fumigates,* they were not able to develop their ideas into a meaningful treatment application [75]. Waksman had been studying the inhibitory effect of certain soil fungi of the Actinomycetale bacterial group which lead to the isolation of a potent antibiotic, actinomycin, which was too toxic for human or animal use. In 1943, however, he and Schatz succeeded in isolating streptomycin from cultures of the Actinomycetale *Streptomyces griseus.* The drug was

able to inihibit the tubercule bacilli and was relatively non-toxic to humans, thus it was introduced as a therapy in 1944. For his work, Waksman was awarded the Nobel Prize in 1952 [75]. The following 15 years saw the rapid discovery and development of anti-tubercular compounds. These included thiosemicarbazone by Gerhard Domagk (1885-1964) in 1940 and para-aminosalicylic acid (PAS) discovered by Jorgen Erik Lehman (1898-1989) in 1946. Streptomycin was combined with PAS as a combination therapy in 1949 to combat emerging streptomycin resistant strains resulting from streptomycin monotherapy. One of the major first-line drugs, isoniazid, was developed in 1912 [76] but its anti-tubercular actvity was only described in the 1950's. Thereafter followed pyrazinamide (PZA, 1954), cycloserine (1955), and ethambutol (1962).

Rifampin is also a first-line drug used in the treatment of tuberculosis and is derived from rifamycins which are molecules with antibiotic properties isolated from *Nocardia mediterranei*. A stable synthetic compound, rifampin (or rifampicin) was later developed by Lepetite Laboratories in Italy and introduced as a potent anti-tubercular drug in 1968 [77]. More recently, the aminoglycosides such as capreomycin, viomycin, kanamycin, and amikacin, and the newer quinolones (e.g. moxifloxacin, levofloxin, ofloxacin, and ciprofloxacin) have been developed to augment anti-tuberculosis therapies.

The current treatment regime for tuberculosis consists of a two-phase multi-drug approach. The multi-drug discipline was introduced to overcome the ability of *M. tuberculosis* to rapidly develop resistance to drugs used in mono-therapy. The first phase is an intensive one in which most (90%) of the bacilli are killed and the second phase, known as the continuation phase, is the period in which the last persistent bacilli are eradicated [78]. The major drug arsenal used to treat

the disease is the so-called first-line drugs which display the highest bactericidal activity. These include isoniazid, rifampin, pyrazinamide, ethambutol and streptomycin.

Isoniazid appears to be largely responsible for the rapid killing of the bacteria in the intensive phase whilst rifampin seems to target persistent bacteria during the continuation phase [79]. For patients infected with fully susceptible *M. tuberculosis,* a 6-month short course regimen called DOTS (Directly Observed Treatment, Short-course), recommended by the WHO, is administered which is comprised of rifampin and isoniazid, pyrazinamide and either ethambutol or streptomycin for two months followed by isoniazid and rifampin for the remaining 4 months (Table 3: Tuberculosis, a comprehensive clinical reference).

Drug	Recommended Dose and Range (mg/kg body weight)	
	Daily	Three times weekly
Isoniazid	5 (4 – 6)	10 (8 – 12)
Rifampin	10 (8 - 12)	10 (8 – 12)
Pyrazinamide	25 (20 – 30)	35 (30 – 40)
Ethambutol	15 (15 – 20)	30 (25 – 35)
Streptomycin	15 (12 – 18)	15 (12 – 18)

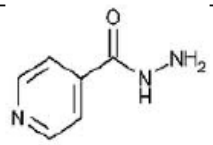
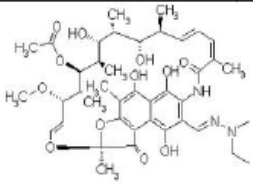
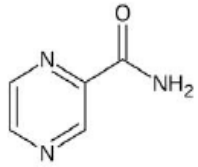
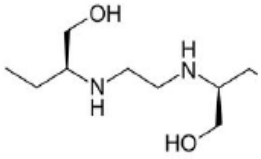
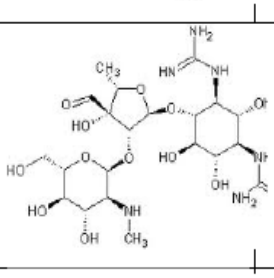
*(WHO, 2009)

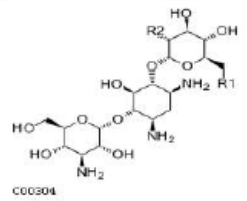
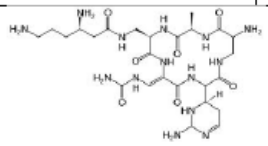
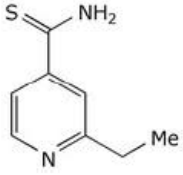
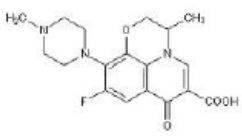
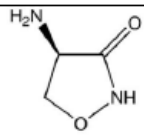
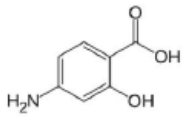
***Table 3**: Daily and three-times weekly dose as recommended by the World Health Organization (WHO)**

This regimen has an approximate 95% success rate in curing pulmonary tuberculosis provided that patients fully comply. In the event that a patient develops drug resistant tuberculosis or is infected with a drug resistant strain, second-line drugs (Table 4), which are generally

bacteriostatic and have adverse side-effects, are employed to treat the disease in the DOTS-PLUS regime, which can last up to 24 months.

Table 4: *First and second-line drugs currently used in the treatment of tuberculosis are given with their respective targets, structures, modes of action and the genes in which mutations can occur to confer resistance.*

Drug		Structure	Target	Mode of Action	Genes in which mutations confer drug resistance
First-line drugs	Isoniazid		Cell wall	Activated INH inhibits mycolic acid biosynthesis.	*kat*G, *inh*A, *ahp*C, *ndh*, *kas*A
	Rifampicin		RNA synthesis	Binds to RNA polymerase to prevent mRNA synthesis and consequent protein production.	*rpo*B
	Pyrazinamide		Unknown	Direct mode of action unknown, however, accumulation of the drug may cause non-specific damage.	*pnc*A
	Ethambutol		Cell wall	Prevents arabinogalactan synthesis.	*emb*CAB gene cluster
	Streptomycin		Translation	Binding of drug to ribosomes inhibits protein synthesis.	*rrs*, *rps*L

Second-line drugs	Kanamycin and Amikacin (aminoglycosides)		Translation	Binding of drug to ribosomes inhibits protein synthesis.	*rrs*
	Capreomycin and Viomycin		Translation	Inhibits protein synthesis by binding to 30S and 50S ribosomal subunits.	*rrs*
	Ethionamide and Prothionamide		Cell wall	Pro-drug which when activated inhibits mycolic acid biosynthesis similar to isoniazid.	*inh*A promoter, *eth*A, *eth*R
	Ofloxacin and Ciprofloxacin(fluoroquinolones)		DNA structure replication	Inhibits DNA gyrase and DNA topoisomerase IV to prevent DNA supercoiling and replication.	*gyr*A, *gyr*B
	Cycloserine		Cell wall	Inhibits cell wall synthesis, however, direct mode of action is unknown.	*alr*A promoter, *Ddl*
	p-Aminosalicylic acid		Folate metabolism	The direct mode of action remains obscure but it has been shown to disrupt intracellular folate levels.	*thy*A

1.6. Problems in eradication of Tuberculosis

Not long after the introduction of chemotherapy (streptomycin) as a treatment for tuberculosis, bacilli resistant to the therapeutic agent were observed. Resistance to a drug arises from rare, spontaneous mutations that enable the bacteria to survive in its presence. This selective pressure can allow the resistant strain to become dominant in the infected host, which would result in treatment failure in the case of continued mono-therapy. The use of multi-drug therapies was

established to curb the generation of drug-resistant strains, however, in recent years an increase in strains resistant to numerous drugs has occurred. This observation can be ascribed many reasons, the foremost of which is the accumulation of a series of mutations in a single strain due to the selective pressure of inadequate drug therapy [80]. Access to and administration of adequate therapy is a global problem and is complicated by patient noncompliance. Due to the extended time period required to treat tuberculosis and adverse side effects from the chemotherapy, many patients (especially in resource poor settings) prematurely abort therapy which leads to the acquisition of drug-conferring mutations [81]. Bacteria resistant to both isoniazid and rifampin are considered to be multi-drug resistant (MDRTB) and patients infected with these strains should be treated with a cocktail of second-line drugs in a specialised treatment regime. An alarming development is the relatively recent identification of extensively drug-resistant tuberculosis (XDR-TB). These are MDR-TB strains which are resistant to any flouroquinalone and at least one of the injectable second-line drugs, making these strains virtually untreatable.

The HIV/AIDS pandemic has massive implications for global tuberculosis control. Individuals living with HIV are immuno-suppressed and as such are at high risk for tuberculosis. They are susceptible to the reactivation of latent bacilli and progress more rapidly to active disease after infection, a fact which has contributed to the resurgence of tuberculosis in recent years. Tuberculosis is responsible for approximately one in four deaths that occur among HIV positive patients worldwide and it is the most common cause of death by opportunistic infection in HIV positive people in low income countries [82]. In addition, diagnosis of tuberculosis in HIV infected patients is made more difficult by the atypical presentation of the disease [83]. A further complication is that current tuberculosis and HIV/AIDS therapies, when co-administered, can

display intolerability and toxicity [83]. Co-infection with tuberculosis also increases the rate of HIV replication which increases the risk of mortality in these patients [84].

1.7. Latency: Bottle neck in Tuberculosis eradication

In the 21st century, we face the problems of billions of people with latent *M. tb* infection. Latency has been defined as the "presence of any tuberculosis lesion which fails to produce symptoms of its presence" [85]. Despite the immune system's ability to clear much of the *M. tb* and arrest an infection, the lungs may still contain small caseous foci. The first evidence of latent *M. tb* in the caseous foci was the reoccurrence of an *M. tb* infection with non drug resistant bacilli after treatment with a regimen of antibiotics [86]. The nature of latency was further elucidated by chemoprophylaxis treatment, which showed that the longer the period of treatment, the lower the chances of *M. tb* reactivation [87]. Since susceptibility to antibiotics required some level of growth and metabolism, it was suggested that there was some degree of growth and metabolism of *M. tb* during latent state. Therefore, persistent bacilli in a lesion are likely to be in a steady state in which intermittent replication is balanced by immune system destruction. The balance of this steady state will determine active disease versus latent infection.

Latency is achieved by cell-mediated immune response which restricts the growth of *M. tb* bacilli. The restriction, however, does not eliminate the pathogen, leaving the *M. tb* bacilli as a present danger to reactivate years later. Secondary infection occurs as the reactivation of an old lesion with latent bacilli at the apical zone of the lungs [88]. Individuals infected with *M. tb* have a 10% chance during their life time to develop active tuberculosis from a latent infection. 5% of the infected population will develop the disease after 5 years and the others will suffer from it at some point during their lives [89]. Often, the precipitating factor for latent tuberculosis

reactivation is a waning immunity, which takes place mostly in the elderly at an estimated rate of 5% per year until complete disappearance of immunity [90]. Factors such as corticosteroids, immunosuppressant, HIV and other factors that lower resistance are a danger for reactivation as well.

The contribution of exogenous reinfection to the incidence of secondary tuberculosis has been largely ignored because it was assumed that the primary infection would provide protection against secondary infection. However, there is skepticism about the idea of dormant bacilli waiting to reactivate due to immunosuppression. Often, *M. tb* primary complex is sterile within five years, suggesting that secondary infection is an infection with exogenous bacteria [91]. It has also been documented that reinfection of some immunocompetent individuals occur with new strains of *M. tuberculosis*. This indicates that immunity to tuberculosis can be incomplete, and that reinfection, at least in areas where tuberculosis is prevalent, probably has a greater role than previously appreciated [92]. The dynamic nature of mycobacteria is highlighted by work showing that exogenously infected *M. marinum* in zebrafish, enters pre-existing granulomas by specific mycobacteria-mediated mechanisms that direct infected macrophages into granulomas [93].

Active *M. tb* lesions generally contain detectable populations of acid-fast, easily culturable bacilli, but *M. tb* from tubercles of post-chemotherapy, sputum-negative patients often fail to be cultured [94]. Extending culture incubation time from weeks to many months enables fully drug sensitive *M. tb* from closed lesions of drug-treated patient to be cultured, proving that bacilli from latent tubercles are still viable [95]. The difficulty in eradicating *M. tb* from a latent infection with drugs has also spurred ideas that alternate forms of *M. tb* may exist, such as protoplast, L-forms (forms without a cell wall), or spores, that may go undetected *in vivo* and are

difficult to culture [96, 97]. Conditions within closed lesions such as a lowered oxygen tension, long chain fatty acids, lactic acid, and other bacteriostatic agents are hypothesized to reduce bacterial metabolism and render the tubercle bacilli resistant to drugs.

Several studies have been carried out to understand the *in vitro* sustainability of *M. tb* in closed, necrotic lesion. Limited amounts of *M. tb* have been shown to survive twelve year incubation at 37^0C in a sealed culture vessel, suggesting the capability of long term persistence in nutrient limited or anaerobic environment [98]. Taking into account that the *M. tb* bacilli are surrounded by layers of immune cells and a fibrotic layer in the granuloma structure, Wayne hypothesized a microaerobic environment for *M. tb in vivo*. Dormancy as a result of metabolic adaptation to anaerobic conditions was proposed and modeled in a system of limited oxygen tension known as the Wayne's *in vitro* hypoxia model [99, 100]. Wayne observed that *M. tb* adapts to oxygen restriction by altering its metabolism to obtain energy through alternate processes [101]. The condition of limited oxygen in the granuloma is supported by gas concentration measurements of cavities from the lungs of living tuberculosis patients: blocked cavities, where the overall pressure is negative, is enriched for carbon dioxide, 10.5% on average versus 3.5% for open cavities, partially depleted for oxygen, 6.3% on average versus 17.8% in open cavities [102].

Latency has been modeled *in vivo* as well to account for additional stresses in a granuloma such as low pH, high concentration of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) activated macrophages [103]. The Cornell model was the first animal model for dormant bacilli. This model involves partial clearance of *M. tb* infection by incomplete chemotherapy to induce the latent state [104]. However, the granuloma and inflammatory response disappear after chemotherapy and hence the conditions of this model do not resemble those observed in humans. The low-dose mouse model of latent tuberculosis, known as the

chronic or plateau model, involves aerosol infection or infection by intravenous routes. This model resembles human latency because the host immune response contains the infection, but larger amounts of bacteria accumulate in the mouse than in humans which leads to a steady accumulation of pulmonary damage [105]. Although these models have their limitations, they are good sources to learn about the metabolic state of persistent mycobacteria and host immunity. Thus overall it shows that latency is playing the important role in throughout the infection of mycobacterium tuberculosis which is well shown in Fig 1.13.

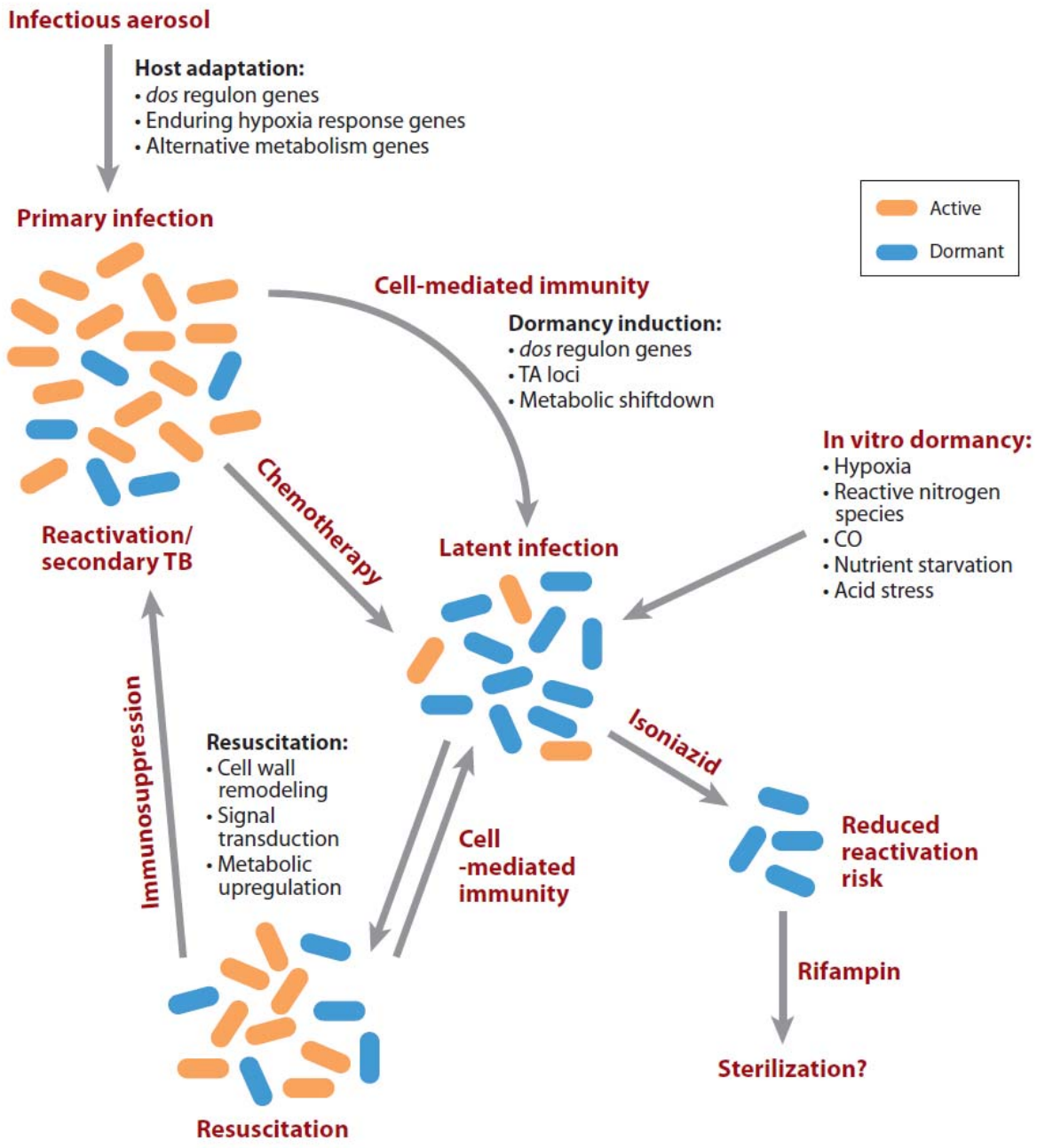


Figure 1.12: A model for human Mycobacterium tuberculosis infection. M. tuberculosis enters a host and upregulates the dos regulon and other stress survival genes to establish a primary infection, which is dominated by actively dividing cells. Some dormant cells can be generated through the induction of toxin-antitoxin (TA) loci or other growth inhibitors to downregulate metabolism. Cell-mediated immunity clears out actively dividing cells preferentially, leaving a predominantly dormant population to account

*for the paucibacillary latent state. A similar state can be achieved through in vitro studies using a variety of host stress conditions. During latency, some bacteria may resuscitate (possibly through a peptidoglycan signaling pathway) to form scouts. Normally, these bacteria are cleared by the immune system, but in an immunocompromised host the bacteria continue to replicate and cause reactivation. Treatment with isoniazid, which targets actively dividing bacteria, reduces reactivation risk by targeting the resuscitated scouts. Rifampin or other dormancy-active antibiotics may help clear the remaining dormant subpopulation and lead to tissue sterilization. [**Adopted from Chao et al., 2010**]*

1.8. Thesis objectives

It is well known that nitrogen metabolic pathway is active during latency of bacteria and because of this reason this pathway is become the attractive pathway for potential drug target [106]. When mycobacteria infects human lung, alveolar macrophages becomes activated and engulf the infected bacilli and then releases reactive nitrogen intermediates (RNI) and reactive oxygen species (ROS) to kill the pathogen. Although most of bacilli were killed by the release of these killer agents but some were escaped from this effect by either spontaneous or enzymatic conversion of NO and ROS to stable non-killer product nitrate with the help of superoxide dismutase and catalase enzyme present in mycobacteria [107]. Nitrate so formed can acts as source of nitrogen and alternate electron donor during dormancy where oxygen is completely absent [108]. Here the role of first gene of nitrogen metabolic pathway, nitrate reductase comes into picture. It been observed that nitrate reductase enzyme activity is up regulated during dormancy which suggests its active role in survival of mycobacterium in dormancy. Evidence for the role of NR in virulence was reported when immunodeficient SCID mice infected with the *M. bovis* BCG narG mutant showed smaller granulomas with fewer bacteria than those infected with the wild-type strain [109]. Another enzyme of nitrogen metabolic pathway, Glutamine synthatase

(GS) is also been extensibly studied and observed that this enzyme extracellular released in pathogenic form of tuberculosis like *M. tb* , M. *bovis* but not in saprophytic, non-pathogenic bacteria like *M. smegmatis* and *E. coli* [110]. These finding suggests its active role in pathogenic bacteria and use of specific inhibitor of GS shows decreased in survival both *in vitro* as well as *in vivo* conditions.

Overall nitrate reductase as well as glutamine synthatase both are playing very active and important role in survival of mycobacterium during dormancy. But the role of nitrite reductase which is an important enzyme and converting nitrite into ammonia in *E. coli* and several other organisms is so far not reported in *mycobacterium sp*. [111]. This enzyme can play important role in dormancy which is only the connecting link between nitrate to ammonia conversion and the ammonia acts as source of amide for different metabolic synthesis like protein, nucleotide and other nitrogen intermediates [112].

The objectives of this thesis are first analyzing the presence of functional nitrite reductase in *mycobacterium sp*. Once the presence of nitrite reductase is confirmed then role of this gene under different conditions will be analyzed. This analysis will shed the light on dependency and importance of nitrite reductase for the growth and survival of mycobacterium during active and well as in persistence stage. Another major intent of the thesis was to isolate, purify and characterize the nitrite reductase (NirBD), in order to better understand the mechanism of the enzyme as well as to develop enzyme based screening protocol which could be used to search inhibitory molecules of nitrite reductase.

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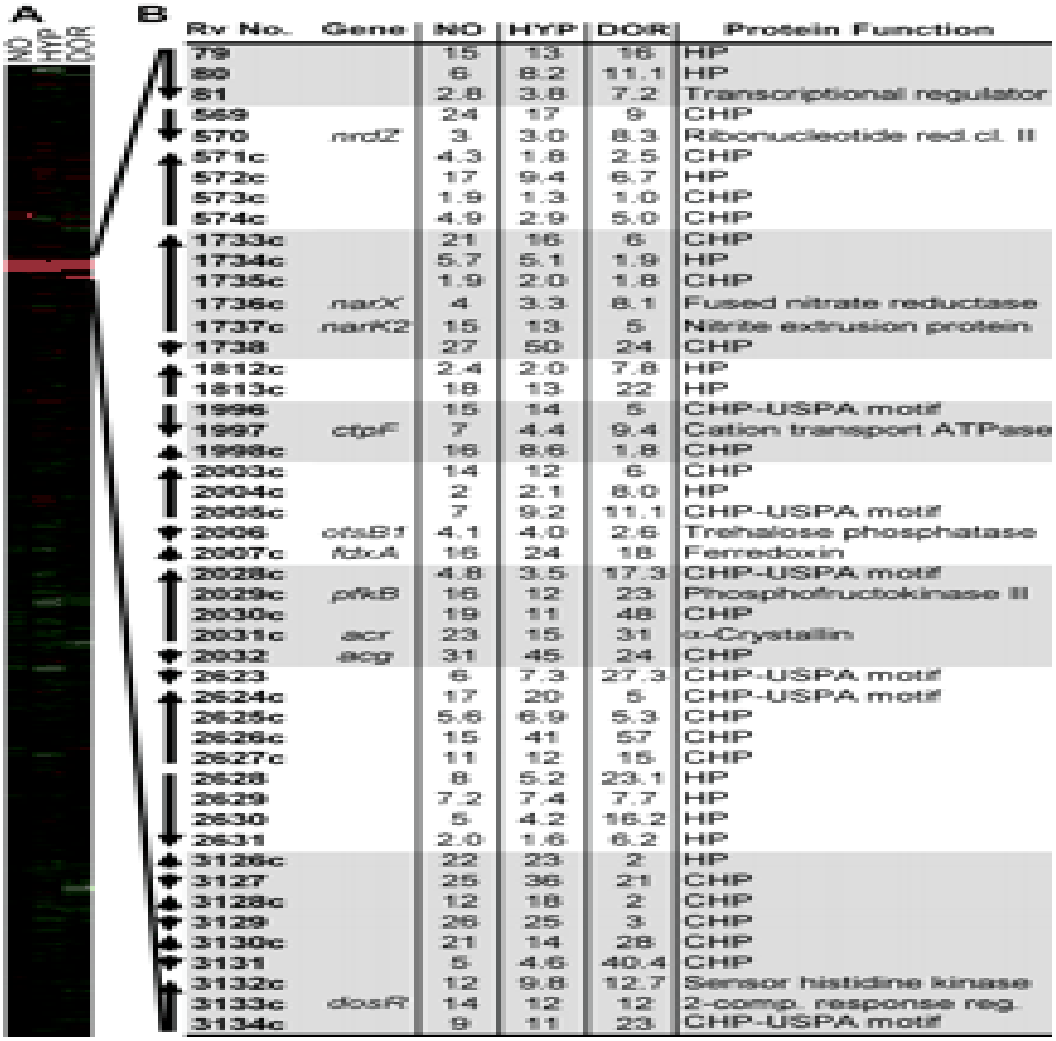
CHAPTER TWO

"*Functional nitrite reductase gene in mycobacterium sp.*"

2.1. Introduction

Tuberculosis is the 2nd leading cause of death with annual death of 2 million and more than 1/3rd world population are harboring this pathogen in latent form [1]. It is well established fact that latency is the major bottleneck in complete elimination of tuberculosis from this globe [2]. Henceforth it becomes very important to know the different mechanisms of bacilli which is playing crucial role in inducing and later on maintaining long term latency inside the host. Unfortunately still we are clueless about the real mechanisms which are important during latency [3]. The complete genome sequencing of *Mycobacterium tuberculosis* in 1998 and later *M. smegmatis* in 2000 has given up new hopes and opportunities to study different molecular mechanism responsible for latency [4]. This marked the beginning of the so called post genome era, the main characteristic of which are large scale studies of genome functional activity. The information on the bacterial genome organization allowed constructing macro- and microarrays containing fragments of a majority of ORFs, which enabled analysis of the pathogen transcription profile variations under different conditions. It's no wonder that the first study of the *M. tuberculosis* transcriptome using microarray technology was carried out in the first year after the publication of the genome sequence [5]. In as little as 5 years, there have been published many reports with the results of using microarrays for *in vitro* mycobacterial transcriptome analysis [6, 7]. These reports mainly emphasize the role of different metabolic pathways which are up or down regulated under *in vivo* condition inside the host macrophages. The outcome of these reports suggests that lipid metabolism, energy metabolism, protein biosynthesis, DNA repair, transcriptional factor, virulence factor, cell wall membrane and transporter genes are playing important and crucial role in attaining and maintaining dormancy [8, 9, 10].



Rv No.	Gene	NO	HYP	DOR	Protein Function
79		15	13	18	HP
80		6	8.2	11.1	HP
81		2.8	3.8	7.2	Transcriptional regulator
569		24	17	9	CHP
570	nrdZ	3	3.0	8.3	Ribonucleotide red.cl. II
571c		4.3	1.8	2.5	CHP
572c		17	9.4	6.7	HP
573c		1.9	1.3	1.0	CHP
574c		4.9	2.9	5.0	CHP
1733c		21	16	6	CHP
1734c		5.7	5.1	1.9	HP
1735c		1.9	2.0	1.8	CHP
1736c	narX	4	3.3	8.1	Fused nitrate reductase
1737c	narK2	15	13	5	Nitrite extrusion protein
1738		27	50	24	CHP
1812c		2.4	2.0	7.8	HP
1813c		18	13	22	HP
1996		15	14	5	CHP-USPA motif
1997	ctpF	7	4.4	9.4	Cation transport ATPase
1998c		16	8.6	1.8	CHP
2003c		14	12	6	CHP
2004c		2	2.1	8.0	HP
2005c		7	9.2	11.1	CHP-USPA motif
2006	otsB1	4.1	4.0	2.6	Trehalose phosphatase
2007c	fdxA	16	24	18	Ferredoxin
2028c		4.8	3.5	17.3	CHP-USPA motif
2029c	pfkB	16	12	23	Phosphofructokinase III
2030c		19	11	48	CHP
2031c	acr	23	15	31	α-Crystallin
2032	acg	31	45	24	CHP
2623		6	7.3	27.3	CHP-USPA motif
2624c		17	20	5	CHP-USPA motif
2625c		5.6	6.9	5.3	CHP
2626c		15	41	57	CHP
2627c		11	12	15	CHP
2628		8	5.2	23.1	HP
2629		7.2	7.4	7.7	HP
2630		5	4.2	16.2	HP
2631		2.0	1.6	6.2	HP
3126c		22	23	2	HP
3127		25	36	21	CHP
3128c		12	18	2	CHP
3129		26	25	3	CHP
3130c		21	14	28	CHP
3131		5	4.6	40.4	CHP
3132c		12	9.8	12.7	Sensor histidine kinase
3133c	dosR	14	12	12	2-comp. response reg.
3134c		9	11	23	CHP-USPA motif

Figure 2.1: Dormancy regulon. Comparison of microarray expression results from M. tuberculosis strain exposed for 40 min to 50 µM of the NO donor DETA/NO (NO), and strain H37Rv exposed for 2 h to 0.2% O2 (hypoxia-HYP) and at day 4 during the gradual adaptation, to low O2 , resulting in an in vitro dormant state (DOR). (Adopted from Voskuil et al. 2003)

According to data obtained in studies of *M. tuberculosis* gene expression *in vivo* condition, energy metabolism of mycobacteria is undergoing a significant transformation during infection process (Fig 2.1) [11]. A characteristic of this transformation is a gradual decrease in the level of the type I NADH dehydrogenase (*nuoA-N*) gene expression and increase in expression of the

nitrate reductase gene cluster narGHJI and of the narK2 gene, the product of which is a nitrate transporter protein [12, 8, 13]. Such a metabolic shift most probably suggests that ETC is being reoriented to the using of nitrate electrons as acceptor. Also, in most cases, aa3 type cytochrome c oxidase (ctaBECD) and cytochrome c reductase (qcrCAB) gene expression is downregulated [14, 8, 15]. This unique property of narGHJI to provide energy in absence of oxygen makes its truly respiratory enzyme which role could be important for the survival of the bacilli during dormancy [16]. In an anaerobic environment, many bacteria are able to use nitrate as a final electron acceptor in place of oxygen for the maintenance of a proton motive gradient to continue growing. Historically, *M. tuberculosis* has been differentiated from *M. bovis* by the fact that only *M. tuberculosis* can reduce significant amounts of nitrate (NO_3^-) to nitrite (NO_2^-) [17, 18]. Nitrate reductase activity occurs at a low level during the aerobic growth of *M. tuberculosis* and increases significantly upon entry into the microaerobic NRP-1 stage [17]. The nitrate reductase enzyme activity, which is membrane bound enzyme increases 10-fold during shiftdown from actively aerobic growing cells to anaerobic dormant bacilli. This indicates the important role of nitrate reductase enzyme during dormancy however its role in the virulence of *M. tuberculosis* has not been investigated. However, immunodeficient SCID mice infected with an *M. bovis* BCG *narG* mutant showed smaller granulomas with fewer bacteria than those infected with the wild-type strain [19]. The mutant produced tissue damage in the lungs of immunocompetent mice but was cleared from many organs, unlike the wild-type strain [20]. The 50% lethal dose of a *Salmonella enterica* serovar Typhimurium nitrate reductase-deficient mutant was increased in mice relative to the wild-type strain, but its virulence was not completely attenuated [21]. These all studies shows that nitrate reductase enzyme is membrane bound respiratory type enzyme and plays an important and crucial role in dormant stage of mycobacteria.

Nitrite, the product of nitrate reductase enzyme which converts the nitrate into nitrite comes in extracellular environment through the specific transporter NarK2. NarK2 is acting as antiporter for nitrate and nitrite and its activity is increased during anaerobic condition as compared to aerobic condition [22]. That's the reason nitrate reductase enzyme activity increases during dormancy although its gene expression remains same. It has been seen that the nitrite which is extracellarly released and never utilized from medium inspite of complete used up of nitrate which suggests that nitrite reductase is non-functional gene during both aerobic and anaerobic conditions in *Mycobacterium tuberculosis* [19].

Our concern here is that if nitrite reductase is truly non-functional, then how bacilli is surviving in dormant model in presence of nitrate only? As for long term survival in dormancy there is requirement of different protein like nitrate reductase, alpha crystalline protein, TACO etc which can support its survival in dormancy [22, 23, 24]. And for protein synthesis in mycobacteria, nitrate has to convert in nitrite which is reduced to ammonia by functional nitrite reductase and formed ammonia acts as source of amide for glutamate to form glutamine which is further required for synthesis of other essential amino acids [25]. Further clue of functional nitrite reductase, NirBD gene is both *M. tb* and *M. smegmatis* comes from the amino acid similarities with nitrite reductase NirBD of *E. coli* which shows 40% and 50% identity [4]. It is well established that there is increased expression of NirBD type nitrite reductase in *E. coli* presence in presence of nitrite under anaerobic conditions and mutant form of NirB shows decease enzyme activity during anaerobic conditions [26]. All these studies suggest that NirBD is playing important role in *E. coli*. Also recently, the presence of NirB is shown in *M. tuberculosis* where in presence of nitrite, nitrite reductase NirB gene get induced under aerobic condition which shows its assimilatory role, further NirB mutant was unable to grow in nitrite under aerobic

condition which shows it as an essential enzyme [27]. While another type of nitrite reductase NirA which is now proofed as sulfite reductase is shown to be a potential drug target as NirA mutant form cannot survive inside the host macrophages [28]. All these recent studies on nitrite reductase shows its importance and functionality in *Mycobacterium tuberculosis* which could be an important gene, might plays an important role during dormancy. But so far no studies related to nitrite reductase were carried out to know about its physiological role in *mycobacterium sp.* under different conditions.

Our main objectives of this chapter which includes:-

1) To check out the growth of *mycobacterium sp.* under different conditions in presence of nitrite as sole nitrogen source
2) To find out involvement of NirB under different physiological conditions
3) Differential expression of NirB, if any under different conditions

2.2. Results

2.2.1. *Growth and utilization of M. tb and M. smegmatis in aerobic and hypoxia condition in presence of nitrate/nitrite as sole nitrogen source*

Mycobacterium can grow well in presence of nitrate as sole nitrogen source under aerobic as well as hypoxia induced dormant culture and nitrate reduced to nitrite by presence of functional nitrate reductase [29]. It has been suggested that further assimilation of nitrite is not seen in mycobacteria and nitrite so formed are excreted to the medium. To check the utilization of nitrite, we grown *M. tb* and *M. smegmatis* both in defined medium in presence of nitrite (2 mM) as sole nitrogen source and analyze its growth curve (Fig 2.2).

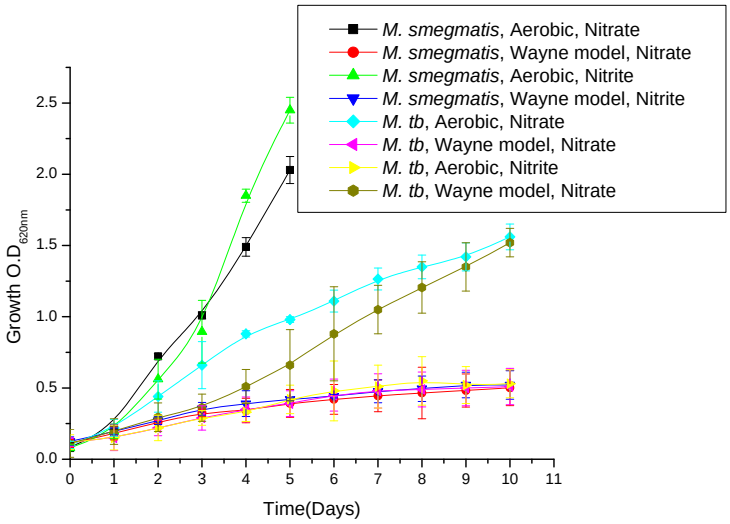


Figure 2.2: Growth analysis curve of M. tb and M. smegmatis under aerobic and hypoxia induced Wayne model in presence of nitrate and nitrite as sole nitrogen source

We observed that both species grew well in presence of nitrite and its growth is comparable with the nitrate (10 mM) growth curve (Nitrate taken as (+) ve control in our study) under aerobic

condition. Also, we measured the assimilation of nitrite in time dependant curve by *M. tb* and *M. smegmatis* and seen that more than 1.5 mM of nitrite has been utilized by both organisms. Although the rate of utilization of nitrite is slow in *M. tb* as compared to *M. smegmatis*. Further we checked the growth and rate of utilization curve in presence of nitrite under Wayne model for *M. tb* and *M. smegmatis.* We observed that bacilli in presence of nitrite shows the characteristics growth curve of NRP-1 and NRP-2 during shiftdown aerobic to anaerobic conditions and comparable with mycobacteria grown in presence of nitrate under similar condition (Fig 2.3) [30]. Interestingly, rate of nitrite utilization is similar in both organisms which indicate the presence of functional nitrite reductase gene present in genome of both organisms.

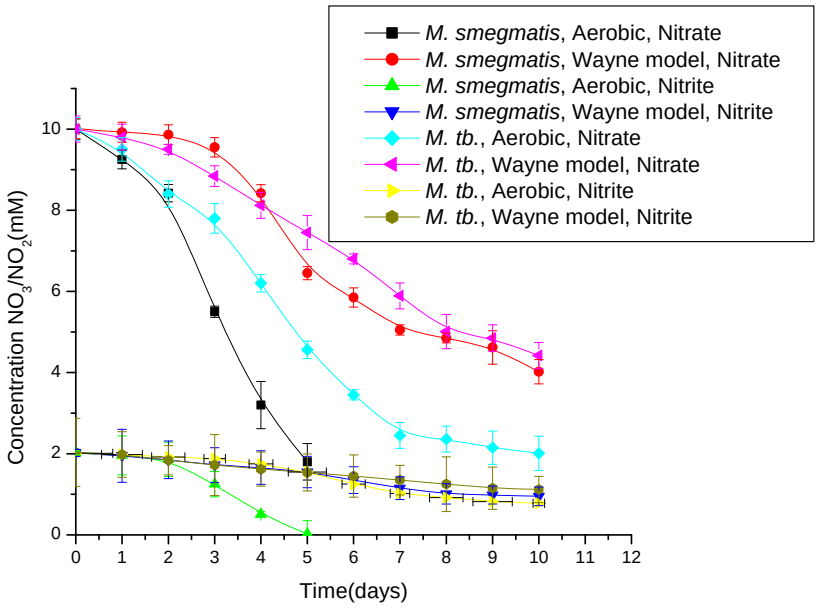


Figure 2.3: Utilization curve of M. tb and M. smegmatis under aerobic and hypoxia induced Wayne model in presence of nitrate and nitrite as sole nitrogen source

2.2.2. Reverse Transcriptase-PCR (RT-PCR) study of nitrogen metabolic genes

To check the expression of nitrite reductase gene at transcript level, we done RT-PCR study on *M. tb* and *M. smegmatis* in presence of nitrite as sole nitrogen source, from aerobic as well as hypoxia induced dormant cultures. We concluded through the genome analysis that the reduction of nitrite to ammonia can be done by two different types of nitrite reductase, NirBD and NirA (Fig 2.4).

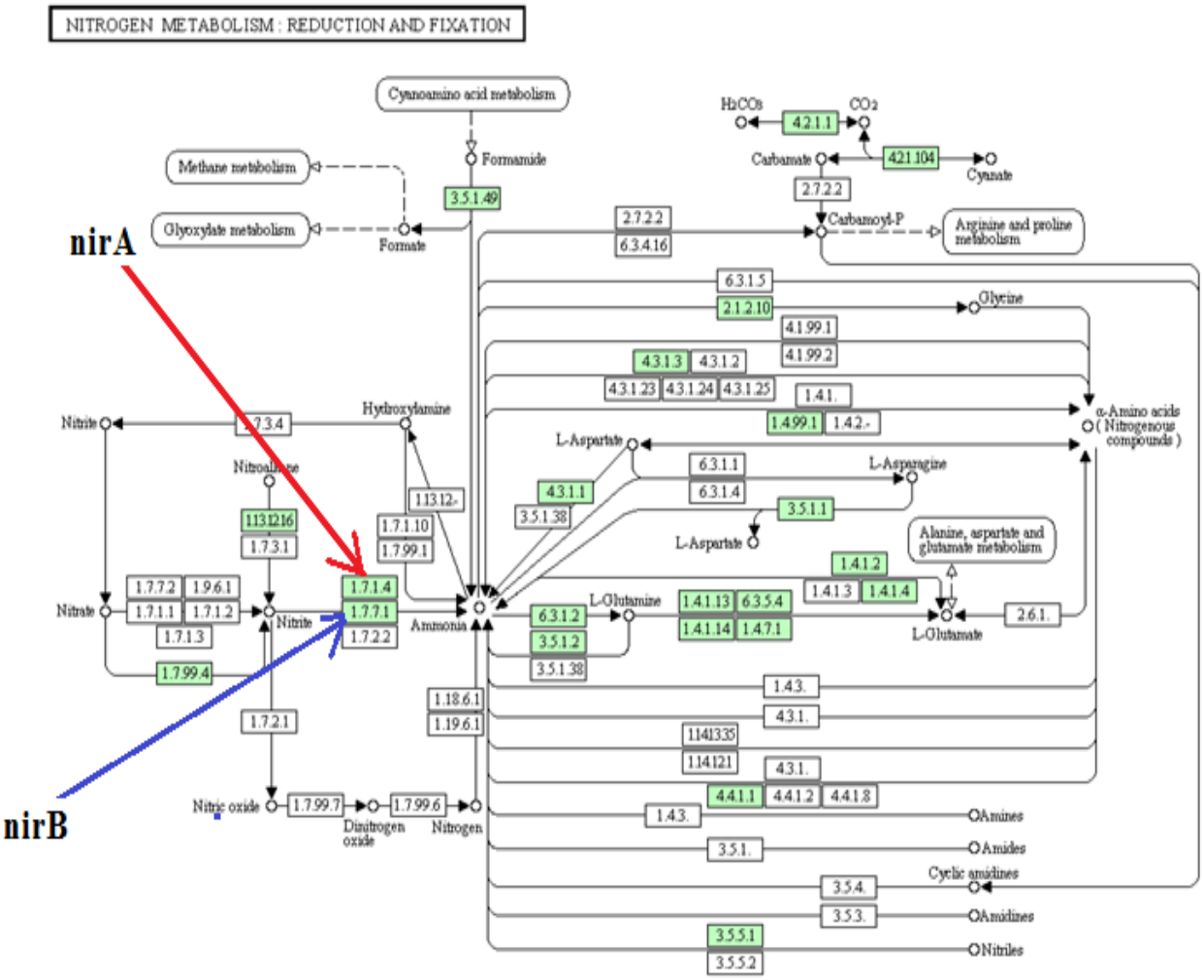


Figure 2.4: The nitrogen metabolic pathways shown in M. tb, where 1.7.1.4 shows NirA while 1.7.7.1 shows NirB type nitrite reductase present in genome of M. tb (Adopted from KEGG data bank)

For the analysis of differential expression, we designed specific primers against both type of nitrite reductase gene. The cDNA was prepared from the total RNA isolated from bacilli

grown in presence of nitrite from both aerobic and hypoxia conditions. Further cDNA was used as template for PCR amplification using NirA and NirB gene specific primers and the amplified product was run on 2% agarose gel (Fig 2.5).

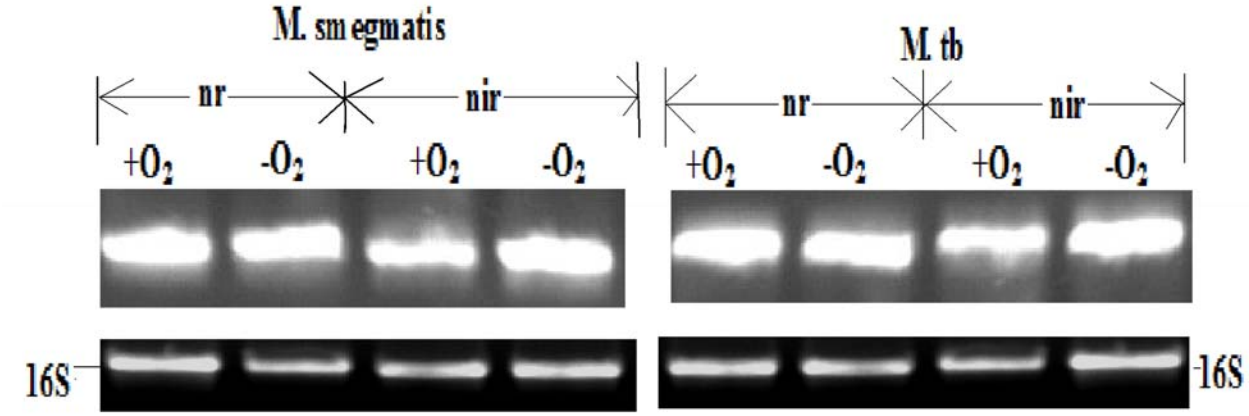


Figure 2.5: Gene expression profile of nrG and NirB gene in mycobacteria under aerobic and hypoxia induced Wayne model

We observed the single and distinct band of expected amplification size in that PCR sample where NirB primer was used while no amplification in NirA primer sample. Further each amplified PCR product was confirmed as amplification of specific gene after BLAST the sequence obtained after sequencing with the available nucleotide sequence in NCBI data bank. It is thus confirmed from the RT-PCR that NirB type nitrite reductase specifically expressed in *M. tb* and *M. smegmatis,* when grown in presence of nitrite as sole nitrogen source under both aerobically and hypoxia dormant culture.

To detremine whether NirBD gene get induce even for assimilation of intracellular nitrite produced by nitrate reductase, when *M. tb* and *M. smegmatis* are grown in presence of nitrate as sole nitrogen source, RT-PCR study was done. For this study, bacilli were grown in nitrate

(10 mM) both in aerobic and hypoxia induced dormant culture and total RNA was isolated and reverse transcribed to get cDNA. The prepared cDNA was used for the RT-PCR study using gene specific primers for narG (nitrate reductase), NirB (nitrite reductaseBD), NirA (nitrite reductaseA) and glnA (glutamine synthatase) genes to analyze the nitrogen metabolic pathway from nitrate to glutamine (Fig 2.6).

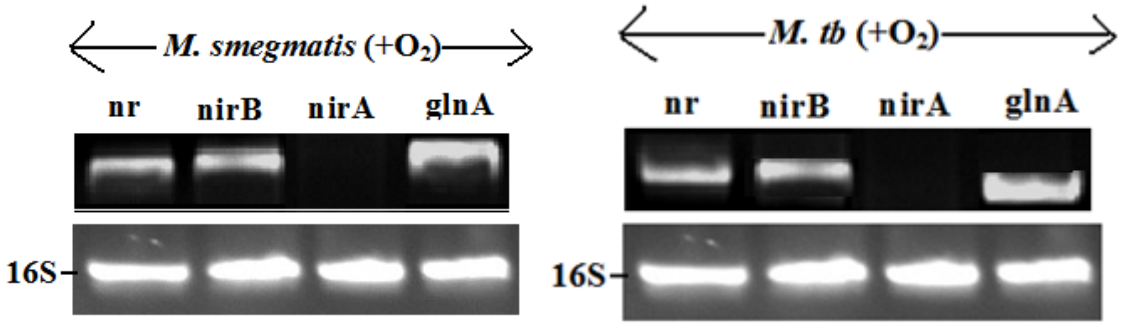


Figure 2.6: Gene expression profile of different genes involved in nitrogen metabolic pathway of M. tb and M. smegmatis, when nitrate is used as sole source of nitrogen in growth medium

The PCR result shows amplification in all samples where specific primer of narG, NirB and glnA was used except in NirA sample, where no amplification was observed. This again shows that NirB gene is the specific nitrite reductase which expressed in presence of intracellular nitrite also along with narG and glnA. Also this is the first direct proof at gene expression level that mycobacteria have the complete functional nitrate reduction pathway where nitrate is converted to nitrite by narGHJI gene and nitrite produced, channelized further to NirBD type nitrite reductase and reduced to ammonia, while again ammonia is converted to glutamine by glutamine synthetase (glnA) gene. And finally glutamine acts as the donor of amine group to different metabolites such as synthesis of purines, pyrimidines, different amino acids, glucosamine and p-benzoate.

Another type of nitrite reductase, NirA is present in mycobacteria, located in operon (ID 261106) consist of 3 genes and all are related to sulfur metabolic pathway (ferrodoxin sulfite reductase, phosphoadenosine phosphosulfate reductase, secreted protein. This suggested that NirA type nitrite reductase may also involve in sulfur metabolism and further shown that NirA gene reduces sulfite to sulfide rather than nitrite to ammonia, although mechanism of enzymatic reduction is similar for both type of nitrite reductase, NirA and NirB. Also it is observed that the sulfite reductase NirA gene expression increases in bacterial residing within infected macrophage [28].

We are highlighting here that all the recent studies related to NirA in mycobacteria, where NirA is shown as sulfite reductase rather than nitrite reductase, uses Magnesium Sulfate ($MgSO_4$) as magnesium source in growth medium. We are suggesting that the addition of $MgSO_4$ in medium itself becomes the source of sulfate which unwontedly induced the expression of NirA gene. Similar results were observed, when $MgSO_4$ was added in growth medium and seen the induced expression of NirA type nitrite reductase irrespective of presence or absence of nitrogen/sulphur source used in growth medium. We observed constitutive expression of NirA even when cells were grown either up or downstream metabolite of nitrite/sulfite i.e in presence of nitrate/sulfate or ammonia/cystenine. When $MgSO_4$ was replaced by $MgCl_2$ as source of magnesium in define media. we interestingly observed that NirB gene expression only in those sample in which nitrate or nitrite is used as sole nitrogen source while no amplification in presence of ammonia while NirA gene get expressed in all samples where $MgSO_4$/Sulfate/Cysteine was act as sulfate source with asparagines as sole nitrogen source (Result summarized in Table 2.1).

Conditions	**$MgCl_2$**		**$MgSO_4$**	
Gene Expression / **Conditions**	**NirA**	**NirB**	**NirA**	**NirB**
Nitrate	No	Yes	Yes	No
Nitrite	No	Yes	Yes	No
Ammonia	No	No	Yes	No
Asparagine	No	No	Yea	No
Cysteine/sulfate/sulfite/asparagine	Yes	No	Yes	No
Cysteine/sulfate/sulfite/nitrate/nitrite	Yes	Yes	Yes	Yes

Table 2.1: Conditional expression of nitrate reductase and nitrite reductase genes in presence of different combination of nitrogen source separately when $MgCl_2$ is changed to $MgSO_4$

2.2.3. Specific activity of Nir

To check the enzyme activity of nitrite reductase (Nir) in both aerobic and anaerobic for *M. tb* and *M. smegmatis* cells in presence of nitrite, we performed the Nir enzyme assay. Nitrite reductase assay was performed in cytoplasmic extract as well as membrane extract after cell lysis to check the localization of nitrite reductase. The nitrite reductase activity was observed only in cytoplasmic fraction; hence we concluded that Nir is cytoplasmic enzyme. We observeded more than 4-fold increase in nitrite reductase enzyme specific activity in hypoxia induced dormant cells as compared to aerobic grown cells (Table 2.2).

Sp. activity of enzyme*	***M. smegmatis***	***M. tb***
NR ($+O_2$)	6.6 (±0.27)	12.0 (±0.48)
NR($-O_2$)	7.33 (±2.93)	9.52 (±3.8)
Nir($+O_2$)	23.4 (±1.02)	29.02 (±1.16)
Nir($-O_2$)	87.89 (±3.52)	120.45 (±5.023)

*Table 2.2: Specific activity of nitrate reductase and nitrite reductase enzymes in presence of nitrate and nitrite as nitrogen source under aerobic and anaerobic Wayne model conditions (*µM of NO_3^- /NO_2^- converted to product per min/mg of total protein)*

2.2.4. Real Time PCR study

Real time PCR was used to measure the exact fold difference of induced nitrite reductase gene at transcript level during aerobic, anaerobic and macrophage infection model. We observed that the level of RNA expressed for NirB in hypoxia condition and infection model is more than 32-fold and 10-fold respectively as compared to aerobic condition. We also correlated with the expression of nitrate reductase which expression is not increasing in either of the conditions [22]. Also we did the quantitative measurement of narK2, specific transporter of nitrate across the membrane in mycobacteria, which is hall mark of hypoxia condition, when cells were grown in nitrate as nitrogen source, increases 28 fold which is consistently with previous reported work (Fig 2.7) [22].

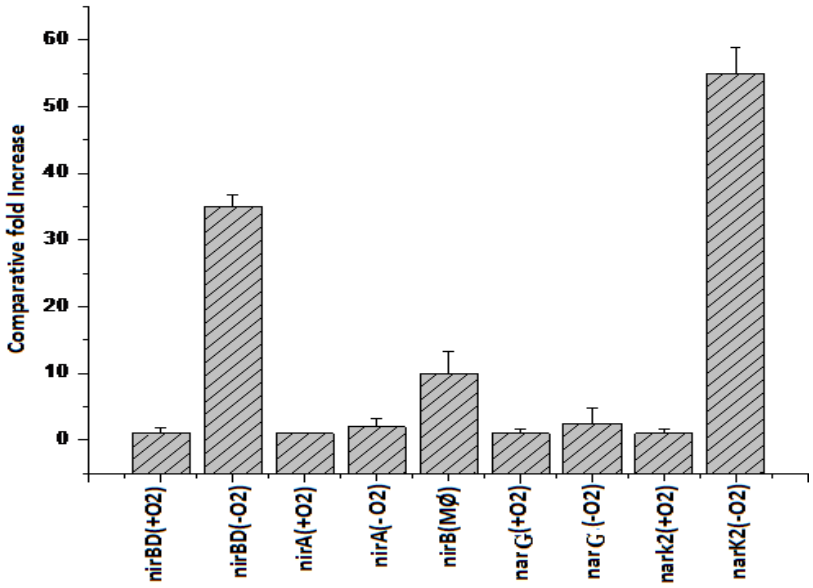


Figure 2.7: Relative fold difference at transcript level in comparison with aerobic condition of genes while 16S gene used as internal control (Results shows the average of 3 identical experiments with ±SD).

2.2.5. Effect of inhibitors of bacilli residing inside the THP-1 macrophages

So far our *in vitro* results show that nitrite reductase is active during both aerobic and anaerobic conditions and its expression increases during hypoxia induced dormant condition. Earlier finding from our laboratory also indicated that *M. tuberculosis* infection leads to generation of hypoxic condition within macrophages. To check the role of nitrate metabolism within infected macrophages, we applied para mercuric benzoate (p-MB) (Vega et al.,) and L-MSO (Harth et al.,) which is specific inhibitor of nitrite reductase and glutamine synthatase repectively and observed that the bacterial CFU count fall after 4th day of post infection (Fig.2.8). This study clearly indicates that nitrite reductase as well as glutamine synthatase are also activily involved for survival of bacilli inside the macrophages and there is an active role of functional nitrogen metabolic pathway inside the host macrophages.

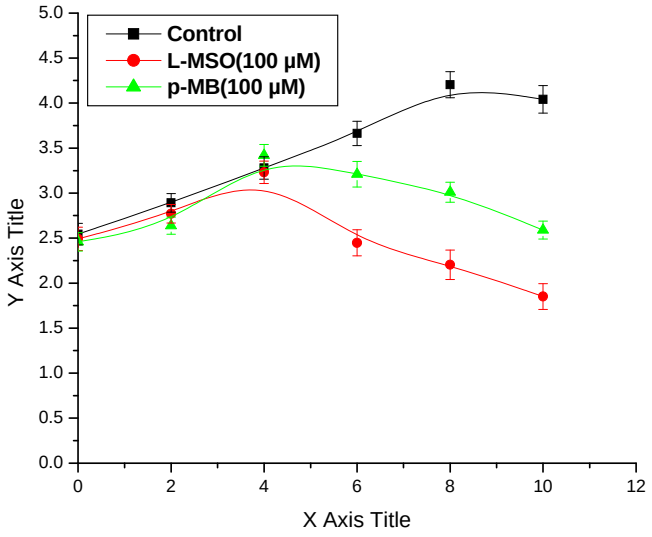


Figure 2.8: Effect of nitrate metabolic pathways inhibitors on the growth and survival of M. tuberculosis in THP-1 macrophage. P-MB (Nitrite reductase inhibitor) and L-MSO (Glutamine synthatse inhibitor) was added at the time of inoculation and effect on growth was examined by lysing the THP-1 macrophage at different time interval and determining the cfu/ml with control (Without any inhibitor). (The Experiments were carried out three times results are the means ± SD).

2.3. Discussion

Here in this study we are analyzing the biochemical role of nitrite reductase in *mycobacterium sp.,* which is not studied till now. Earlier studies shows that nitrite reductase is nonfunctional in mycobacterium as nitrite was excreted out in medium when *M. tb* was grown in presence of nitrate during both aerobic and anaerobic conditions and this extracellular nitrite was not utilized even after complete assimilation of nitrate (10 mM). In our assumption, if nitrite reductase is truly non functional then how bacteria is utilizing and growing in presence of nitrate as sole nitrogen source. As for nitrate assimilation, nitrate reduced to nitrite and nitrite is further converted to ammonia by nitrate reductase and nitrite reductase respectively, while ammonia incorporated to different intermediates of biosynthetic pathway. Hence, we hypothesized here that there must be utilization of nitrite by mycobacterium to support nitrate assimilatory pathway. To prove our hypothesis, we have grown *M. tb* and *M. smegmatis* in presence of 2 mM nitrite as sole nitrogen source and observed that bacilli were growing and utilizing the nitrite although there is less growth and utilization rate as compared to nitrate. This may be because of toxic effect of nitrite which leads to slow replication of bacilli and can be bacteriostatic effect on mycobacterial growth [31]. This could be one of the reason why mycobacteria is not/slowly utilizing the execrated nitrite as the it has been shown that nitrite concentration more than 3 mM in extracellular medium has inhibitory effects for the cells [32]. Also we observed that 2 mM nitrite supported the bacilli growth in dormant Wayne model with similar growth pattern as mycobacterial cells grows in presence of nitrate. Thus nitrite can also support the bacilli in achieving dormancy as nitrite can act as alternative electron accepter in absence of oxygen and nitrate [33].

Assimilated nitrite is further reduced to ammonia by nitrite reductase and we observed that Nir enzyme can be encoded by two different genes NirA and NirBD localized at different position in genome and expressed in sulfur and nitrogen source specifically. Interestingly, recent studies suggest that NirA is sulfite reductase which converts sulfate/sulfite to sulfide. Our results also supports that NirA is not nitrite reductase as it always induced whenever magnesium sulfate is present in medium irrespective of presence or absence of nitrogen source. When we replaced $MgSO_4$ with $MgCl_2$, then the type of reductase (NirA/NirBD) become specific, as in presence of nitrate/nitrite leads to induction of NirBD type specifically, while sulfate/sulfite induced NirA type. Over all we suggest from these results that as $MgSO_4$ is important component in most of the growth medium, its use can unwontedly expressed NirA and may mislead the overall interpretations of conditional expression and importance of NirA gene in specific. Thus we done all studies in presence of $MgSO_4$ as well as in $MgCl_2$ and observed that NirBD is playing an important role mainly in nitrogen metabolic pathway.

We observed the presence of functional NirBD gene in *M. tb* and *M. smegmatis* which is inducible in presence of extracellular nitrite in growth medium and also in intracellular nitrite produced during nitrate assimilation, both under aerobic as well anaerobic conditions. Expression of induced NirB during assimilation of nitrate by mycobacteria may be similar to the environment persist inside the infected host macrophages as the killing agents of activated macrophage i.e. NO and ROS combine together to form peroxynitrite which further convert to nitrite and is utilized by pathogen for its own survival inside the macrophage [34]. This is further confirmed by the induction of NirB gene expression by more than 10-fold in mycobacteria isolated from infection macrophages along with NirB expression increased during anaerobic condition specifically by more than 30-times when compared with aerobic

condition. This high level of nitrite reductase gene can co-relate by increased level of nitrite reductase enzyme activity by more than 4-times during anaerobic conditions. Thus over all these result shows that nitrite reductase can play an important role during survival and maintaining the dormancy.

2.4. Materials and Methods

2.4.1. Bacterial strains, chemicals and media –

Mycobacterium tuberculosis H37Ra (ATCC 25177) and *Mycobacterium smegmatis* mc^2 155 were obtained from Astra Zeneca, India. All the chemicals were purchased from Sigma, USA unless mentioned. A defined medium containing 0.5 gm Potassium dihydrogen ortho phosphate, 0.25 gm Sodium citrate, 60 mg Magnesium chloride, 2 mM nitrite and 2 ml glycerol in 100 ml of distilled water at pH 6.6 ± 0.2 used throughout the study, magnesium chloride was replaced with same conc. of magnesium sulfate where mentioned [36]. The stock cultures were maintained at -70°C and sub cultured once in liquid medium before inoculation to an experimental culture.

2.4.2 Cultivation of aerobic and dormant bacilli

For aerobic cultivation, The bacterial cultures were grown in 20 ml defined medium in 100 ml flask under aerobic conditions in a shaker incubator (Thermo Electron Corporation Model 481) maintained at 150 rpm and 37^{o}C till logarithmic phase ($O.D._{620}$ ~ 1.0) was reached.

For the cultivation of anaerobic dormant bacilli, Wayne 0.5 HSR model was followed wherein 20x125 mm tubes with total volume of 25.5 ml. Inoculums size used here was about 10^5 cells per ml by diluting the culture upto 0.008 A_{620} [30]. After putting 8 mm magnetic spin bars, the tubes was made airtight using rubber septa. The culture was gently stirred at 100 rpm on a magnetic stirrer platform.

2.4.3 Estimation of nitrate and nitrite in liquid culture

Nitrate concentration in the culture was determined by a method based on salicylic acid nitration [37]. Briefly, 50 µl of the culture was added with 200 µl of 5% salicylic acid solution prepared in concentrated sulfuric acid. The solution was incubated for 20 min and 4.75 ml of 2 N Sodium chlorides was added to develop yellow color. Absorbance of the sample was read at 410 nm and nitrate concentration was determined by comparing standard nitrate curve.

Concentration of nitrite in the whole cell culture was estimated by Griess method [38]. Briefly, 1 ml of the culture was added with 1 ml of 1% sulphanilic acid (prepared in 20% HCL) and 1 ml of 1% NEDD solution (prepared in DW). The tubes were incubated for 15 min to develop pink color. Absorbance was measured at 540 nm and nitrite concentration was calculated by using nitrite standard curve.

2.4.4. Preparation of cells extract and Nir assay

For nitrite reductase enzyme assay, spheroplast solution consisting of Lysozyme (0.002 % w/v in DW), D-cycloserine (0.0006 % w/v in DW), Glycine (1.4 % w/v in DW), EDTA (0.2 % w/v in DW) and Lithium chloride (0.1 % w/v in DW) was added aseptically at the respective final concentrations in the growing culture and incubated for 1 hr [39]. After incubation bacterial cells were pellet down using centrifugation at 10,000 rpm for 5min at 4^{o}C. The pellet was washed twice with Potassium phosphate buffer (50 mM, pH 6.6 ± 0.2) and pellet was resuspended in same buffer containing protease inhibitor. The prepared solution was sonicated in water bath for 5 min at 50 kHz. The obtained lysates was centrifuged at 15,000 rpm for 30 min at 4^{o}C. The supernatant was separated in another tube while the cell debris contained membrane fraction was resuspended in same buffer containing protease inhibitor. After determining the protein

concentration, samples were used for Nir assay by following earlier described method of nitrite or ammonia estimation.

Methyl Viologen nitrite reductase activity was assayed done as described by Ida et al. [40]. The reaction mixture in a final volume of 1 ml contained: 75 mM Tris-HCl (pH 8.0), 2 mM KNO_2, 1.5 mM Methyl Viologen, 20 mM potassium dithionite (freshly dissolved in 0.29 M $NaHCO_3$) and an appropriate amount of the enzyme. The reaction was carried out for 5 min at 37°C and stopped by vigorous shaking to obtain complete oxidation of the excess reductant. Nitrite disappearance was determined after a 100-fold dilution of the reaction mixture by the diazo coupling method [38]. The concentration of nitrite utilized by the enzyme in reaction sample was calculated from the standard graph obtained using different dilution concentration of nitrite. Protein quantification was done by following Bradford method using BSA as standard protein [41].

2.4.5 RNA isolation from aerobic, hypoxia dormant culture and infected macrophages

For aerobic culture, bacterial cells were grown upto mid logarithmic phase and total RNA was isolated using spheroplast method [42]. Briefly, the spheroplast solution was added directly to growing culture in mid logarithmic phase and incubated for 1 hr. After incubation, cells were pellet down and resuspended in Trizol reagent. The resulted solution was mild sonicated in water bath sonicater for 2 min on ice box. The resulted suspension was used for total RNA precipitation using chloform: isoamyl method.

For hypoxia induced dormant culture, spheroplast solution was aseptically added to Wayne model using 0.5 ml syring on 7^{th} day. The dormant cells were pellet down after centrifugation at

10,000 rpm for 5 min at 4^{o}C. The pellet was resuspended in Trizol reagent and total RNA was precipitated as mentioned above.

For bacilli residing in infected THP-1 macrophage cell line, THP-1 cells (10^5 cells/ml) were suspended in MEM medium containing 10% FBS in a 75 cm^2 culture flask and incubated at 37^{o}C for 1 day. Phorbol myristate acetate (20 nM) was added to THP-1 culture and incubated for 12 hrs to convert monocytes into macrophages. Macrophages were infected with *M. tuberculosis* (10^7 CFU/ml) at MOI (multiplicity of infection) of 1:100. Then, macrophages were washed 12 hours post infection (PI) with Phosphate Buffer Saline (PBS) and incubated in CO2 incubator at 37^{o}C and 5% CO_2. Spheroplast solution was added to MEM medium on 5th day of PI. On 7th day of PI, cells were washed twice with PBS and then Trizol reagent was added and kept for 15 minutes at room temperature. After incubation, Trizol solution was pipetted out in separate tube and centrifuged at 15,000 rpm at 4^{o}C for 10 minutes to separate the bacterial cell as pellet while supernatant consisting of RNA from macrophages. Collected supernatant was processed for isolation of RNA from macrophages using chloroform: isoamyl (24:1) alcohol precipitation method. The pellet was resuspended in Trizol reagent and processed for mycobacterium RNA as mention earlier.

2.4.6 Quantification of mRNA levels

1 µg of total RNA from mycobacteria and Thp1 macrophage were treated with Dnase-I (Sigma) and then incubated at 70^{o}C according to manufacturers' instruction. 250-300 ng of DNase-I treated total RNA was used for cDNA synthesis using random primer and enhanced avian reverse transcriptase provided in "first strand cDNA synthesis kit" (Sigma) at 25^{o}C for 10 minutes followed by incubation at 45^{o}C for 50 minutes. The resulted c-DNA was used as

template for PCR or real time PCR amplification. The PCR was carried out as per manufacturer's instruction by using Taq DNA polymerase provided in "PCR core kit" (Sigma) in a total volume of 50 µl. Primers used for the PCR study and its amplification conditions are mentioned separately (Table 1). Amplification product of PCR was first analyzed on 2% agarose gel and then by using NCBI blast software after nucleotide sequencing of the PCR product.

Real-time quantitative PCR was performed with the Brilliant SYBR green QPCR Master Mix kit (Stratagene, La Jolla, Calif.). Reactions were performed in a volume of 25 µl, and the reaction mixtures consisted of a 0.05 µM concentration of primers (Table 1), 12.5 µl of 2X master mix, and 2.5 µl of cDNA. The control with no RT was included in each run. An additional sample with RNA diluted 1:10 was also included to measure 16S rRNA. Amplification was performed in the ICycler (Bio-Rad, Hercules, Calif.) with sampling during elongation. The samples were subjected to PCR as follows: (i) an initial denaturation step of 2 min at 95°C (ii) 40 cycles, with 1 cycle consisting of 30 s at 95°C, 1 min at 66°C and 1 min at 72°C (iii) an extension step of 10 min at 72°C. A melting curve analysis was then performed. All samples were run on a 2% agarose gel to verify that only a single band was produced. Each gene was analyzed from three independent RNA samples.

Table 2.3. List of the primers and its amplification parameters used for gene expression studies in M. tb and M. smegmatis

Genes	Primer sequence	Annealing temp.(^{o}C)	Amplification size (bp)
narG(*M.tb*)	F 5'-ACTACGCCGACAACACCAAGTTCGCCGACG-3' R 5'- AGCGGCGCACATAGTCGACAAAGAACGGAA-3'	68	158
narG(*M.smeg.)*	F 5'- ACGAGGTTCCGTACTTCACCGATT-3' R 5'- TCAGATCGAAGACCGTACACACGA-3'	66	450
NirB(*M.tb*)	F 5'-GTCCCGGTTCGTTTCCTTCG-3' R 5'- CGCGGGATACCAATGGACAC-3'	55	155
NirB(*M.smeg.)*	F 5'-AAGGGATTTCGGCCTCTACACCAA-3' R 5'-AGAACATGAGGAACCGGTCGATGT-3'	66	480
glnA(*M.tb*)	F 5'-AACCTTGCCGCTGTGCCTAA-3' R 5'-TAGGTACCTGATACGGTACCTTACGC-3'	63	385
glnA(*M.smeg.)*	F 5'-ATCCGGTTCCCACCAGCACTTT-3' R 5'- TCACCGAAGTTCTTCTGCTCGTACC-3'	66	545
nirA(*M.tb*)	F 5'-TTCATGCCGTAACAGGATTA-3' R 5'-AACCTTGGCCTTACGGTAGACCA-3'	63	400
nirA(*M.smeg.*)	F 5'-AGTTCAGGGAAGTGCTGGAAACCGAGTA-3' R 5'-AGCTCAGCTTGCAGAACTCGATAC-3'	61	350
narK2(*M.tb*)	F 5'-TGCTTCGTGATGCACCCTACTTTCGGCCCA3' R 5'-CCGCCGAACACGATCGCGTACAGAAACGAC3'	63	350
16S*	F 5'-ATGCATGTCTTGTGGTGGAAAGCG-3' R 5'-TTCACGAACAACGCGACAAACCAC-3'	58	350

(*16S gene PCR was done for 25 cycle while PCR of other genes were done for 35 cycles)

2.5. References

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CHAPTER-THREE

Nitrite dependent nitric oxide production induces dormancy in Mycobacterium smegmatis

3.1. Introduction

Infection by *Mycobacterium tuberculosis*, the causative agent of tuberculosis, entails a long-term intimate relationship between bacteria and host [1]. One of the major contributing factors to the success of *M. tuberculosis* as a human pathogen is its ability to persist in the dormant state within the human host for decades, with subsequent reactivation later in life [2]. After infection, *M. tuberculosis* initially multiplies within the host macrophages until circulation of infected macrophages is restricted to form calcified granuloma [3, 4]. Gamma interferon (IFN-γ) of macrophages is perhaps the most important and intensively studied cytokine that can activate antimycobacterial mechanisms of murine mononuclear phagocytes [5]. The essential role of IFN-γ in the resistance of mice to mycobacterial infections was clearly demonstrated by reports that IFN-γ and IFN-γ receptor gene knockout mice cannot control *M. tuberculosis* infection *in vivo* [6]. IFN-γ alone was reported to activate murine macrophages to kill or inhibit *M. tuberculosis*, *M. bovis*, *M. avium*, and *M. leprae* under in vitro condition [7]. IFN-γ alone, or IFN-γ together with lipopolysaccharide (LPS) or tumor necrosis factor alpha (TNF-α), can activate murine macrophages to kill or inhibit mycobacteria by the induction of nitric oxide [8], which is produced from oxidation of L-arginine by inducible nitric oxide synthase (iNOS) [9]. iNOS was maximally induced in murine mononuclear phagocytes stimulated with IFN-γ and LPS or with IFN-γ and TNF-α [10]. The induction of nitric oxide has been directly related to the capacity of IFN-γ-activated in vitro murine macrophages to inhibit or kill *M. tuberculosis*, *M. avium*, and *M. leprae* [7]. Moreover, inhibition of nitric oxide production under *in vivo* conditions increased the mortality, bacillary burden, and tissue damage in mice infected with virulent *M. tuberculosis* [11]. Furthermore, IFN-γ and IFN-γ receptor gene knockout mice that failed to produce nitric oxide were unable to restrict the growth of *M. tuberculosis* or *M. bovis in vivo* [12]. An inducible

nitric oxide synthase (NOS) within activated macrophages generate copious amounts of nitric oxide (NO) to poison pathogenic microorganisms [13]. NO exerts its cytotoxic and genotoxic effects through its ability to damage DNA and iron–sulphur centres of key enzymes such as aconitase [14] and inhibits terminal respiratory oxidases [15]. *M. tuberculosis* has evolved successful strategies to cope with the hostile cellular environment of the macrophage phagolysosome. However, the molecular mechanisms by which it is able to do so are only beginning to be unraveled.

Mycobacterium tuberculosis carries two haemoglobin like protein called HbN and HbO [16]. The functions of these haemoglobins may be diverse which is currently unknown. *M. tuberculosis* HbN is a dimeric haemoglobin that binds oxygen co-operatively but suggested that NO is converted into the nitrite with the help of these proteins. Its high affinity for oxygen binding [17] and the structural characteristics of its distal haem pocket [18] suggested that its purpose may not be just oxygen delivery. It was speculated that HbN might be involved in oxygen-sustained detoxification of NO, providing a defense mechanism to the bacillus against macrophage generated reactive nitrogen species (ROS) [19]. Indeed, some invertebrate Hbs and microbial flavohaemoglobins have been shown to exhibit detoxification functions. For example, it has been demonstrated that haemoglobin from the parasitic nematode, *Ascaris lumbricoides*, uses endogenously produced NO to detoxify oxygen [20]. In contrast, two-domain flavohaemoglobins (flavoHbs), carrying an N-terminal haemoglobin (Hb) domain associated with a flavin-binding reductase module, have been shown to exhibit NO dioxygenase activity [21]. Further, the presence of flavoHbs protects bacteria and yeast from growth inhibition against NO-releasing agents [22] and pure gaseous NO. Recently, single-domain, dimeric haemoglobin (VHb) from the bacterium *Vitreoscilla* , carrying a heterologous reductase domain of *Ralstonia*

eutropha , was shown to metabolize NO and protect the respiration of recombinant *E. coli* from NO inhibition [23]. It was proposed that unique structural features of VHb allow it to associate with a flavoreductase in *Vitreoscill* to produce a flavoHb-like protein. Recently available microbial genome sequences have revealed the presence of HbN homologues in several other mycobacterial species, such as *M. bovis*, *M. avium*, *M smegmatis*, etc., suggesting that these haemoglobins may be playing vital function(s) in the cellular metabolism of mycobacteria [24].

Initially nitrite was considered as a product of NO metabolism, not a source of NO, in tissues. In 1995, it was observed that nitrite could be a prominent source of NO also in biological tissues under conditions of intracellular acidosis, as occur following the onset of ischemia with a lack of tissue perfusion [25]. Several alternative pathways of nitrite-dependent NO generation have been observed to occur in biological systems. NO formation could occur by the simple process of nitrite disproportionation [25]. Several enzymatic systems were reported to be involved in nitrite reduction with NO generation in biological systems. Xanthine oxidase (XO) has a similar structure to bacterial nitrite reductase [26]. XO reduces nitrite to NO at the molybdenum site of the enzyme with the help of xanthine, NADH, or aldehyde substrates serving to provide the requisite reducing equivalents [27-29]. This NOS-independent NO production could serve as a source of NO under ischemic conditions where NO production from NOS is significantly impaired [28]. Aldehyde oxidase (AO) is a cytosolic enzyme that plays an important role in the biotransformation of drugs and xenobiotics [30]. The amino acid sequences of AO and XO are remarkably similar, with 86% homology, and they belong to the same family of molybdenum-containing proteins with two iron-sulfur clusters, a flavin cofactor, and a molybdopterin cofactor [31].

However, no prior research has been done to investigate whether AO has the similar ability to catalyze nitrite reduction to NO. Furthermore, the mechanism, magnitude, and quantitative importance of AO-mediated nitrite reduction in biological systems have not been studied. In addition to the molybdenum enzymes XO and AO, ubiquinone/cytochrome bc1 complex of the mitochondrial electron transport chain was also identified as a site where nitrite is reduced [32]. Nitrite can also accept electrons from cytochrome P-450 (CP) of liver microsomes [33]. In the presence of excessive nitrite, significant NO generation from CP was detected, suggesting that CP-mediated nitrite reduction can be a source of NO [34]. It is now certain that there are multiple pathways of NO generation from nitrite in biological systems, but questions remain concerning the localization, magnitude, mechanism and biological significance of these NOS-independent sources of NO under physiological or pathological conditions. To characterize the mechanism and magnitude of NO generation from nitrite in tissues and blood, electron paramagnetic resonance (EPR) spectroscopy, chemiluminescence NO analyzer, NO electrode, immunoassays of cGMP formation and more recently use of DAF-DA fluorescence dye in plants and bacterial systems were performed [35-A, 35-B]. These studies demonstrate that NO is generated largely from nitrite reduction in tissues not in the blood. Hemoglobin in red cells is observed to function as a trap rather than a functional source of NO. The molybdenum containing enzymes, XO and AO were shown to be important sources of nitrite-derived NO in tissues with this nitrite-dependent NO generation regulated by pH, oxygen tension, nitrite, and reducing substrate concentrations.

Within the purview of this study, we have shown that nitrite negatively control the growth of *Mycobacterium smegmatis* when added in the medium at high concentration under aerobic

condition. We are interested to analyze the reason behind this detrimental effect of nitrite on the growth of mycobacterium in depth in this chapter.

3.2 Results

3.2.1. Growth and utilization analysis in presence of nitrite as sole nitrogen source

During the course of our investigation about the utilization of nitrite, the optimum concentration of nitrite required for growth of *M. smegmatis* was observeded 2 mM (Fig. 3.1).

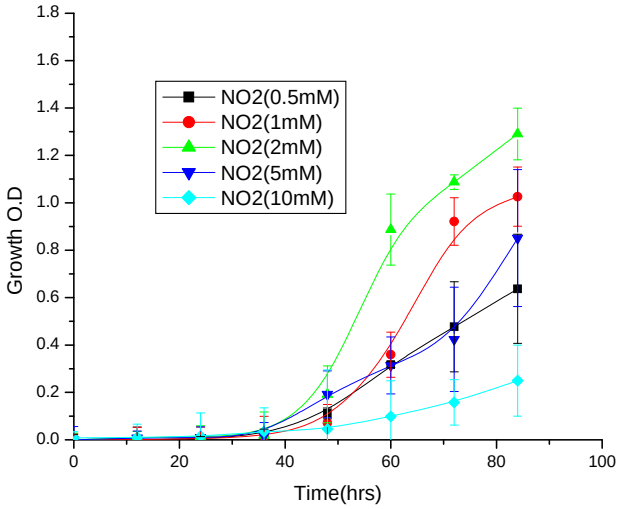


Figure 3.1: Growth optimization curve in different concentration of nitrite as sole nitrogen source in M. smegmatis under aerobic condition

For further investigate the detrimental effect of nitrite on *M. smegmatis* growth and survival, bacilli were grown in presence of varying nitrite concentrations (2-10 mM) in *M. pheli* medium under aerobic condition (Fig 3.2). The growth curve analysis clearly indicated that the cells density was inversely proportional to the nitrite concentration used in the medium. Thus, it indicates that 2 mM is showing maximum growth while growth was very limited in 10 mM concentration of nitrite.

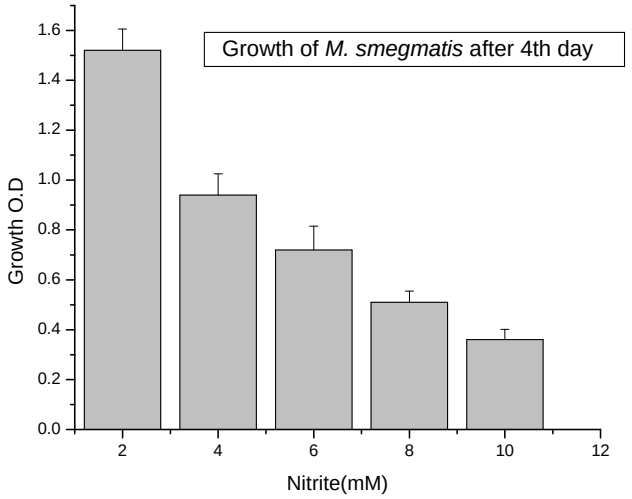


Figure 3.2: Effect of nitrite on growth of M. smegmatis under aerobic condition. The log phage cells were inoculated in M. phili medium in presence of nitrite (0.5-10 mM) as sole nitrogen source. The growth of bacilli was measured at O.D$_{620}$. The result was expressed as an average of three identical experiments carried with standard deviation.

As nitrite is metabolized to other assimilatory products in the metabolic pathway, it becomes necessary to identify the extent of nitrite utilized by the same culture. The extent of nitrite utilized clearly indicated that most prominent inhibitory effect of nitrite utilization seen at its highest applied concentration (Fig. 3.3).

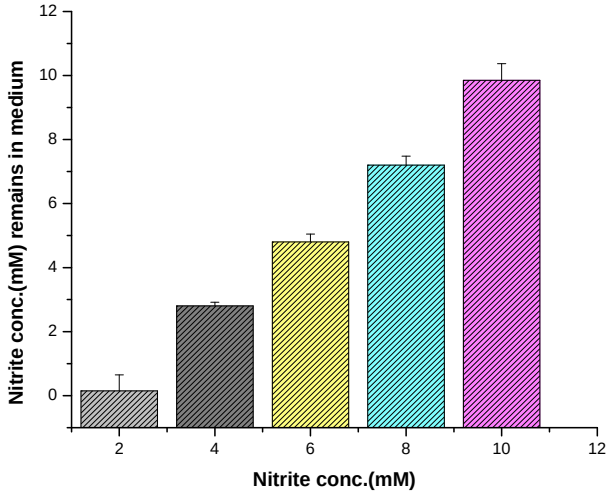


Figure 3.3: Nitrite concentration remains in medium after 4th of utilization by M. smegmatis under aerobic condition

Above mentioned results clearly indicated that the active factor/s responsible for arresting the growth of the bacilli was directly linked to nitrite and its concentration reached a significant level to produce an effect only at higher than 2 mM in the aerobic culture medium. Nitrites are highly water-soluble, and they can undergo the following reversible acid-base reaction.

By convention, HNO_2 represents molecular nitrous acid in the aqueous phase (aq.), which can reversibly dissociate into H^+ and NO_2^- ions or partition to the gas phase (g), where nitrous acid is designated as HONO. The equilibrium gas-phase concentration over an aqueous solution of nitrous acid, [HONO]*, is determined by the acidity (pH value) and nitrite concentration of the solution [36]. [HONO]* is a key parameter controlling the exchange of HONO between the gas and aqueous phase. When [HONO]* is higher than the actual gas-phase concentration, [HONO], nitrous acid will be released from the aqueous phase; otherwise, gaseous HONO will be deposited. It was suggested that higher concentration of nitrite has toxic effect as it form nitrous acid after combing with water which is acidic in nature hence leads to acidity of medium, could be one of the possible reasons for detrimental effect seen at higher concentration of nitrite.

To check first the detrimental effect might be because of low pH at higher concentration of nitrite used in growth medium, we measured the pH of medium at 0^{th} day and on 4^{th} day i.e. on the day of initiation and termination of experiment respectively (Fig. 3.4). The result showed that pH of the medium did not change at all during the progression of experiments, hence we concluded that pH had no role on the nitrite toxicity of the bacilli.

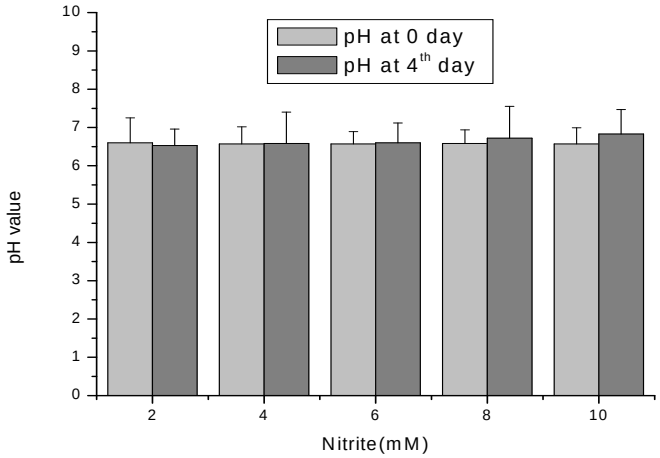


Figure 3.4: Comparison of pH change on 0th and 4th day of experiment with different nitrite concentration

3.2.2. Effect of specific scavenger of ROS and RNI

It was earlier reported that nitrite can be source of reactive nitrogen intermediates (RNIs) [36]. Nitrite could be converted to nitric oxide by the numerous pathways involving haemoglobin [37], myoglobin [38], xanthine oxidoreductase [39], ascorbate [40], polyphenols [41] and protons [42]. Nitrite (NO_2^-) is formed in the body via the oxidation of nitric oxide (NO) or through the reduction of nitrate (NO_3^-). The non-enzymatic reaction of NO with oxygen in tissues is relatively slow, whereas its oxidation by the multi copper oxidase ceruloplasmin present in plasma in eukaryotic system is rapid. Commensal bacteria in the oral cavity and gastrointestinal tract contribute to nitrite formation via a one-electron reduction of nitrate.

Reduction of nitrite to NO occurs in blood and tissues and proceeds through several enzymatic and non-enzymatic pathways, some of which are listed below. The acidic reduction of nitrite results in the generation of NO but also other nitrogen oxides, with nitrosating (N_2O_3) and nitrating (nitrogen dioxide, $^{\bullet}NO_2$) properties. In the presence of ascorbic acid or polyphenols, the acidic reduction of nitrite is greatly enhanced with less generation of N_2O_3 and $^{\bullet}NO_2$.

Oxidation of nitrite occurs in the red blood cell and results in the formation of nitrate and methaemoglobin (Met-Hb).

Nitrite reduction:

Deoxyhaemoglobin/myoglobin

$NO_2^- + Fe^{2+} + H^+ \rightarrow {}^{\bullet}NO + Fe^{3+} + OH^-$

Xanthine oxidoreductase

$NO_2^- + Mo^{4+} + H^+ \rightarrow {}^{\bullet}NO + Mo^{5+} + OH^-$

Protons

$NO_2^- + H^+ \rightarrow HNO_2$

$2\ HNO_2 \rightarrow 2\ N_2O_3 + H_2O$

$N_2O_3 \rightarrow {}^{\bullet}NO + {}^{\bullet}NO_2$

Ascorbate

$NO_2^- + H^+ \rightarrow HNO_2$

$2\ HNO_2 + Asc \rightarrow 2\ {}^{\bullet}NO + dehydroAsc + 2\ H_2O$

Polyphenols (Ph-OH)

$NO_2^- + H^+ \rightarrow HNO_2$

$Ph\text{-}OH + HNO_2 \rightarrow Ph\text{-}{}^{\bullet}O + {}^{\bullet}NO + H_2O$

Thus it is clear that nitrite can be the source of different reactive nitrogen intermediates which could be formed by enzymatic or non-enzymatic process. Now, the mechanism of NO production as well as its detection needed to prove the actual reason behind the nitrite toxicity of *M. smegmatis*. Hence, we used p-caumaric acid (pCA) and rutine hydrate (RH), well known scavenger of RNI to block the possible detrimental effects of nitrite on the growth of *M. smegmatis* (Fig. 4.0) [43, 44]. The result indicated that in presence of pCA and rutine hydrite,

cells regain its growth and its O.D reached more than 1.2 while the control sample without any scavenger at 0.2 only even after 4th day after inoculation (Fig. 3.5).

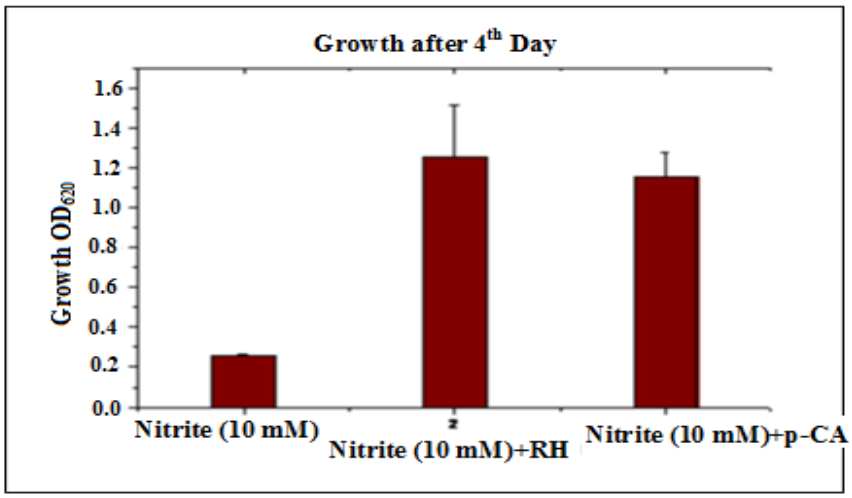


Figure 3.5: Growth O.D at 4th day of M. smegmatis in presence of 10 mM nitrite with RNI scavengers (p-CA & RH).

Further we checked the utilization of nitrite in presence of p-CA and RH which indicated assimilation of more than 6 mM of nitrite while more than 9 mM nitrite remained in the medium where scavenger was not added (Fig. 3.6). This clearly proved that higher nitrite leads to production of RNI which could have detrimental effect on growth of bacilli.

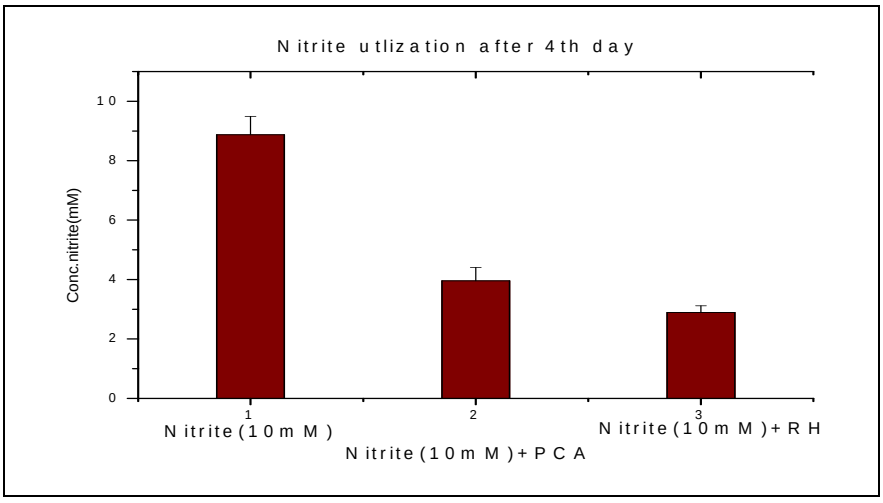
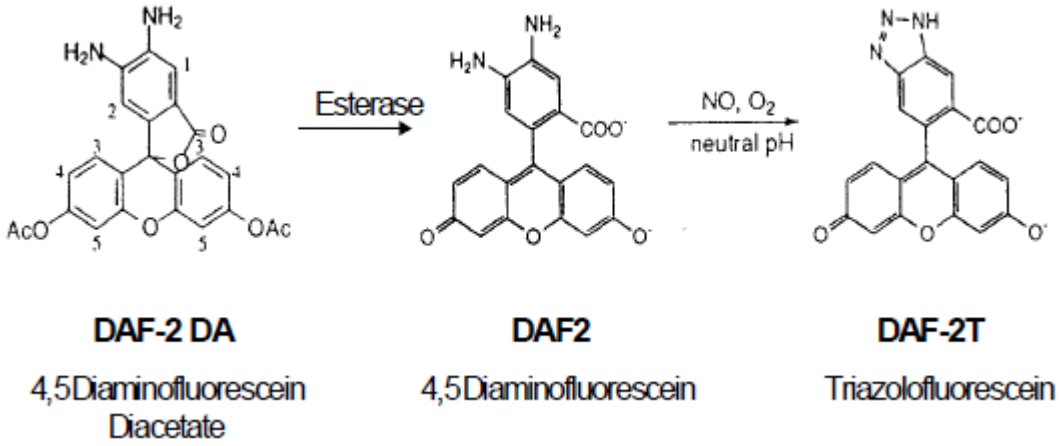


Figure 3.6: Concentration of nitrite remains un-utilized in medium on 4th day by M. smegmatis under aerobic condition in presence of RNI scavengers.

3.2.3. Evidence of Nitric oxide production in presence of nitrite

Until recently nitrite was thought to be an inert oxidative breakdown product of endogenous NO synthesis but in the past few years research have focused on the reduction of nitrite back to NO in the circulation as a possible mechanism for hypoxia vasodilatation [45]. Nitrite has evolved into an endogenous signaling molecule and regulator of gene expression that may not only serve as a diagnostic marker but also find its role as a potential therapeutic agent of cardiovascular disease. Here, we used DAF-DA dye, specific for detecting NO production in *M. smegmatis* under aerobic condition.



The fluorescence microscopic picture clearly indicated NO production within the *M. smegmatis* cells specifically in presence of 5 mM and 10 mM nitrite while 2 mM of nitrite concentration shows no nitric oxide production (Fig 3.7).

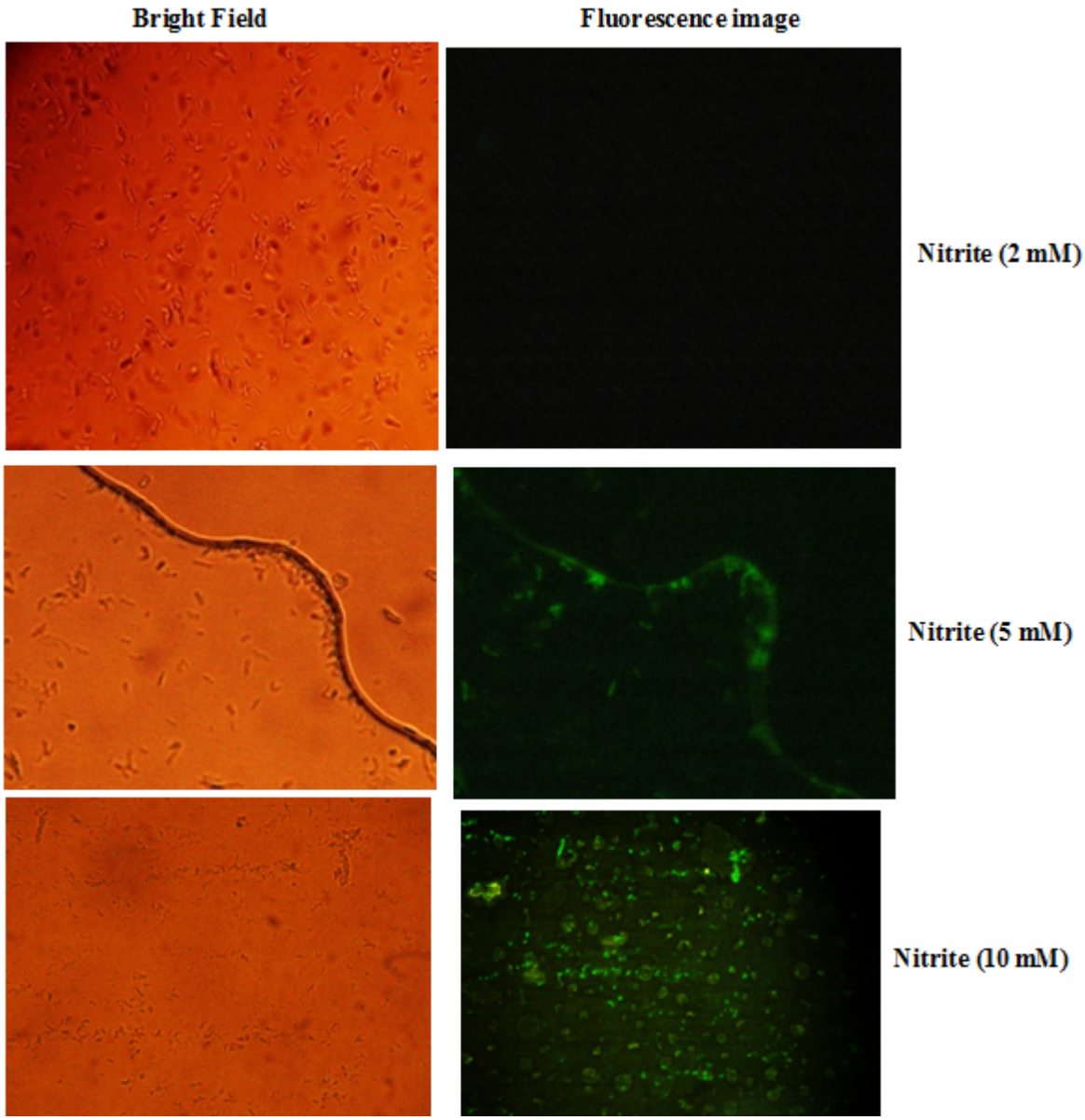


Figure 3.7: Microscopic images of M. smegmatis grown in different nitrogen sources. A: 2 mM nitrite, B: 5 mM nitrite, C: 10 mM nitrite. While panel 1 & 2 shows bright filed and its Florescence images respectively.

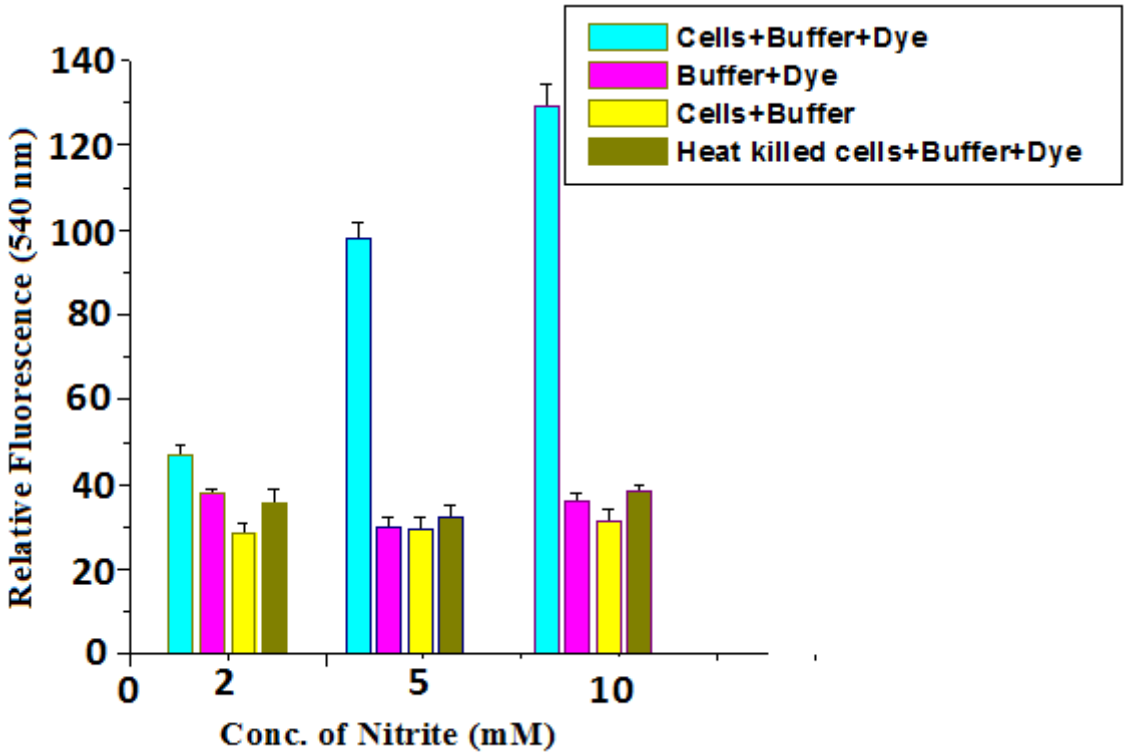


Figure 3.8: Relative fluorescence studies of M. smegmatis grown in different nitrite concentration along with controls.

Further we did the quantitative estimation of relative fluroscence which clearly demonstrate that NO production take place in presence of nitrite at 5 mM and 10 mM concentration while very low fluorescence at 2 mM (Fig. 3.8). In overall, these pictures as well as quantitative measurement of fluorescence clearly demonstrated that there is nitric oxide production within aerobic *M. smegmatis* cells specifically in presence of higher level of nitrite in the medium.

3.2.4. Inhibition of biofilm formation: Evidence of NO production

Microorganisms possess many ways to ensure their survival, such as sporulation and biofilm formation. In nature, most microbes live as communities in biofilms, a conglomeration of

bacteria and other microbes embedded in a self-produced and secreted matrix of extracellular polymeric substances (EPS). The EPS could be composed of polysaccharides, proteins, nucleic acids and lipids. The biofilm functions as a protective hydrated barrier between the bacterial cells and their environment. It facilitates survival of the bacterial cells under harsh conditions and environmental insults such as ultraviolet radiation, physicochemical stresses, desiccation and insufficient supply of nutritive resources [47].

Recently, it was observed that both the fast-growing nonpathogenic *M. smegmatis* and the slow-growing opportunistic pathogen *Mycobacterium avium* are able to slide and can form biofilm and in both species this ability correlates with the presence of GPLs [48], a class of glycosylated peptidolipids present in the outermost layer of the cell envelope [49].

Recently in *Pseudomonas aeruginosa*, cell lysis and dispersal of biofilm was linked to both the activation of prophage and the generation of oxidative or nitrosative stress inside the microcolonies [50]. Oxidative stress results from either endogenous production of or exogenous exposure to reactive oxygen intermediates (ROI), which include superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the extremely reactive hydroxyl radical ($HO\cdot$) [51]. Also polysaccride intercellular adhesion (PIA) and biofilm formation was observed to be inhibited in *Stephylococus aures* when nitrate and nitrite was added in growth medium. Nitrite, either as the endogenous product of respiratory nitrate reduction or after external addition, caused repression of the *icaADBC* gene cluster responsible for biofilm formation. Moreover, preformed biofilms could also be eradicated by the addition of nitrite in the medium, likely because of the result of the formation of toxic acidified nitrite derivatives and biofilm formation was abrogated by the addition of nitric oxide (NO) scavengers, suggesting that NO is directly or indirectly involved in the process [52].

In order to check the inhibition of biofilm formation by *M. smegmatis*, cells were grown in presence of nitrate and nitrite in the medium (Fig. 3.9).

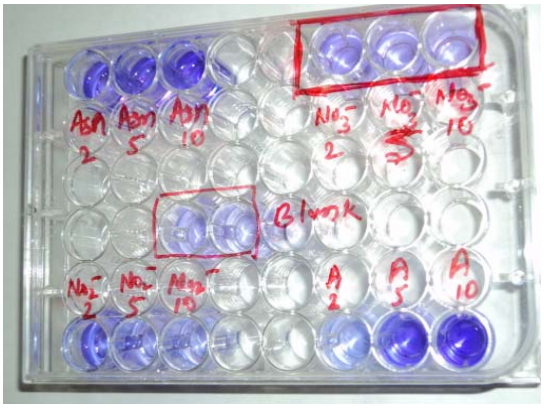


Figure 3.9: Crystal violet assay on M. smegmatis grown in 96 well microplate under aerobic condition in presence of different nitrogen source in varied concentration. Asn,NO_3^-, NO_2^-, A represents: Asparagine, nitrate, nitrite and ammonia used in 2,5, and 10 mM concentration as sole nitrogen source for biofilm formation experiments respectively.

It was observed that nitrate and nitrite increasingly affected biofilm formation in *M. smegmatis* as the concentration was increased from 2 mM to 10 mM (Fig. 3.10). Interestingly, the inhibitory effect on biofilm formation was not observed when asparagines or ammonia even at higher concentration which indicates that asparagines and ammonia are supporting the growth of *M. smegmatis*.

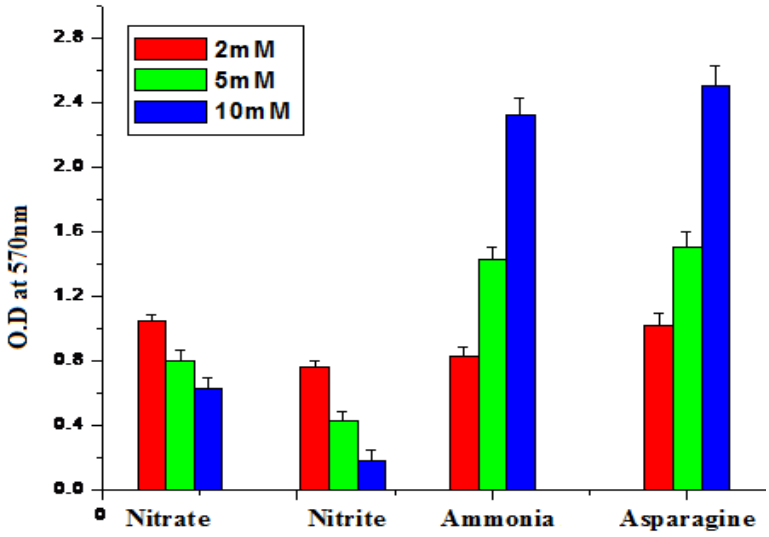


Figure 3.10: Relative O.D. of Crystal violet assay on M. smegmatis grown in 96 well plate under aerobic condition in presence of different nitrogen source in varied concentration. Asn,NO_3^-, NO_2^-, A represents: Asparagine, nitrate, nitrite and ammonia used in 2,5, and 10 mM concentration as sole nitrogen source for biofilm formation experiments respectively.

The spectrophotometer result also shows that as the concentration of nitrate/nitrite increases from 2 mM to 10 mM, there is inhibition in biofilm formation which indirectly indicates the production of nitric oxide at higher concentration of nitrate/nitrite while no any inhibition in biofilm formation at higher asparagines/ammonia concentration during growth of *M. smegmatis*.

3.2.5. Expression of dosSR regulon

The two-component response regulator, dormancy survival regulator (dosR) encoded by Rv3133c, but not a sensor histidine kinase encoded by the adjacent gene Rv3132c, is required for induction of *acr* by hypoxia [53]. Microarray expression profiling was used to determine the regulation of NO/dormancy/ hypoxia-induced gene expression controlled by dosR. Inactivation of Rv3134c, the first gene of a presumed three-gene operon, fully or partially reduced induction

of each of the 48 genes in response to NO as well as during low O_2– induced dormant state. Complementation of the mutant with Rv3134c/*dosR* largely restored this effect. It was also recently demonstrated that hypoxia fails to induce any of the 48 genes when *dosR* is selectively disrupted [54]. Together, these results demonstrated that NO, hypoxia and adaptation to an in vitro dormant state induce a common set of 48 genes by dosR.

Further analysis regarding the production of nitric oxide at higher concentration of nitrite, which is one of among the several signals for induction of dormancy, dosSR gene expression was studied (Fig. 3.11). As expected, the dosS, dosR and hspX genes were induced in presence of nitrite and upregulated as the concentration of nitrite is increasing in the growth medium.

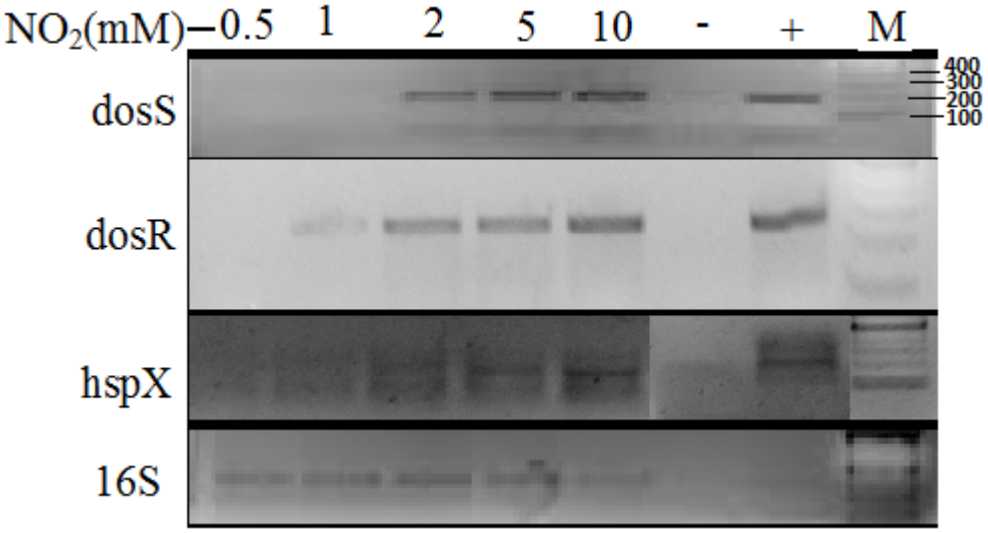


Figure 3.11: Gene expression profile of dos-SR regulon which includes dosS, dosR and hspX genes in M. smegmatis under aerobic condition at different nitrite concentration with (+) ve: genomic DNA as template and (-) ve: DNA pol. negative PCR samples. 16S gene expression as internal control while M shows marker

The expression of three important genes of dos-SR regulon was clearly an indication prevailing stressed condition within the cells due to NO production at higher concentration of nitrite which leads to shift of active bacilli into dormant stage.

3.2.6. Cell morphological study of bacilli

The existence of dormant MTB cells was originally proposed to account for the presence of acid-fast, but transiently 'non-culturable' bacteria in closed pulmonary lesions [55]. Later, Khomenko and colleagues observed filterable or mini-forms of MTB that remained within the tissues of guinea pigs upon the completion of anti-TB treatment [56]. After several months of therapy, viable MTB could no longer be detected by standard plating procedures, although microscopic examination of organ homogenates filtered through 0.2–0.7 mm filters revealed the presence of electron-dense forms with a rounded shape and an average diameter of 0.25 mm [57]. Direct administration of these forms to guinea pigs induced the development of TB, and after several passages, MTB could be isolated by standard culture methods.

Periodic examinations of these cultures by phase-contrast microscopy revealed accumulation of atypical cells with significantly altered morphology. After 14 days of incubation, 10–20% of the cells in the population were rods with easily distinguishable 'bulges' at the cell ends. During further incubation, cells became racket-like, and after 1.5 months, 50–70% of the cell population was represented by almost round (ovoid) cells with central highly refractive regions. The percentage of ovoid cells did not change significantly up to 5 months of further incubation. The length to width ratio of these cells was 1.0–1.4 (average diameter 0.8–1.4 mm), in contrast to 5–7 for stationary-phase cells [58]. Hence, it is well accepted that dormant mycobacterial cells has typical structure from normal rod like thick structure to round coccoid structure via dumble shape to racket shape which are hall mark of shift down of cells from active to dormant form.

In order to check the effect of nitrite on *M. smegmatis* morphology under aerobic condition, scanning electron microscopic study was carried out (Fig. 3.12). The results show that cell changes its morphology from normal rod shaped (nitrite 2 mM) to dumble shaped at higher

concentration of nitrite (5 mM) to complete round/ovoid shape at 10 mM nitrite on 4th day of their growth. While cells in 10 mM nitrite regained its growth only after 7th day, its cells morphology becomes changed from round to dumble shaped. Hence these images clearly indicates that higher concentration of nitrite induces dormancy in *M. smegmatis* and as the nitrite concentration is within its physiological range, cell regain its growth and thus morphology is changing from dormant form-round shape to normal-rod shape through intermediate dumble shape structure.

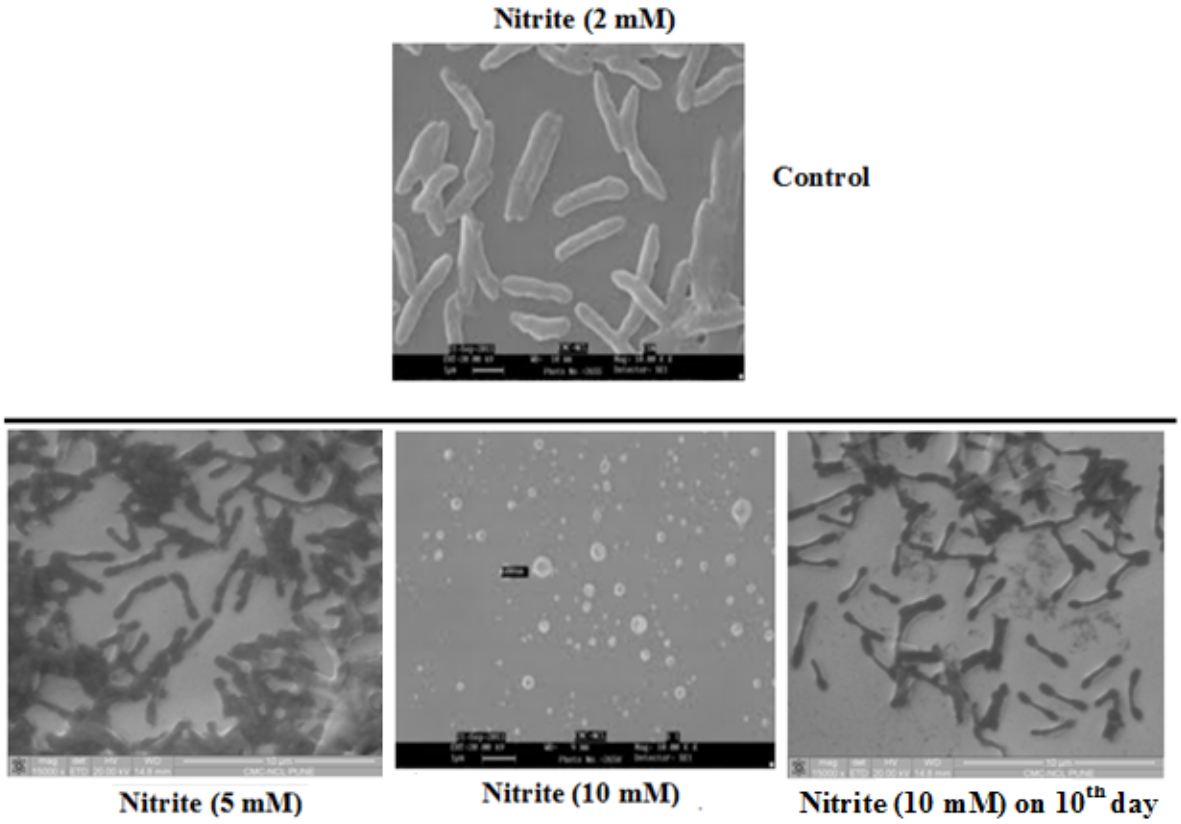


Figure 3.12: Electron microscopic images of M. smegmatis grown in different nitrite concentration on 4th day of their growth

3.3. Discussion

Voskuil et al., has shown that in presence of DETA-NO (NO producer) *Mycobacterium tuberculosis* undergo dormancy and its CFU count does not change significantly as compared control hence its show bacteriostatic condition, while nitrite at higher concentration is bactericidal for mycobacterium as well as other organisms. In our study, we have shown that exposure of bacilli to higher level of nitrite in the medium will lead to dormancy instead of helping growth under aerobic conditions. Nitrite is unique to the nitrogen oxides in its redox position between oxidative (NO_2 radical) and reductive (NO radical) signalling and its relative stability in blood and tissue [59]. The generation of NO by these pathways is greatly enhanced during hypoxia and acidosis, thereby ensuring NO production in situations for which the oxygen-dependent NOS enzyme activities are compromised [60]. The formation of NO within the *M. smegmatis* bacilli was initially indicated from: 1) nitrite concentration dependent growth inhibition, 2) the reversal of nitrite effect on growth of the bacilli by NO scavenger's p-CA and RH (Fig 1-3). It was not very clear whether pH could influence this conversion of nitrite to NO although our results suggested no change in the pH of the medium during these experiments (Fig 3). Other denitrifying bacteria like *Pseudomonas sp.*, specific enzyme representing the gene cd1 type nitrite reductase K present in the genome which converts nitrite to NO while any denitrifying gene in mycobacterium is not reported [61].

Further, formation of NO from nitrite was confirmed from 1) microscopic elucidation of intracellular fluorescence of DCAFH-DA, 2) growth retardation in presence of increased amounts of nitrite in the medium (Fig 1-2) and 3) inhibition of biofilm synthesis by M. *smegmatis* culture in presence of nitrite (Fig 9 and 10). This biofilm formation in staphylococcus

culture was observed to be inhibited in presence of nitric oxide in part due to the inhibition of *icaADBC* gene cluster playing important role in the process [52].

It was well known that NO along is capable of inducing dormancy in mycobacterium [62]. This induction of dormancy is mainly carried out through the activation of a cascade of genes dos-SR regulon and its products [63]. The devSR (dosSR) two-component system plays a crucial role in the adaptation of mycobacteria to hypoxia and NO induced dormant conditions. Approximately 48 genes of *M. tuberculosis* were reported to be induced due to the exposure to such conditions. Hence, the expression of dosS, dosR and hspX in presence of nitrite directly indicated the production of nitric oxide which leads to shift down of bacilli into dormant phase (Fig. 3.11). It is well known that in dormant phase the mycobacterium shutdown most of its synthesis machinery. The scanning electron microscopic picture of these nitrite treated cells clearly showed change in bacterial shape from rod shape (control or 0.5 mM nitrite) to complete round oval shape when treated with 10 mM of nitrite which was similar to the shape suggested for the dormant bacilli (Fig 12).

Thus, the overall study had focused on the detrimental effect of exposing *M. smegmatis* to higher concentration of nitrite on its growth. This detrimental effect is mainly because of nitric oxide production at higher concentration of nitrite within the cells and this NO production was sufficient enough to lead to shift down of actively grown aerobic mycobacterium into dormant form. Involvement of Nitrite reductase, Xanthine oxidase or some other enzyme could not be ruled out. For this, probably we need to knock out each of these genes involved in nitrite metabolism within *M. smegmatis* bacilli.

3.4. Materials and Methods

3.4.1. *Bacterial strains, chemicals and media*

Mycobacterium smegmatis mc^2 155 were obtained from Astra Zencea, Bangalore India. All the chemicals were purchased from Sigma, USA otherwise mentioned. A defined medium containing 0.5 gm Potassium dihydrogen orthophosphate, 0.25 gm Sodium citrate, 60 mg Magnesium chloride, with nitrate/nitrite/ammonia as sole nitrogen source and 2 % (v/v) glycerol in 100 ml of distilled water at pH 6.6 ± 0.2 were used throughout the study [64]. The stock cultures were maintained at -70°C and sub cultured once in liquid medium before inoculation to an experimental culture.

3.4.2. Cultivation of aerobic bacilli

For aerobic cultivation, bacterial cultures were grown in 20 ml defined medium within 100 ml flask under aerobic conditions in a shaker incubator (Thermo Electron Corporation Model 481) maintained at 150 rpm and 37^{o}C till logarithmic phase ($O.D_{620}$ ~ 1.0) was reached.

3.4.3. Estimation of nitrite in medium

Concentration of Nitrite in the whole cell culture was estimated by Griess method [65]. Briefly, 1 ml of the culture was added with 1 ml of 1% sulphanilic acid (prepared in 20% HCL) and 1 ml of 1% NEDD solution (prepared in DW). The tubes were incubated for 15 min to develop pink color. Absorbance was measured at 540 nm and nitrite concentration was calculated by using nitrite standard curve.

3.4.4. Fluorescence microscopic study

For fluorescence microscopic studies, aerobically grown cells were centrifuged at 10,000 rpm for 5 min at 4^{o}C. The medium was discarded and washed twice with PBS buffer using 10,000 rpm for 5 min at 4^{o}C. The cell pellet was used for the assay for NO production following the protocol mentioned in manuals provide in kit (FANOS, Sigma). After incubation with dye and NO detection buffer for 15 min at room temperature in dark, cells were washed twice with PBS buffer. Finally the washed cells were resuspended in 100 µl of PBS buffer and the fluorescence microscopic or absorbance studies were carried out. The excitation and emission wavelength were 450 nm and 540 nm respectively.

3.4.5. Estimation of Biofilm formation using crystal violet assay

The biofilm formation protocol was adapted from an earlier method developed by O'Toole and Kolter [66]. *M. smegmatis* was grown in *M. pheli* medium with different nitrogen source in 96 well polystyrene plates under aerobic condition up to 4th day in rotary shaker incubator. After 4th day, media were discarded and 1% solution of crystal violet was added to stain the attached cells. After 10 to 15 min of incubation at room temperature, the wells were rinsed with water, and the biomass of attached cells (biofilm) was quantified by solubilizing the dye in 2 ml of 95% ethanol. The absorbance was measured at 600 nm with a spectrophotometer.

3.4.6. Reverse Transcriptase PCR studies

For RT-PCR study, *M. smegmatis* was grown in aerobic condition in presence of nitrite in *M. pheli* medium. Mid-logarithmic phase culture was used for total RNA isolation using spheroplast method [67]. Total RNA was converted to cDNA using single strand synthesis kit

(Sigma) and the 1st step at 25°C for 10 min followed by incubation at 45°C for 50 min. The resulted cDNA was used as template for PCR. The PCR was carried out as per manufacturer's instruction by using Taq DNA polymerase provided in "PCR core kit" (Sigma) in a total volume of 50 µl. Primers used for the PCR study and its amplification conditions are mentioned separately (Table 1). Amplification product of PCR was first analyzed on 2% agarose gel and then by using NCBI blast software after nucleotide sequencing of the PCR product.

3.4.8. Sample preparation for scanning electron microscope (SEM)

For scanning electron microscope, cells were pellet down at 10,000 rpm for 10 min at room temperature. Pellet cells were washed two times with PBS and finally resuspended in glutaraldehyde solution and incubated for 30 min at room temperature. After incubation, cells were again washed with DW and resuspended in 50 µl PBS. 5µl of cells were dropped on silicon wafers and kept for drying at room temperature. After dry, sample was used for scanning electron microscopic study at 5000X magnification.

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CHAPTER FOUR

A) Cloning, expression and characterization of Mycobacterium smegmatis NirB

4A.1. Introduction

Mycobacterium tuberculosis, the primary etiologic agent of tuberculosis, is one of the world's leading causes of death, killing two million peoples annually, worldwide and one-third of world population carrying the bacilli in the latent form [1]. This, compounded with the emergence of drug-resistant strains and the HIV epidemic in both developing and industrialized countries, underscores the need for an effective drug against this disease [2]. Although combination chemotherapy is effective in the treatment of tuberculosis, the treatment is arduous and requires stringent compliance to avoid the development of multidrug-resistant strains of *M. tuberculosis* [3]. A greater understanding of biology and immunology of this pathogen are high priorities of tuberculosis research for developing new strategies to combat *M. tuberculosis*.

Mycobacterium tuberculosis, along with other pathogenic mycobacteria, is a very active reducer of nitrogen sources both under aerobic and hypoxia induced Wayne model as well as *ex-vivo* macrophage infection model [4]. Nitric oxide (NO) and reactive oxygen species (ROS) which are produced inside host macrophages to kill the pathogen could be converted to nitrate by either enzymatic or non-enzymatic processes [5]. The nitrate thus formed, could act as source of nitrogen which is converted to nitrite by membrane bound nitrate reductase [6]. The nitrite is further reduced to ammonia by NADH type nitrite reductase, the only known pathway present in mycobacterial for the assimilation of nitrate in the medium.

Nitrite reductase (Nir) is an important enzyme because of the presence of single pathway for the utilization of nitrite accumulated during nitrate respiration as well as alternate ATP generator in absence of both oxygen and nitrate in the medium [7]. Recently it has been shown that NirB gene expression increased during the anaerobic condition in *E. coli* suggesting an important role

played by the gene product in mycobacterium under the condition mentioned. Unfortunately, there is no three dimensional structure related information about mycobacterium NirB available to get an insight about the enzyme which could help in developing novel drugs against tuberculosis.

The genome sequence of *M. smegmatis* is cracked in 2000 and immediately after that research shifted to post genomic era where analysis of different functional genes present in organism, genome functional activity and pathways analysis started in full swing [8]. Post genomic studies have helped in better understanding the physiological state of pathogen and suggested a number of potential drug target. Nitrate reductase and Glutamine synthatase are two important enzyme of nitrogen metabolic pathway which are among the suggested drug target against mycobacteria [9, 10]. Since we observed the increased expression of nitrite reductase during anaerobic and ex vivo macrophages model (Chapter-2), we are suggesting nitrite reductase also as potential drug target. The gene information of NirB from *M. smegmatis* available in KEGG data bank [http://www.genome.jp/kegg/genes.html] shows that the protein is a heteromer of two subunits, larger subunit has 859 amino acid long having molecular weight of ~91.5 kDa suggest that this domain might be playing crucial and important role in nitrite metabolizing activity as the functional catalytic domain while smaller subunit is made up of 122 amino acid having molecular weight of ~13 kDa. NADH dependant NirB is cytoplasmic enzyme reduces nitrite into ammonia requiring 8 electrons supplied from NADH act as electron donor.

The purification and properties of Nitrite reductase (NirB) from *Escherichia coli* has been the subject of extensive investigation, but nothing is known about its counterpart in *M. smegmatis* [11]. Though the enzyme present in the *M. smegmatis* was observed more than 80% homologous

to NirB of *E. coli*, yet this genetic difference could bring a substantial divergence to the structure as well as function of the enzyme [12]. It was therefore of interest to isolate, purify and characterize soluble form NirB from *M. smegmatis* in order to understand the basic role of this enzyme during anaerobic dormant stage.

In part A of this chapter, we described the cloning and expression of NirB gene from *M. smegmatis* and its partial characterizations.

4A.2. Results and Discussion

The rise in worldwide incidence of tuberculosis and emergence of multidrug-resistant strains of mycobacteria have emphasized the need for better understanding of the biology of the organism, and develop new strategies for combating the disease through the finding of novel drug targets [2]. Nitrite reductase expression increases both at gene as well protein level during hypoxia induced Wayne model and macrophage infection model system which indicates that this gene can be potential drug target. For in depth study, we cloned and over expressed NirB and purified in larger quantity which can be helpful in the characterization of proteins as well as development of NirB dependant assay which leads to screening of different compounds which can act as novel inhibitor against mycobactrial NirB.

4A.2.1. Cloning and expression of NirB

Open reading frame (ORF) of NirB was cloned successfully in pET 102-d topo vector and cloned plasmid was transformed into *E. coli* BL-21 star cells. The cloned plasmid clearly shows the gel shift as compared to 'no' insert plasmid (Fig.4A.1).

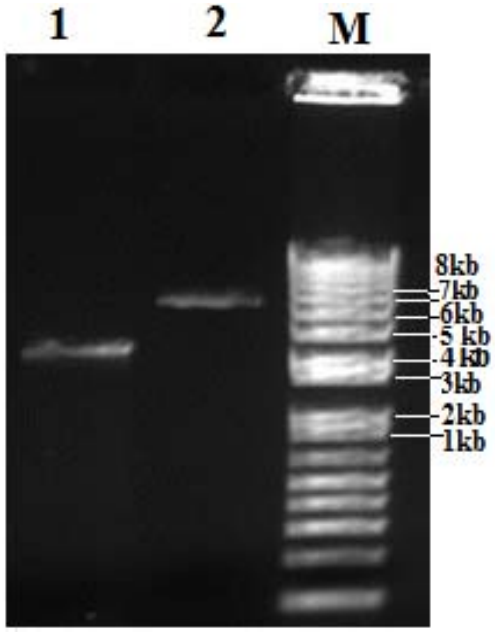
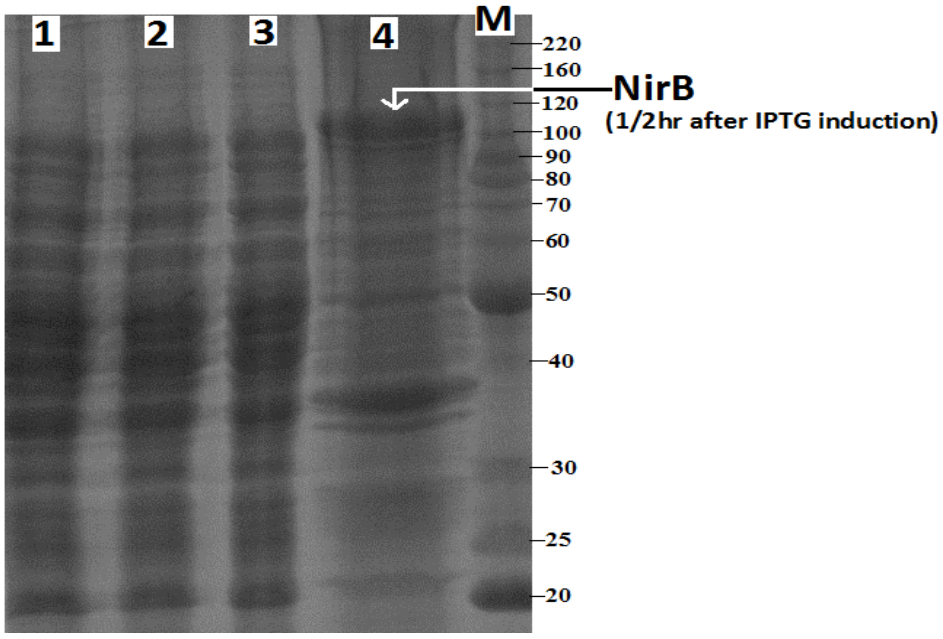


Figure 4A.1: Gel image of plasmid showing the clear shift in which NirB inserted (+)ve. Lane 1 showing (-)ve insert while lane 2 showing (+)ve and M: Marker.

Further the cloned gene was expressed by inducing with IPTG (100 mM). After induction, cell extract was prepared by lysis under non denaturing conditions and Nir activity was determined. The expression of cloned protein was increased by more than 15-fold after IPTG induction as compared to non-induced protein. The cell lysate was further separated as soluble and pellet fraction after centrifugation at high speed and again Nir activity was done. We observed the NirB activity mostly in pellet fraction which is more than 10-fold higher as compared to soluble fraction. The protein profile of un-induced and induced, pellet and supernatant was shown in SDS PAGE (Fig. 4A.2). The gel image clearly shows an over-expressed protein band in induced pellet lane and induced protein corresponds to ~105 kD molecular weight when compared with marker, which shows the nitrite reductase NirB subunit (90.1 kD) along with 16 kD tag on C-terminal of vector. Further purification of NirB is under progress in our lab.



SDS protein profile of Nitrite reductase (NirB) protein expression. Lane 1: Un-induced Supertant fraction, Lane 2: Un-induced Pallet fraction, 3: Induced Supernatant fraction, 4: Induced Pallet fraction & M shows protein marker (Invitrogen).

For maximum induction of NirB expression, recombinant E. coli cells were induced with IPTG (100 mM) for different time interval and we observed that maximum expression of NirB protein was after 2 hr of induction with IPTG, which was further used for characterization purpose.

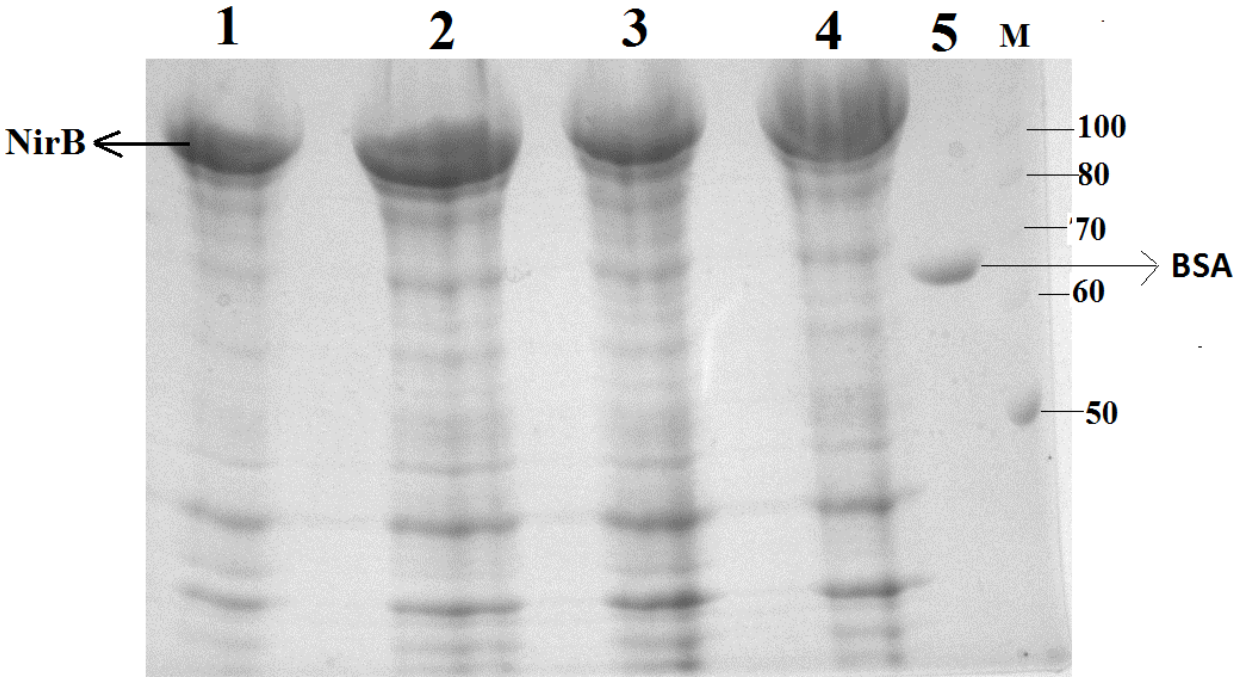


Figure 4A.2: Protein expression profile of NirB after induction with IPTG for different time period. Lane 1, 2, 3, 4 represents the pallet resuspension of IPTG induced culture for 1 hrs, 2hrs, 3hrs and 4hrs repectively. Lane 5 shows standard protein BSA while M shows the protein marker (BenchMark Protein Ladder, Invitrogen).

4A.2.4. Partial characterization of enzyme

1. Molecular weight determination:

Molecular weight of nitrite reducatse was determined on SDS-PAGE gel by comparision of rate of migration with protein of known molecular weight marker and indicated the value of ~90 kDa. The obtained molecular weight is good agreement with the molecular weight calculated from

the amino acid sequence of *M. smegmatis* nitrite reducatse available in KEGG pathways (excession no.MSMEG_4527).

2. Incubation temparature of nitrite reducatse assay

The incubation temparature of nitrite reducatse assay was standarlized by using partially purified enzyme and shows that at 45^{o}C temparature, the activity of enzyme was maximum, when all the parameters were fixed.

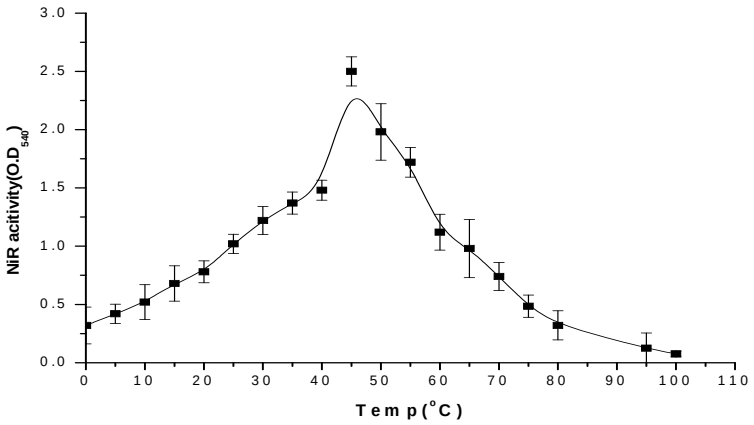


Figure3: Effect of different incubation temperature on nitrite reductase assay.The enzyme assay containing 1.5 ml of Potassium phosphate buffer (10mM, pH6.5), 200 ul of 0.05% methyle viologen, 100 ul of enzyme solution,10ul of sodium nitrite(10mM), 100 ul of 0.08% sodium dithionite dissolved in 0.08% sodium bbicarbonate and incubated for 30 min at 45 ^{o}C.

3. Incubation time for enzyme assay

The standarizaration of incubation time was done using enzyme activity kinetices for different time period. The result shows that the maxuimun activity of enzyme after 30 minutes of incubation of assay mixture.

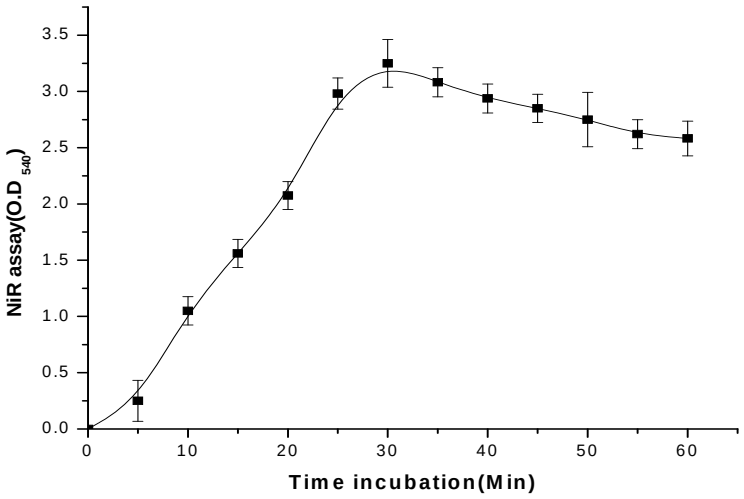


Figure 4: Time dependant curve of incubation of Nir assay

4. Optimum pH of nitrite reductase assay

To know the stability of enzyme activity at particular pH, different pH of potassium phoshate buffer was tested and observed that the enzyme activity of Nir was best at pH 6.5.

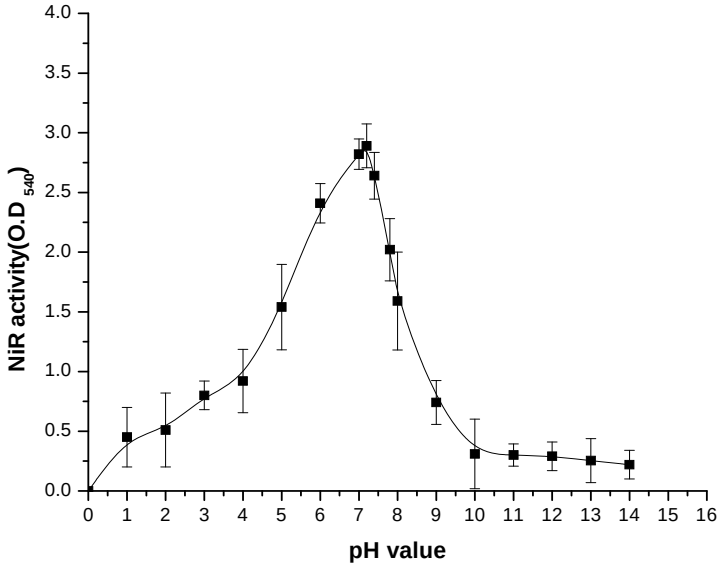


Figure 5: Effect of pH on nitrite reductase assay

5. Determination of optimum concentration of potassium phosphate buffer

Optimum concentration of potassium phosphate buffer was determined by taking the different concentration of buffer and nitrite reductase was checked. It was observed that at 10 mM concentration of potassium phosphate buffer, activity of enzyme was maximum.

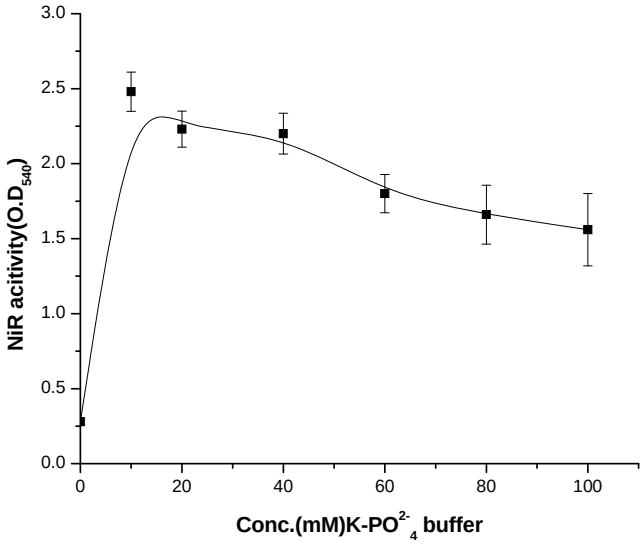


Figure 6: Effect of different concentration of potassium phosphate buffer on Nir assay

6. Temparature stability of nitrite reductase enzyme

To know the thermal stabilty of nitrite reductase, different temparature shock was given to enzyme for 1 min. and then enzyme activity was checked. It was observed that enzyme activity was stable at 80^{o}C and after that its activity started decling down but never lost completely even at 100^{o}C.

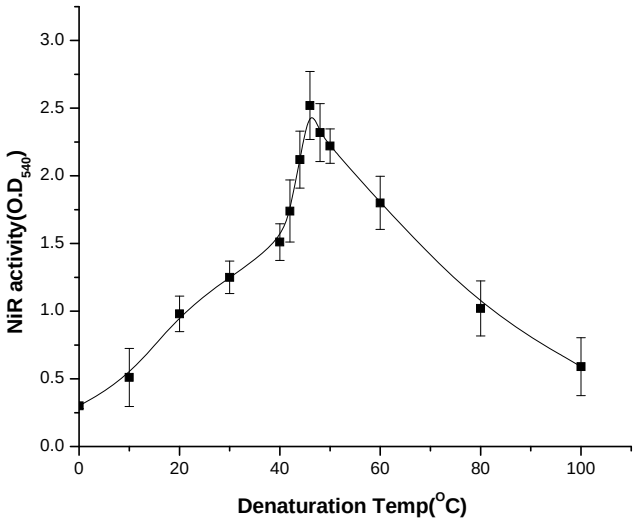


Figure 7: Graph showing the heat stability on Nir enzyme

8. Effect of metals ions on enzyme activity

Effect of different metals ions were tested on the nitrite reductase activity at final concentration of 50µM in enzyme assay mixture. It shows that ferric ion (Fe^{2+}) inhanced the activity by 3-times as compared with control which indicats that nitrite reductase can aslo accept the electron from ferrous ions for its activity apart from NADH.

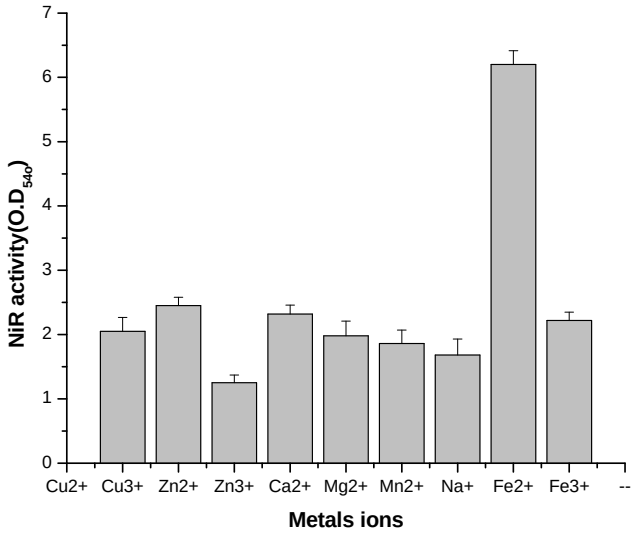


Figure 8: Bar graph showing the effect of different metal ions on nitrite reductase assay

The Stability of the enzyme was first examined in various buffers, and other stabilizing agents to determine its native environment and optimum preservation conditions. Among different buffers, it was observed to be most stable in 10 mM potassium phosphate buffer (pH 7.2). Nitrite reductase activity was optimal at pH 7.2 (Fig. 5.6). It maintained more than 50% of the optimal activity from pH 5 to 8 and still detectable activity in the range of 2-10. This indicated the enzyme has ability to adapt to a wide range of pH and functioning in environment at different physiological pHs. The optimal temperature of the enzyme was observed to be 45^{o}C and more than 50% of it was retained in the range of 20-60^{o}C (Fig. 5.7). Activity was almost completely lost at below 10^{o}C and above 70^{o}C. In case of *E. coli* nitrite reductase, optimum temperature was reported to be 23^{o}C (21). This indicated that the extreme temperature adaptability and thermo tolerant nature of *M. tuberculosis* nitrite reductase. Different metal ions were examined thereafter to determine their effect on nitrite reductase activity. There was significant effect seen of Fe^{2+}

metal ions on nitrite reductase activity. This may be because of ferrous ion can be electon donar which is required for reduction of nitrite to ammonia and specifically ferrous ion because of presence of iron-sulfure clusture present in nitrite reductase which leads to inhancement of reaction. It has been seen the effect of ferrous ion inhances the enzyme activity by more than ~3-fold.

Altogether, this work and the subsequent characterization studies should contribute to the understanding of the important role played by nitrite reductase during nitrogen metabolism during active growth as well as in latency and allow us to explore the development of new anti-mycobacterial agents that target nitrite reductase during latency in *M. tuberculosis*

4A.3. Materials and Methods

4A.3.1. Bacterial strains, chemicals and media

Mycobacterium smegmatis mc^2 155 was obtained from Astra Zeneca, Bangalore India. All the chemicals were purchased from Sigma, USA unless mentioned. A defined medium containing 0.5 gm Potassium dihydrogen ortho phosphate, 0.25 gm Sodium citrate, 60 mg Magnesium sulphate, 10 mM asparagines and 2 ml glycerol in 100 ml of distilled water at pH 6.6±0.2 was used throughout the study [13]. The stock cultures were maintained at -70°C and sub cultured once in liquid medium before inoculation to an experimental culture.

4A.3.2. Cultivation of aerobic M. smegmatis and E. coli

For aerobic cultivation, The bacterial cultures were grown in 20 ml defined medium in 100 ml flask under aerobic conditions in a shaker incubator (Thermo Electron Corporation Model 481) maintained at 150 rpm and 37^{o}C till logarithmic phase ($O.D._{620}$ ~ 1.0) was reached.

E. coli cells were grown in sterilized LB (Luria-Bertani) broth medium. The cells were kept in sterile conical flask shaking with a speed of 200rpm at 37^{o}C under aerobic condition in a shaker incubator till logarithmic phase ($O.D._{600}$ ~ 0.6) was reached.

4A.3.3. Genomic DNA isolation from M. smegmatis

M. smegmatis was used for isolation of genomic DNA (g-DNA) using standard protocol [14]. Briefly, 2ml of culture (O.D~1.0) was centrifuged at 10,000 rpm at room temperature for 5 min to pellet down the cells. After discarding the medium, cells were resuspended in 550 µl of Solution A (2 mg/ml of Lysozyme in Tris EDTA buffer, 10 mM, pH 8.0) and vigorously mixed before incubation for 1 hr in a water bath maintaied at 37^{o}C. After incubation, 70 µl of

Solution B was added (10 mg/ml proteinase K in 10% of SDS solution made in DW) and mixed properly before heating at 60^{o}C for 10 mins. After heating, 80 µl of 5M NaCl was added in cell suspension and mixed by shaking it. 100 µl of CTAB+NaCL (10% CTAB dissolved in 5M NaCl solution) was added and again mixed it vigorously and heated again at 60^{o}C for 10 mins. After incubation, 500 µl chloroform and isoamyle mixure (24:1) was added and mixed gently 4-5times and then centrifuged at 10,000 rpm for 10 mins at room temperature. Upper clear phase was taken out without disturbing or touching middle layer into another tube. 500 µl of isopropanol was added in it and again mixed it very gently up and down for 4-5 times and genomic DNA can be visualized as woolen treads in mixture. The mixture sample was kept for incubation at -20^{o}C for 1 hr.

After incubation, sample was centrifuged at 12,000 rpm for 15 min at room temperature which will lead to precipitation of g-DNA as pellet. The pellet was washed with 70% ethanol and air dried for 5 min. After proper drying, g-DNA was resuspended in 50 µl TE buffer (10 mM, pH 8.0) and stored at 4^{o}C.

4A.3.4. Primer designing

Primers were designed using the gene sequence data available in KEGG pathway for *M. smegmatis* nitrite reductase (MSMEG_277) using ITDNA primer designing software. For full length amplification of NirB subunit of nitrite reductase, CACC was added before initiation codon in forward primer while stop codon was removed from the reverse primer.

Forward Primer Sequence: 5'-CACCATGCAGTCAACGAGAAACGTCGT-3'

Reverse primer Sequence: 5'-TGACATCTCCTGTAGTGGCCGGATCGT-3'

The designed primers were checked for annealing temperature, GC content, self ligation, and hairpin formation etc with the help of ITDNA software and optimized the parameters for amplification.

Volume	**Reagents**	**Final Conc.**
5 µl	10X Buffer for LA DNA pol.	1X
2.5 µl	Deoxy Ribonucleotide	200 µm
1 µl	Forward Primer	0.5 µm
1 µl	Reverse Primer	0.5 µm
1 µl	LA DNA Pol.	0.1 unit/µl
2.5 µl	DNA template	500 pg/µl
37 µl	PCR grade Water	
Total= 50 µl	PCR reaction mixture	

Table 4.1: The components used for PCR reaction mixture

Steps		**Temp.**	**Time**
1.	Initial Denaturation	95^{o}C	2min
2.	Denaturation	95^{o}C	30sec
3.	Annealing	66^{o}C	30sec
4.	Extension	68^{o}C	2.5min
5.	Repeat step 2-4 for 30 cycles		
6.	Final Extension	68^{o}C	10min
	Hold	4^{o}C	Overnight

Table 4.2: PCR parameter used for amplification of NirB genes

4A.3.6. Preparation of Ligation mixture

The amplified PCR product was gel eluted after cutting the appropriate band from agarose gel using Gel-Elute PCR Kit (Sigma) following the instructions mentioned in manual. The PCR product was quantified using nano-drop spectrophotometer. The ligation mixture consist of cloning vector, salt and PCR product as follows

Reagents*	Chemically Competent *E. coli*	Electrocompetent *E. coli*
Fresh PCR product	0.5 to 4 µl	0.5 to 4 µl
Salt Solution	1 µl	--
Dilute Salt Solution (1:4)	--	1 µl
Sterile Water	add to a final volume of 5 µl	add to a final volume of 5 µl
TOPO® vector	1 µl	1 µl
Total Volume	**6 µl**	**6 µl**

*Store all reagents at -20°C when finished. Salt solution and water can be stored at room temperature or +4°C.

After mixing all the components, ligation mixture was kept in water bath at room temperature for 30 min.

4A.3.7. Transformation into E. coli

For transformation, 6 µl of ligation mixture was added to 500 µl of competent *E. coli* cells (TOP10, provided in kit) and kept on ice for 5 min. After incubation, cells were given heat shock at 42^{o}C for 30-45 sec in water bath and immediately transferred in ice for 5 min.

After incubations, 200 µl of fresh SOC medium (provided in kit) was added to transformed cells and incubated in shaker incubator with speed of 150 rpm for 1 hr at 37^{o}C. After 1 hr of

incubation, cells were spread on preformed 1.5% agar plate with ampicillin (100 µg/ml) under aseptic condition and incubated at 37^{o}C for overnight.

4A.3.8. Plasmid isolation from transformed E. coli cells

For plasmid isolation from the transformed *E. coli* cells, individual colony on agar plate was inoculated in 10 ml LB medium containing 100 µg/ml of ampicilin and grown up to O.D$_{600}$~ 0.6. The cells were then centrifuged at 10,000 rpm for 5 min at room temperature. After centrifugation, the cell pellet was processed for plasmid isolation using plasmid extraction kit (Sigma) following instruction provided in manual. The plasmid was then qualitative analyzed on 1% agarose gel and quantified on nano-drop spectrophotometer.

4A.3.9. E. coli cell lysis

The cell culture was centrifuged at 10,000 rpm for 5min at room temperature. After discarding the medium, the lysis buffer (50 mM potassium phosphate, pH 7.8, 400 mM NaCl, 100 mM, KCl, 10% glycerol, 0.5% Triton X-100, 10 mM imidazole) was added and mixed properly with the cell pellet. The cell suspension in lysis buffer was sonincated by applying 80 kz for 30 sec for 3 cycles on ice. The lysed cells were then centrifuged at 15,000 rpm for 30 min at 4^{o}C to separate soluble supernatant from membrane or un-dissolved portion as pellet. Both fractions were subjected for protein estimation, enzyme assay or SDS PAGE.

4A.3.10. Analysis of protein by SDS-PAGE

The expression profile of cloned NirB was monitored by using both supernatant as well as pellet fractions as protein samples in SDS-PAGE (sodium dodecyl sulfate-Poly acryl amide gel electrophoresis). Briefly, protein samples were first mixed with 1X loading buffer containing 5%

β-meceptoethanol (Sigma). The samples were then heated for 10 min at 80^{o}C. 30 µl of each sample was then loaded onto 12.5% acrylamide-bis-acryleamide in Tris-buffer precast polyacrylamide gel and electrophoresis was carried out using mini-cell system (Amersham, USA). After electrophoresis gel was subjected to Comassie blue staining for overnight. Protein bands seen on the gel after destaining with DW: Ethanol: Acetic acid in 45:45:10 ratio.

4A.3.11. Nitrite reductase enzyme assay

Methyl Viologen nitrite reductase activity was assayed by the method of Ida et al., [15]. The reaction mixture in a final volume of 1 ml contained: 75 mM Tris-HCl (pH 8.0), 2 mM $NaNO_2$, 1.5 mM Methyl Viologen, 20 mM potassium dithionite (freshly dissolved in 0.29 M $NaHCO_3$) and an appropriate amount of the enzyme. The reaction was carried out for 5 min at 37°C and stopped by vigorous shaking to obtain complete oxidation of the excess reductant. Nitrite reduction was determined after a 100-fold dilution of the reaction mixture by the diazo coupling method [16]. The concentration of nitrite utilized by the enzyme in reaction sample was calculated from the standard graph obtained using different dilution concentration of nitrite.

4A.3.12. Protein estimation

Protein quantification was carried out by following Bradford method using BSA as standard protein [17]. The protein concentrations of samples were determined from standard graph.

4A.4. References

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CHAPTER FOUR

B) Spheroplast method for total RNA Isolation from Mycobacteria

4B.1. Introduction

Mycobacterium tuberculosis is one of the most successful human pathogens that cause tuberculosis, resulting in 2 million deaths and 9 million new cases annually [1]. More than one-third of human population carries this pathogen in the latent form due to its successful survival in the host system, which can re-activate at any time, particularly when the host immune system is being compromised [2]. Very little is known about the conditions prevailing in latent phase of bacilli or within infected macrophages. Transcript analysis at different phases of its survival within host system could become extremely helpful to understand its pathogenesis and latency [3]. Considering the unstable nature of RNA, efficient isolation of total RNA from the bacilli is very crucial for transcript analysis. Tough cell wall structure with thick lipid coating makes the lysis difficult for RNA isolation from mycobacteria [4]. The different methodology so far reported for mycobacteria are based on enzymatic lysis of cell wall [5], mechanical disruption by French pressure [6] or bead beater [7] and nitrogen decompression techniques [8]. Most of these methods are either time consuming or use harsh treatments which inadverantly lead to degradation of RNA and finally affecting its yield. A few of these methods even produce aerosol droplets during cell disruption of pathogenic *M. tuberculosis* making the surrounding environment contagious [9].

In chapter 4 (Part B), we report a very simple and high yielding technique of total RNA isolation from mycobacteria by using spheroplast method that makes the cell wall of bacilli weak by inhibiting cell-wall synthesis. Our proposed method is also observed to be equally advantageous in extracting RNA from bacilli grown in infection model without any cross contamination from host macrophages.

4B.2. Results and discussion

4B.2.1 Isolation of total RNA from aerobic and hypoxia-induced dormant mycobacterium cultures

Apart from the short half life of mRNA due to its inherent fragile nature, cell lysis remained a major bottleneck for successful RNA isolation from different microorganisms [10]. This problem became more acute in case of mycobacteria due to its complex cell wall structure, composed of peptidoglycans, arabinogalactans and mycolic acids along with other complex polysaccharides which make the bacilli very tough to break [6]. Earlier reported methods used frozen or normal cells for lysis by applying sophisticated and expensive instruments like French pressure cell, nitrogen decompression or bead beater followed by precipitation of RNA using kit or chloroform/isoamyl alcohol protocol [11]. To overcome these problems, we used spheroplast method that does not recommend freezing or lysis to get total RNA, hence making this method simple. Suggested method weakens the mycobacterial cell wall effectively and thus increasing its fragility. Therefore even applying mild sonication becomes sufficient to release the total RNA in the Trizol solution. This method is also cost effective in comparison to specific lysis technique using ionic/nonionic surfactant and RNA isolation kit, because spheroplast method requires smaller quantity of D-cycloserine (0.0006 %) and Lysozyme (0.002 %) for RNA extraction [12, 13].

In order to verify the robustness of spheroplast method, *M. tuberculosis, M. bovis BCG* and *M. smegmatis* were used after growing them under different conditions. Almost equal and distinct 23S and 16S rRNA bands along with light background in between representing mRNAs were observed in agarose gel from different samples (Fig. 4B.1).

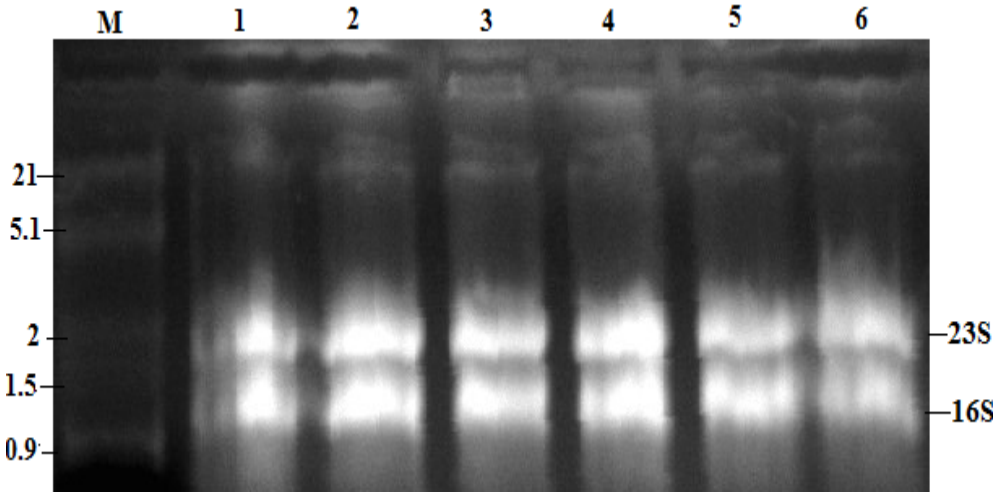

Figure 4B.1: Gel electrophoresis picture of total RNA isolated by spheroplast method from mycobacteria. 10 µg of total RNA from aerobic and hypoxia dormant culture were electrophoresed through a 1 % agarose - 0.66 M formaldehyde gel in MOPS running buffer (20 mM MOPS, 10 mM sodium acetate, 2 mM EDTA, pH 7.0). Lanes 1-3 & 4-6 representing RNA samples from aerobic and hypoxia dormant culture. Lane 1 & 4: M. smegmatis, lane 2 & 5: M. bovis BCG, lane 3 & 6: M. tuberculosis respectively. In Lane M, marker (Biolit Mid-range, 0.5 µg) was loaded.

The purity of RNA was judged from the ratio of $A_{260\ nm}$: $A_{280\ nm}$ that was within 1.9-2.0 indicating that the RNA preparation was free from any contamination. The yield of RNA obtained using spheroplast method was 50 µg/10^7 cells. This high yield could be useful in microarray analysis where 20-200 µg of RNA is required [14]. Our method can be helpful in carrying out real-time PCR, cDNA library preparation, Northern blotting and semi-quantitative PCR.

To further evaluate the quality and intactness of isolated RNA from bacilli grown under different conditions, RT-PCR was done for differentially expressed genes. We have used specific primer of respiratory type nitrate reductase (narG) for in-vitro culture study. The size of the RT-PCR products obtained (>950bp) proved the presence of un-degraded narG transcripts in the RNA preparations (Fig. 4B.2). Sequence and amplification parameters of narG are given in table 1.

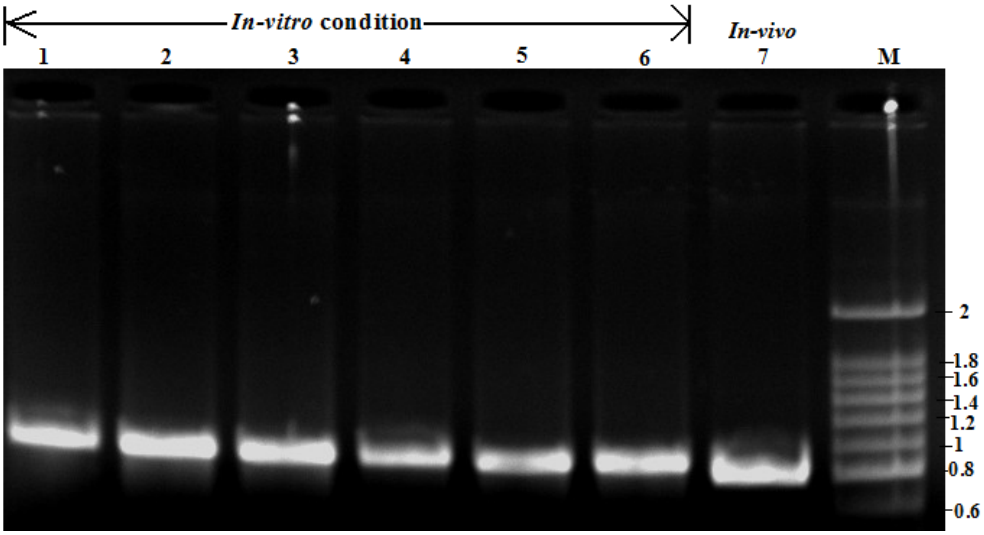

Figure 4B.2: PCR amplification of nitrate reductase (narG) and Rv3804 gene from Mycobacteria grown under different conditions. Lane 1-3 & lane 4-6 shows RT-PCR product (NarG) from aerobic and hypoxia grown culture while lane 7 shows amplification product (Rv3804) of M. tuberculosis from infection model. Lane 1 & 4 M. smegmatis, Lane 2 & 5 M. bovis BCG, Lane 3 & 6 M. tuberculsis while M shows the marker (Hi-media 200bp, 0.5 µg).

Observed effect on bacilli was mainly because of Lysozyme and D-cycloserine present in spheroplast solution. Lysozyme acts on the glycosidic bond between peptidoglycans on bacterial cell wall and make it weak while D-cycloserine blocks the cell wall formation by

inhibiting D-alanine racemase and D-alanyle-D-alanine synthatase [15, 16]. Effect of the spheroplast solution on the viability of bacilli or its generation time was determined by colony forming unit (CFU) and optical density of *M. tuberculosis* from aerobic culture, hypoxia-induced dormant and infection models. The CFU count and doubling time indicated no major adverse effect of spheroplast solution on the bacilli (data not shown). Altogether, spheroplast solution made the tough and thick cell wall complex of mycobacterium significantly weak as a result of which it's cell shape changed from rod like structure to a spherical one [17].

4B.2.2. Isolation of total RNA from intracellular bacilli (Infection model)

It was observed that spheroplast method was equally effective in isolating total RNA from bacilli residing within macrophages. We compared the yield of mycobacterial RNA from infection model using our method with the recently published mechanical cell disruption method [13]. The spheroplast method is showing 50 times higher yield with sharp and intact 23S and 16S rRNA band without any contamination of macrophage RNA which was further confirmed by RT-PCR. Total RNA isolated from both host macrophages and intracellular bacilli were reverse transcribed using random primer and the resulted cDNA was separately used for the amplification of both 16S and actin genes. The result of RT-PCR indicated that there was only 16S rRNA gene amplification product seen in PCR sample when mycobacterial cDNA was used as template while actin gene amplification product was obtained only from macrophage cDNA (Fig. 4B.3).

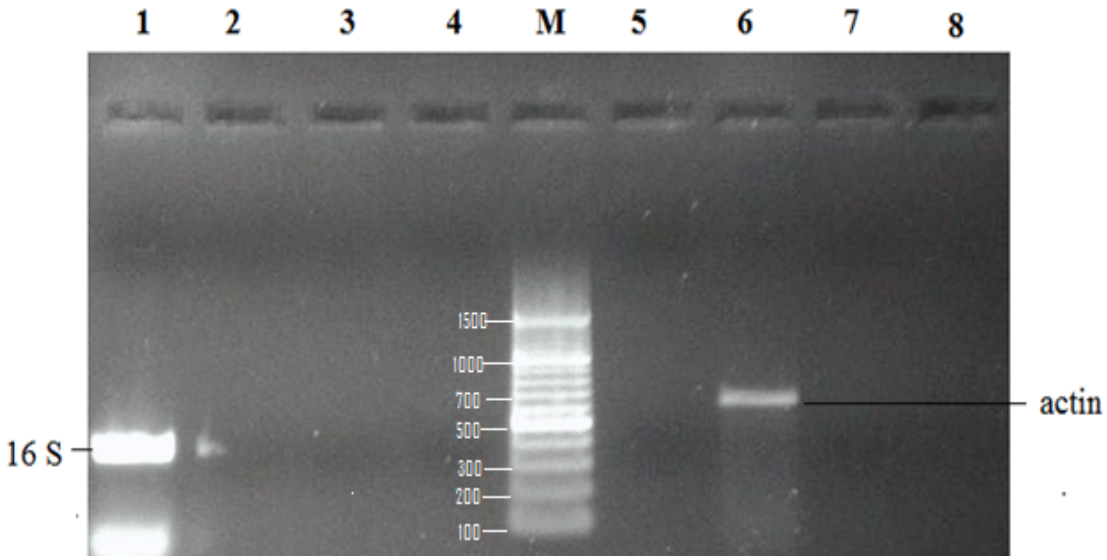

Figure 4B.3: RT-PCR analysis of 16 S and actin gene amplification product from RNA isolated from infection model. 20 ng of PCR products were loaded on 1 % agarose gel. Lane 1 and 6: 16 S and actin from bacilli and macrophages; 3, 4 and 7, 8: reverse transcriptase minus (negative control) and Taq polymerase minus (negative control) 2 and 5: 16 S and actin gene amplification using cDNA template of macrophage and bacilli respectively. M shows the marker (Fermentas 100bp, 0.5 µg).

To further show the intactness of mycobacterial RNA, RT-PCR was done for Rv3804 gene using primer to amplify longer region. Amplification product of 964bp showed the un-degraded transcript in RNA sample obtained from infected mycobacteria (Fig. 4B.2). These results clearly showed that the spheroplast method is equally successful in isolating total RNA from intracellular mycobacteria residing in macrophages.

Further, to evaluate any non-specific effect of spheroplast solution on infected macrophages, fluorescence microscopic study was done using DAPI dye. The results indicated that the integrity of nuclear structure was maintained similar to that of control cells (without

spheroplast treatment), depicting the healthy state of the host cells which further proved that the components of spheroplast solution has specific effect on mycobacterium and not on the macrophages (Fig. 4B.4).

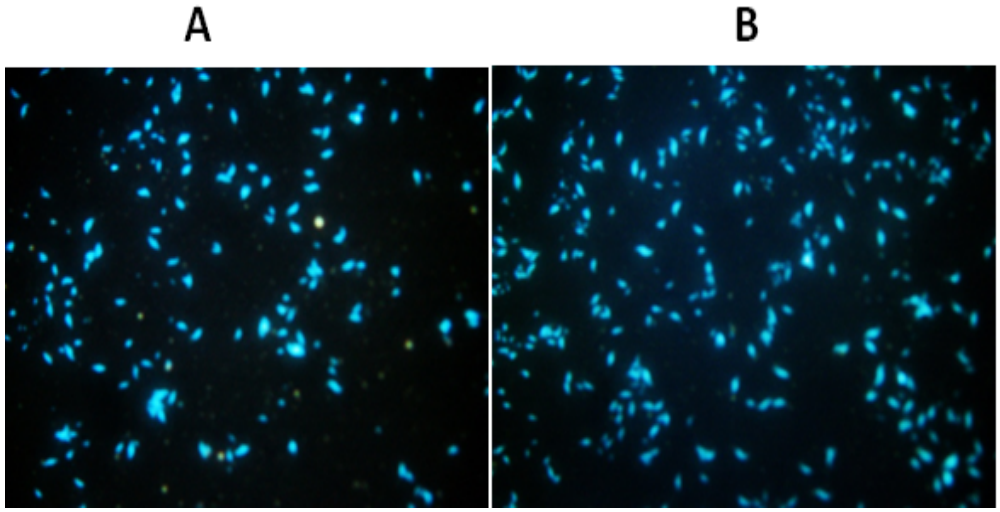

Figure 7: Fluorescence microscopic images of (A) spheroplast solution treated and (B) control macrophages (10^5 cells) at 350 nm excitation and 450 nm emission wavelengths

Since the reported method does not produced fumes or aerosol and droplets during cell lysis, this method could be safely used for other group 3 hazardous organisms like virulent form of Pseudomonas, Rickettsea and Salmonella. Apart from its convenience and ease in carrying out experiments under sterile conditions within biosafety cabinet or in sealed tubes, this method is also useful for DNA and protein purification in large quantity without using any sophisticated technique and expensive equipments [18,19]. Altogether, this high yielding spheroplast method provided an efficient, convenient and cost effective approach to safely isolate total RNA from very tough and potentially hazardous cultures without compromising its reproducibility and quality. This suggested method can be a very useful tool to carry out transcript analysis studies for better understanding of host pathogen interactions

Very recent studies showed that phenotypically different forms of *M. tuberculosis* that existed as spheroplasts were isolated from the patients suffering from pulmonary and extra pulmonary tuberculosis as well as from the organs of guinea pig three weeks after intranasal infection [20, 21]. Successful inter-conversion of spheroplast forms could play an important role in survival of bacilli inside macrophages and its reactivation that leads to chronic infections [22, 23]. .

4B.3. Materials and Methods

4B.3.1. Isolation of RNA from Mycobacterial strain grown in vitro condition

M. tuberculosis H37Ra (ATCC 25177), *M. bovis* BCG (ATCC 35745) and *M. smegmatis* mc^2 177 were aerobically grown up to the late logarithmic phase ($1X10^5$ CFU/ml, $O.D_{620}$~1.0) in a defined medium containing nitrate as nitrogen source [24]. Grown mycobacterial cells were converted to spheroplast using spheroplast solution as described earlier [17]. Briefly, spheroplast solution consisting Lysozyme (0.002 % w/v in DW), D-cycloserine (0.0006 % w/v in DW), Glycine (1.4 % w/v in DW), EDTA (0.2 % w/v in DW) and Lithium chloride (0.1 % w/v in DW) was added aseptically at the respective final concentrations in the growing culture and incubated for an hour. For hypoxia induced non-replicating dormant stage, mycobacterium cells were grown in 0.5 HSR model and the spheroplast solution was added aseptically at NRP-1 stage through a 0.5 gauge syringe and incubated for an hour before harvesting the cells [25].

For harvesting aerobic bacilli, late logarithmic phase (10^7 CFU/ml) culture was centrifuged at 15,000 rpm at 4 °C for 10 min. Cell pellet was resuspended in Trizol reagent and was a given mild sonication in water bath sonicator at 50 kHz for 1 min. using an ice box. Resulted suspension was immediately processed for total RNA isolation by precipitating with chloroform/isoamyl alcohol mixture [26]. Briefly, 200 µl of chloroform was added in suspension obtained after mild sonication and vigorously shaked for 15 seconds. The suspension was then centrifuged at 15,000 rpm at 4 °C for 10 min. resulting in separation of nucleic acids and proteins in upper and lower liquid phases respectively. The upper phase was carefully pipette out in separate tube and 500 µl of isopropanol was mixed by inverting the tube 4-5 times. After incubation for 10 min., the suspension was centrifuged at 20,000 rpm at

4 °C for 15 min. The pellet obtained, was washed with 70% cold ethanol and then air dried for 5 min. The dried RNA pellet was dissolved in 50 µl DEPC treated water which was quantified using nano-drop spectrophotometer (Thermo scientific model no. V 3.7) and further analyzed on 1% denature agarose gel.

For hypoxia induced dormant culture, cells at NRP-1 stage (10^4 CFU/ml) from 0.5 HSR Wayne model were used for total RNA isolation. Wayne tube culture was centrifuged at 15,000 rpm at 4 °C for 3 min to obtain the cell pellet which was resuspended in Trizol reagent and RNA was extracted using the chloroform/isoamyl alcohol as described above.

4B.3.2. RNA isolation from infected macrophages

THP-1 cells (10^5 cells/ml) were suspended in MEM medium containing 10 % FBS in a 75 cm^2 culture flask and incubated at 37 °C for a day. Phorbol myristate acetate (20 nM) was added to THP-1 culture and incubated for 12 hrs. to convert monocytes into macrophages. Macrophages were infected with *M. tuberculosis* (10^7 CFU/ml) at MOI (multiplicity of infection) of 1:100. Then macrophages were washed 12 hours post infection (PI) with Phosphate Buffer Saline (PBS) and incubated in the CO_2 incubator at 37 °C and 5 % CO_2 [27]. Spheroplast solution was added to MEM medium on 5^{th} day of PI and incubated overnight. After incubation, cells were washed twice with PBS and then Trizol reagent was added which was kept for 15 min. at room temperature. Following incubation, Trizol solution was pipetted out in separate tube and was centrifuged at 15,000 rpm at 4 °C for 5 min. to separate the bacterial cells as pellet while supernatant containing RNA from macrophages. Collected supernatant was processed for isolation of RNA from macrophages using

chloroform/isoamyl alcohol precipitation method. The pellet was resuspended in Trizol reagent and processed for mycobacterium RNA as mention earlier.

4B.3.3. Reverse transcriptase (RT)-PCR

For cDNA synthesis, 1 μg of total RNA from mycobacteria and THP-1 macrophage were treated with DNase-I (Sigma) and then incubated at 70 °C according to manufacturer's instruction. 250-300 ng of DNase-I treated total RNA was used for cDNA synthesis using random primer and enhanced avian reverse transcriptase provided in "first strand cDNA synthesis kit" (Sigma) at 25 °C for 10 min. followed by incubation at 45 °C for 50 min. The resulted cDNA was used as a template for PCR amplification. The PCR was carried out as per manufacturer's instruction by using Taq DNA polymerase provided in "PCR core kit" (Sigma) in a total reaction volume of 50 μl. Primers used for the RT-PCR study and its amplification conditions are mentioned separately in the Table 1. Amplification product of RT-PCR was first checked on 1 % agarose gel and then after sequencing, it was analyzed by using BLAST software in the NCBI database.

Table 1: Sequences of primers used for RT-PCR in mycobacteria and macrophages with its amplification parameters.

Genes	Primer sequences*	Amplification product (bp)	PCR parameter# (Annealing temp.)
16S	F 5'ATGCATGTCTTGTGGTGGAAAGCG3' R 5'TTCACGAACAACGCGACAAACCA3'	371	58 °C
Actin	F 5'ATGGATGACGATATCGCT3' R 5'ATGAGGTAGTCTGTCAGGT3'	700	60 °C
narG(M.smeg.)	F 5'CAACTGGAATCTGGACCTGGAGAA3' R 5'TCGATCGACATCAGCAGATCAAGC3'	1059	61 °C
narG(M. tb / M.bovis BCG)	F 5'AAGACGGGATCATCACCTGGGAAA3' R5'TTGACAGCCTTGTCCATCTCCACA3'	1069	63 °C
Rv3804	F 5'ATGCAGCTTGTTGACAGG3' R 5'TGGCACCCAGTGCCCGTT3'	964	61 °C

* *Primers were designed using the IDTDNA primer designer software*.

\# *Initial denaturation of 95℃ for 2 min. followed by 30 cycles of 94℃ (30 sec.), respective annealing temp. (1 min.), 72℃ (1 min.) and final extension of 10 min. at 72℃ were used for amplification of mentioned genes*

4B.3.4. Fluorescence microscopic study

THP-1 cells were grown on cover slip in 8 well microplates and then washed twice with chilled methanol followed by staining with 4', 6-diamidino-2-phenylindole (DAPI) at final concentration of 300 nM [28]. Macrophage cells were kept for 15 min. at room temperature under dark condition. After incubation, cells were washed three times with distilled water and analyzed under the fluorescence microscope (Leitz Wetzlar model, Germany) at 350 nm excitation and 450 nm emission wavelengths (400 X) using 'I_3' filter.

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Appendix-I

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Notes & Tips

A method to extract intact and pure RNA from mycobacteria

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We describe a high-yielding, simple, and aerosol-free protocol for the isolation of RNA from mycobacteria that does not require sophisticated instruments. The method yielded 50 lg of RNA from 10^7 cells, 50 times more than a recently reported method. Our method can extract total RNA from aerobically grown bacteria and from in vitro hypoxia-induced dormant bacilli and mycobacteria residing within infected macrophages.

Isolation of RNA is very tedious and time-consuming and often produces messenger RNA (mRNA)[1] of poor quality with low yield, especially in the case of bacteria whose mRNA has a very short half-life [1]. The isolation of RNA from mycobacteria is even more difficult because of the thick coating of lipids in the cell wall that make the bacilli resistant to lysis. Efficient lysis is very crucial for the successful isolation of RNA [2]. The methodologies currently available for the isolation of mycobacterial RNA are based on enzy-matic lysis of the cell wall [3], mechanical shearing by a French press [4], sonication, use of a bead beater [5], or nitrogen decompression techniques [6]. Enzymatic lysis is time-consuming and can lead to degradation of full-length transcripts. French presses and nitrogen decompression techniques require sophisticated and expensive instruments, and bead beaters and sonication often produce contam-inating fumes or aerosols and so should not be used for airborne organisms such as Mycobacterium tuberculosis [7]. Here we describe a simple, reproducible, and high-yielding method for the isolation of total RNA from mycobacteria by conversion into spheroplasts that are prone to breakage by mild sonication. This technique for the iso-lation of RNA, therefore, is faster and more efficient and does not re-quire sophisticated equipment for the lysis of cells.

A spheroplast solution consisting of lysozyme (0.002% [w/v] in distilled water [DW]), D-cycloserine (0.006% [w/v] in DW), glycine (1.4% [w/v] in DW), ethylenediaminetetraacetic acid (EDTA, 0.2% [w/v] in DW), and lithium chloride (0.1% [w/v] in DW) was asepti-cally added to late exponential phase bacteria grown aerobically in Mycobacterium phlei medium containing nitrate as the nitrogen source and incubated for 1 h [8]. Mycobacterial cells in a hypoxia-induced, nonreplicating dormant stage were grown in a 0.5-HSR (head space ratio) Wayne model where oxygen depletion leads to a gradual shift-down of bacilli into the NRP-1 (nonreplicat-ing persistent-1) stage [9], and then spheroplast solution was added aseptically to bacilli grown in M. phlei medium through a 0.5-ml syringe and incubated for 1 day. After the respective incu-bations, aerobically and hypoxiaally grown bacilli were harvested by centrifugation at 15,000 rpm for 10 min at 4 LC. The pellet was resuspended in Trizol reagent (Sigma) and then mildly sonicated in a water bath sonicator at 50 kHz for 1 min using an ice box. After 10 min of incubation at 37 LC, 200 ll of chloroform/isoamyl alcohol (24:1) was added to the Trizol solution and mixed vigorously for 15 s [10]. Cell debris was removed by centrifugation at 10,000 rpm for 10 min at 4 LC, and the upper aqueous phase was collected in a separate tube. Isopropanol (500 ll) was added to the aqueous phase, and the RNA was precipitated by centrifugation at 20,000 rpm for 15 min at 4 LC. The RNA pellet was washed twice with 70% ethanol, air-dried at 37 LC for 5 min, and then resus-pended in 50 ll of DEPC (diethyl pyrocarbonate) water. Qualitative and quantitative analyses were performed with agarose gel elec-trophoresis and a NanoDrop spectrophotometer. For an infection model, THP-1 cells were grown to 10^5 cells/ml in MEM (minimal essential medium) containing 10% FBS (fetal bovine serum) and then converted into macrophages using phorbol myristate acetate (20 nM) [11]. Macrophages were then infected with M. tuberculosis

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[1] Abbreviations used: mRNA, messenger RNA; DW, distilled water; EDTA, ethylene-diaminetetraacetic acid; PI, postinfection; PBS, phosphate-buffered saline; rRNA, ribosomal RNA; RT–PCR, reverse transcription polymerase chain reaction; cDNA, complementary DNA.

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(10^7 CFU/ml) at an MOI (multiplicity of infection) of 1:100. Macro-phages were washed 12 h postinfection (PI) with phosphate-buf-fered saline (PBS) to remove uninfected bacilli and were kept in a CO2 incubator at 37 LC and 5% CO_2 after the addition of fresh med-ium. Spheroplast solution was then added to the macrophage cul-ture on the 5th day PI and incubated overnight. After incubation, cells were washed twice with PBS, added to Trizol reagent, and then kept for 15 min at room temperature. The infected bacilli were pelleted by centrifugation at 10,000 rpm for 5 min at 4 LC with the macrophage RNA remaining in the supernatant. The supernatant was removed to another tube, and the macrophage RNA was precipitated using chloroform/isoamyl alcohol. The bac-terial pellet was again resuspended in Trizol reagent and processed for precipitation of mycobacterial RNA. The bacterial lysis and iso-lation of RNA was achieved within 1 h, allowing rapid and multiple isolations of RNA from different organisms or growth conditions.

Fig. 1 shows a photograph of an agarose gel containing total RNA from different mycobacterial species (M. tuberculosis H37Ra [ATCC 25177], Mycobacterium bovis BCG [ATCC 35745], and Myco-bacterium smegmatis mc^2 177) grown under aerobic and hypoxia conditions using the spheroplast method. The gel displays equal intensities of the 23S and 16S bands of ribosomal RNA (rRNA) with a light background between the bands. The ratio of A_{260nm}/A_{280nm} of the RNA was within 1.9 to 2.0, indicating that the RNA prepara-tion was free of contamination. We routinely obtained 50 lg of RNA from 10^7 bacterial cells, in excess of 50 times greater than a recently reported method where a silica gel membrane-based spin column was used for mycobacterial RNA isolation [12]. This high yield can be useful in microarray analyses where 20 to 200 lg of RNA is required [13]. The presence of intact mRNA within the total RNA was demonstrated by reverse transcription polymerase chain reaction (RT–PCR) of differentially expressed genes. For RT–PCR, 1 lg of total RNA was reverse transcribed using random primers provided with the First Strand Synthesis Kit (Sigma) following the manufacturer's protocol. The complementary DNA (cDNA) synthesized was used for RT–PCR of the nitrate reductase (NirG) gene for an in vitro study using gene-specific primers (M. tubercu-losis/M. bovis BCG, forward 5^0 -AAGACGGGATCATCACC-TGGGAA A-3^0 , reverse 5^0 -TTGACAGCCTTGTCCATCTCCACA-3^0 (annealing temperature 63 LC); M. smegmatis forward 5^0 -CAACTGGAATC TGGACCTGGAGAA-3^0 , reverse 5^0 -TCGATCGACATCAGCAGATCAAG C-3^0 (annealing temperature 61 LC). All PCRs had an initial denatur-ation step at 94 LC for 2 min followed by 30 cycles at 94 LC (30 s), the respective annealing temperature (1 min), extension at 72 LC (1 min), and a final extension at 72 LC (5 min). The RT–PCR ampli-fied a region of NirG > 1000 bp, indicating the presence of undegraded mRNA (Fig. 2A). Interestingly, the spheroplast method is also applicable to the isolation of mycobacterial RNA from an infection model, where most other methods are either difficult or

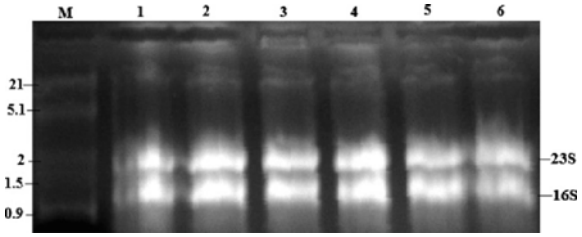

Fig.1. Gel electrophoresis image of total RNA isolated from mycobacteria using spheroplast method. Total RNA (10 lg) from aerobic- and hypoxia-dormant cultures was electrophoresed through a 1% agarose–0.66 M formaldehyde gel in Mops running buffer (20 mM Mops, 10 mM sodium acetate, and 2 mM EDTA, pH 7.0). Lanes 1 to 3 and lanes 4 to 6 represent RNA samples from aerobic and hypoxia dormant cultures, respectively. Lanes 1 and 4: M. smegmatis; lanes 2 and 5: M. bovis BCG; lanes 3 and 6: M. tuberculosis; lane M: marker (BioLit Midrange, 0.5 lg).

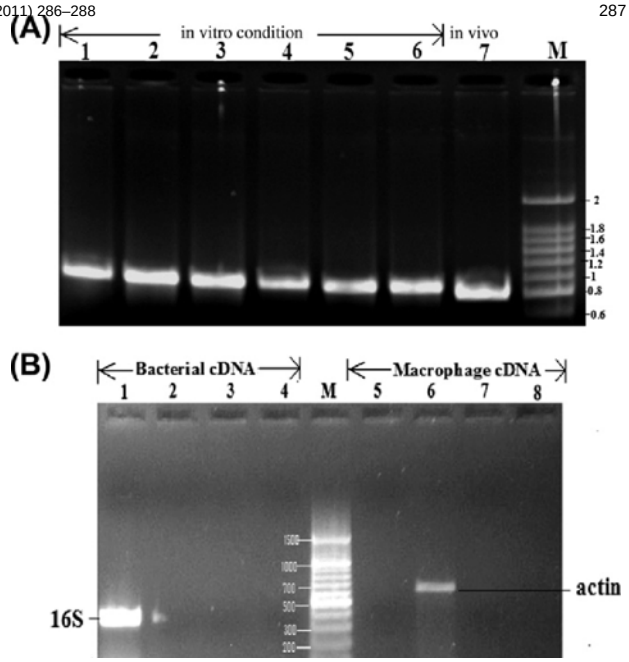

Fig.2. (A) RT–PCR amplification of nitrate reductase (narG) and Rv3804 gene from mycobacteria grown under different conditions. PCR products (20 ng) were loaded on 1% agarose gel containing 0.005% ethidium bromide. Lanes 1 to 3 and lanes 4 to 6 show amplification products of narG gene from aerobic- and hypoxia-grown cultures, respectively (in vitro), whereas lane 7 shows the Rv3804 gene of M. tuberculosis from the infection model (in vivo). Lanes 1 and 4: M. smegmatis; lanes 2 and 5: M. bovis BCG; lanes 3 and 6: M. tuberculosis; lane M: marker (Hi-Media, 200 bp, 0.5 lg). (B) Analysis of cross-contamination during RNA isolation from infection model using RT–PCR. cDNA samples were prepared using bacterial and macrophage RNA as described in the text and were used as template for the PCR study. In lanes 1 to 4, bacterial cDNA template was used; in lanes 5 to 8, macrophage cDNA template was used. Lanes 1 and 5: 16S primer; lanes 2 and 6: actin gene primer; lanes 3 and 4: reverse transcriptase minus (negative control); lanes 7 and 8: Taq polymerase minus (negative control); M, marker (Fermentas, 100 bp, 0.5 lg).

inefficient [14]. Efficient isolation of total bacterial RNA from an infection model and lack of cross-contamination with macrophage RNA were confirmed by RT–PCR. Total RNA isolated from both host macrophages and intracellular bacilli was reverse transcribed using random primers, and the resulting cDNA was separately used for the amplification of 16S rRNA and actin genes (16S: forward 5^0 -ATGCATGTCTTGTGGTGGAAAGCG-3^0 , reverse 5^0 -TTCACGAACAA CGCGACAAACCA-3^0 (annealing temperature 58 LC); actin: forward 5^0 -ATGGATGACGATATCGCT-3^0 , reverse 5^0 -ATGAGGTAGTCTGTCAG GT-3^0 (annealing temperature 60 LC). The RT–PCR indicated that only the 16S rRNA gene was amplified when mycobacterial cDNA was used as template and only the actin gene was amplified from macrophage cDNA (Fig. 2B), indicating the absence of any contam-ination of the bacterial RNA with host RNA. To further show the integrity of the RNA isolated from intracellular mycobacteria, RT– PCR performed on the Rv3804 gene using gene-specific primers (Rv3804: forward 5^0 -ATGCAGCTTGTTGACAGG-3^0 , reverse 5^0 -TGG CACCCAGTGCCCGTT-3^0 (annealing temperature 61 LC), amplifying a fragment 964 bp in length, confirmed the presence of unde-graded transcript (Fig. 2A, in vivo lane) [12].

The suggested spheroplast method specifically affects the mycobacterial cell wall, rendering the normally rod-shaped bacte-ria spherical. This specific effect is due mainly to the lysozyme and D-cycloserine present in the spheroplast solution [15]. In addition, the spheroplast solution has no effect on the viability of the bacilli, generation time, or macrophage integrity (data not shown).

The suggested method is clearly advantageous over other reported methods. It is very simple (obviating the need for mechan-ical instruments for cell lysis), is less time-consuming, and has high reproducibility, all of which make it easily adoptable even in less well-equipped laboratories. The whole process can be performed in sealed tubes or in level 1 biosafety hoods for even biohazardous group 3 organisms such as M. tuberculosis. A further benefit is that the same protocol can be used for the isolation of intracellular mycobacteria without contamination from macrophages. In con-clusion, this high-yielding spheroplast method is efficient, conve-nient, and cost-effective and can be a useful tool in transcript analyses for a better understanding of host–pathogen interactions.

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