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PHYSIOLOGICAL CHARACTERISTICS OF MYCOBACTERIUM SPECIES

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ABSTRACT :

Mycobacterium species are characterized by their unique physiological features, which contribute to their diverse ecological niches and pathogenic potential. Key physiological characteristics include their unique cell wall structure rich in mycolic acids, which imparts resistance to environmental stresses and antibiotics. Mycobacteria exhibit slow growth rates, requiring specialized culture techniques and extended incubation periods for isolation and identification. Metabolically, they are versatile, capable of utilizing a wide range of carbon sources and surviving within host macrophages. These bacteria also demonstrate an intricate lipid metabolism, essential for their survival and virulence. Understanding these physiological traits is crucial for developing effective diagnostic methods, treatments, and control strategies for mycobacterial infections, including tuberculosis and leprosy.

1. INTRODUCTION :

Microbes and microorganisms are small organisms which are not visible to naked eyes because they have a size of 0.1 mm or less. They can, therefore be seen only under the microscope are cosmopolitan in distribution inside the soil, in all types of waters, in air, on dust particles, inside and outside our bodies as well as on other animals and plants.

Microorganisms over the years have proved as fascinating source of naturals products for the industries especially pharmaceutical industries Microorganism are biotechnological valuable which are well exploited for secondary metabolities. **(Diraviyam et al.2010)**

As long as the major challenges in biotechnology and biomedicine remain (e.g.emerging diseases, established diseases, antibiotic resistance, environmental pollution, and need for renewable energy) microbial resources will be of interest to mankind providing sustainable and environmentally friendly solution with the effort of mankind, this natural treasure can become a gainful source of utilization. The best yet to come as microbes moves into the environmental and energy sectors. As stated many years ago by Jackson W.**Foster " Never underestimate the power of microbe" , and by david pariman " If you take care of your microbial friends, they will take care of your future" (Chaudhary et al. 2013)**

1.1 Mycobacterium spp.:

Mycobacteria belong to the family Mycobacteriaceae and are members of the CMN group (Corynebacteria, Mycobacteria and Nocardia). The family Mycoacteriaceae are Gram positive, nonmotile, catalase-positive have a rod like to filamentous morphology and can be pleomorphic. As a group, the produce characteristic long fatty acids. Mycobacteria are acid-fast rods of varable appearance, approximately 0.2 - 0.6 by 1 - 10 micrometer. The genus Mycobacterium consists of 127 species (excluding subspecies) according to the latest approved list of bacterial species. Mycobacterium other then Mycobacterium tuberculoses are commonly referred to as atypically or non- tuberculosis mycobacteria (NTM). Two of these cause disease in normal hosts and are thus primary pathogenic. M. leprae, M. ulcerans. They are often regarded as NTM. The remaining species are considered non-pathogenic or opportunistic pathogens and cause disease when host – defence are compromised.

Mycobacteria can be arranged into four groups according to the **Runyon classification**

Group 1 – **Photochromogens :** slow growers and form pigments when exposed to light (eg. M. kansasii, M. marinum, M.simiae)

Group 2– **Scotochromogens:** slow growers and form pigments in the dark (eg. M.scrofulaceum. M.szulgai, M. gordanae)

Group 3 – **Nonphotochromogens:** slow growers and not pigmented (eg. M.malmoense, M. xenopi, M.avium-complex, M.ulcerans, M.haemophillum)

Group 4 – **Rapid growers** (M.fortuitum, M.chelonae, M.abscessus)

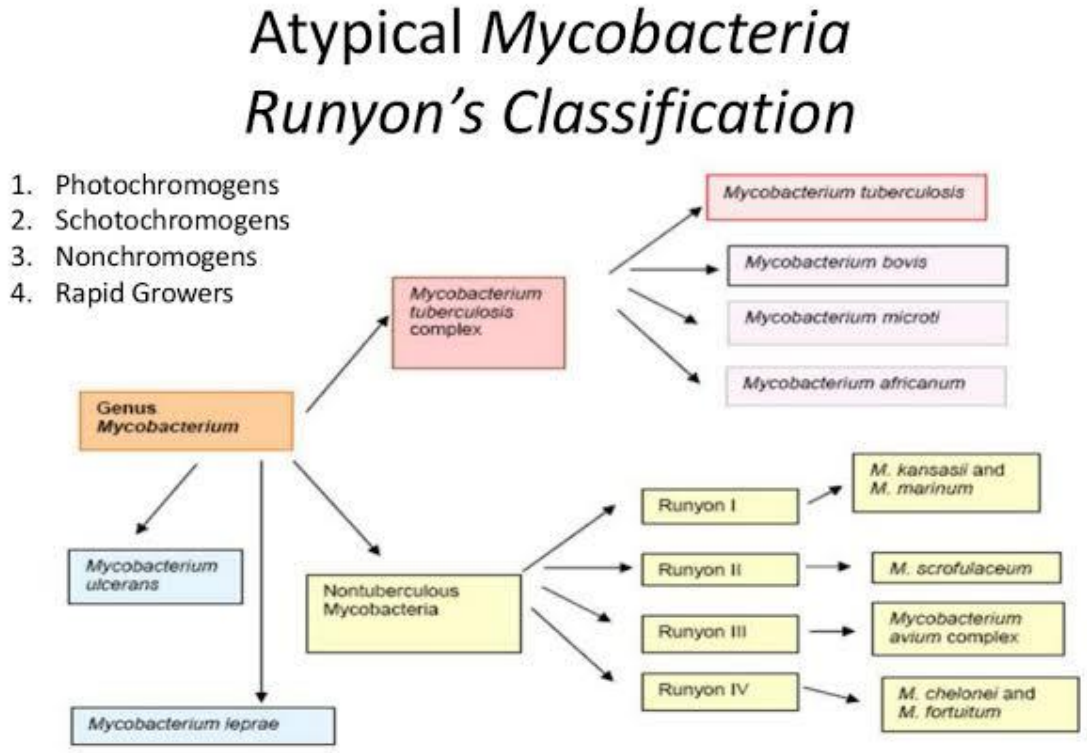

Fig 1: Runyon' s Mycobacterium classification

(http://www.nature.com/nprot/joumal/v4/n2/fig_tab/nprot.2006.126_F1.html)

Most slow growing species have been associated with disease in humans while only few species of group 4 (the ones mentioned above) are disease associated. Since the advent of AIDS and the application of recent developments in molecular biology for the detection and identification of NTM. NTM infections are increasingly detected. This in its turn created a higher awareness for mico-bacterial involvement in a veriety of clinical conditions and NTM diseases have been increasingly recognised in immune competent patient as well.

M. avium subsp. Para tuberculosis present on pastures or barns is the most common non-tuberculous mycobacteria detected in soil (Eisenberg et al, 2009; Pribylova et al. 2011). Soil is easily contaminated by fertilization with manure or liquid dung or by water contaminated by animal faces. Survival of mycobacteria in soil for as long as one year or longer is associated with amoebae or other protozoa or with the shedding of mycobacteria by wild ruminants, wild board, hairs, rabbits and other animals. Mycobacteria from river sediments can be transferred to soil by floods or by the ejections of micro droplets farming aerosols. Any of these transfer mechanisms can used for grazing. (White et al 2010). Other M. avium subsp. were studied to determine sources of infection for patients (Kaevska et al. 2011). A small number of studies were concerned with the detection of M. bovis (Young et al. 2005) or M. leprae in soil. The association was observed between endemicity of leprosy in Africa and India, the distribution of mycobacteria in soil and water with respect to dry or wet season and geographical distribution. The mycobacterial isolates from soil were identified as M. fortuitum, whereas the uncultured sequences obtained from soil DNA fell into a few closely related groups, either M. fortuitum or other fast growing mycobacteria, like M. tokaiense, or M. austroafricanum and M. heidelbergense. However, the method described in this study based on the sequencing of a 473 bp region of the 16SrRNA gene, cannot be used to discriminate many species that are human and animal pathogens, i.e. M. tuberculoses, M. avium, M. bovis and M. leprae, although sequences belonging to this group were identified (Chilima at al 2006; Lavania et al. 2008; Turankar et al.2012). With regard to mic-obacterial diversity in poly cyclic aromatic hydrocarbon-contaminated soil, investigations have revealed the presence of certain species typical for that environment. Cheung and Kinkle (2001) studied the diversity of mycobacteria in petroleum-contaminated soils 16S rRNA sequences were amplified and subjected to temperature gradient gel electrophoresis analysis. All of the sequences belonged to fast-growing mycobacteria, some of them similar to M. monascense and M. chlorophenolicum. A similar study was conducted by Leys et al. (2005). The sequences detected in the contaminated soil belonged to the species M. frederiksbergense. M. austroafricanum, M. petroleophilum and M. tusciae. In a study conducted on heavily contaminated soil in Southern Finland, Denaturation gradient gel electrophoresis revealed that 30% of the clone library of the contaminated soil belonged to the genus Mycobacterium (Bjorklofet al. 2009).

1.2 Structure:

Mycobacterium spp. is a Gram-positive bacteria, characterised by an inner cell membrane and a thick cell wall. Mycobacteria have an outer membrane. They do not have capsules, and most do not form endospores. Mycobacterium marinum and M. bovis have been shown to sporulate, however, this has

been contested by further research. The distinguishing characteristic of all Mycobacterium species is that the cell wall is thicker than in many other bacteria, which is hydrophobic waxy, and rich in mycolic acids/mycolates. The cell wall consists of the hydrophobic mycolate layer and a layer held together by a polysaccharide, arabinogalactan. The cell wall makes a substantial contribution to the hardiness of the genus.

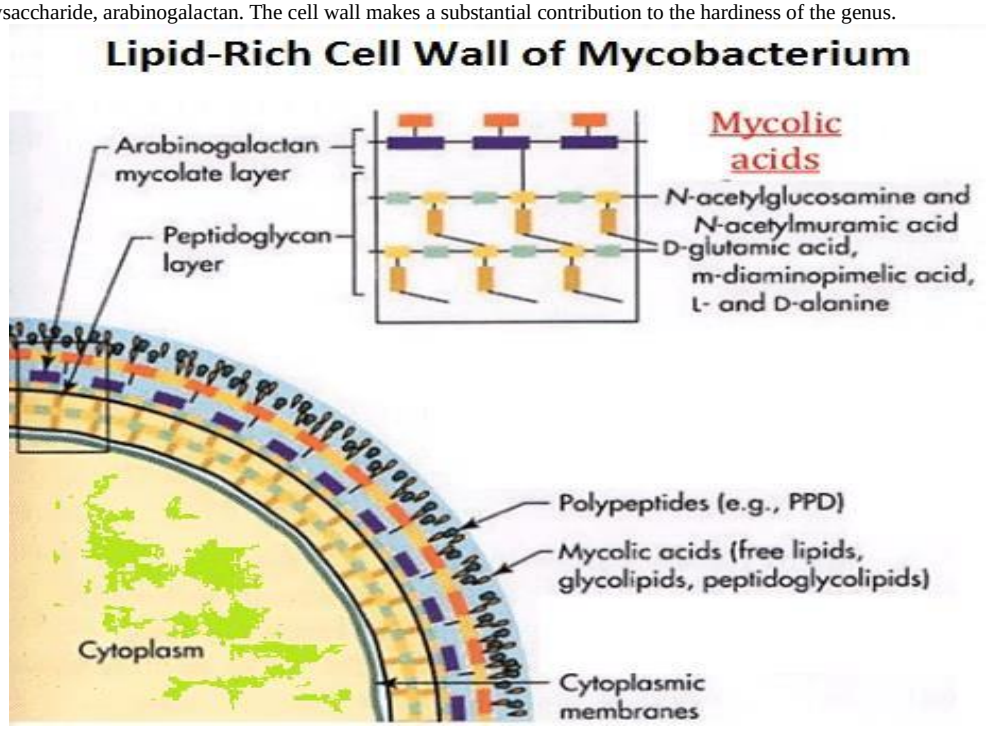

Fig: 2 Cell wall structure of Mycobacterium spp.

(http://www.slideshare.net/deshkar/mycobacteriumspp.)

Although this bacteria is Gram-positive. It has some unique qualities that are divergent from most gram- positive bacteria. Its cell wall contain mycolic acids, long, branched fatty acids that are normally present in acid-fast bacteria. The acids prevent proper gram staining that would normally identify the cell as a gram positive cell because they create a waxy coating so the crystal violet has difficulty interning the cell, therefore making its seem gram negative. The cell wall is also abnormal because it is irregularly thick for a garm-positive bacteria and its hydrophobicity reduces desiccation. This feature in addition to its slow cell growth (in comparison to most other bacteria) attribute to Mycobacterium smegmatis low response to antibiotic Mycobacteria are aerobic and nonmotile bacteria (except for the species Mycobacterium marinum, which has been shown to be motile within macrophages) that are characteristically acid fact.

1.2.1 STRUCTURES EXTERNAL TO THE BACTERIAL CELL WALL

Examination of a prokaryotic cell reveals various component structures. Some of the structures are naturally common to almost all prokaryotes, but some structures are present in only certain species. Some of these structures are external to the cell wall, whereas are internal to the cell wall. All the structures found external to the bacterial cell wall have a function in protection, attachment, motility or conjugation. Some of these structures are described below:

THE GLYCOCALYX

Some bacrerial cells secrete on their surface an adhering layers of viscous (sticky), gelatinous polymer called the glycocalyx. The bacterial glycocalyx is composed of polysaccharide, polypeptide or both. Its chemical composition varies widely with the species. For the most part, it is made inside the cell and secreted to the cell surface. It is formed by various bacilli and cocci , but not by spiral bacteria. If this layer is thick, tightly bound to the cell wall and can be visualised by light microscopy using special staining methods, it is termed capsule. If the layer is to thin to be seen by light microscopy, it is termed microcapsule, and if it is less tightly bound, unorganised, flowing materials, embedding many cells in a common matrix, the material is called slime layer

THE FLAGELLA

Numerous bacilli and spirilla and few species of cocci are capable of independent motion by the use of flagella (singular flagellum). The flagella of prokaryotes are whip-like, long filamentous and helical appendages that protrude through the plasma membrane and cell wall. They are much thinner then the flagella or cilia of eukarya.Flagella are so thin that they cannot be observed directly with a bright field microcscope, but must be stained with special techniques designed to increase their thickness.

In gram- positive bacteria, the outer set of rings is absent. The M ring is embedded in the inner membrane, and the S-ring is attached to the inside of the thick peptidoglycan layer. The chemical composition of basal body is unknown.

The hook it connects the basal body with the main filament or shaft. The hook of thr Gram-positive bacteria flagellum is longer than that of the Gram – negative flagellum. The hook is composed of protein subunits (monomers) arranged in a helical fashion

The main filament or shaft it is also composed of protein subunits arranged in a helical fashion. The protein of the filament is known as flagellin. The bacterial flagellum grows at its tip rather then at its base. Flagellin monomers synthesised within the cell are belived to pass along the hollow centre of the flagellum and are added to the distal end of the filament.

PILI (FIMBRIAE)

Pili (singular, pilus) or fimbriae (singular, fimbria) are hollow,non-helical, filamentous appendages that are thinner,shorter and more numerous than flagella. Although a cell may be covered with upto 1, 000 fimbriae, they are only visible in an electron microscope due to their small size. Their size ranges from 0.007 to 0.008 nanometer width and 0.5 to 2.0 namometer length, depending on the type of pilus. Like flagella, pili appear to originate in basal bodies and pierce the cell wall and capsule (if present).With a few exception, they have been observed only on Gram-negative rods. They donot function in motility, since they are found on non- motile as well as motile species. However, they are involved in attachment processes and in bacterial mating. It should be noted that some microbiologists use the word fimbriae for bacterial structures used for attachment and reserve the word pili for structures functioning in genetic transfer. But, usually the two terms are used interchangeably.

1.2.2 THE CELL WALL

Beneath the external structures such as capsules, sheaths, pili and flagella, and external to the fragile cytoplasmic membrane is the cell wall. It is a complex, semi-rigid structure present in almost all prokaryotes, excepting the mycoplasmas (eubacteria) and Thermoplasma (archaeobacteria). Some of the major functions of the bacterial cell walls are:

1.To protect the cell against osmotic shock, that is, the cell wall prevents bacterial cells from rupturing when the osmotic pressure inside the cell is greater than that outside the cell.

2.It helps to maintain the shape of a bacterium.

3.It serves as a point off anchorage for flagella and pile.

4.It is usually essential for bacterial growth and division. Cells whose walls have been completely removed (i.e. protoplasts) are incapable of normal growth and division

5.The cell wall is relatively porous allowing passage of molecules into and out of the cell (molecular sieving).

6.Clinically, the cell wall is important because it contributes to the ability of some species to cause disease and is the site of action of some antibiotics.

The cell wall constitutes a significant portion of the dry weight of the cell. Depending on the species and culture conditions, it may account for as much as 10 -40% of the total weight.

GRAM-POSITIVE EUBACTERIAL CELL WALL.

The cell wall of Gram-positive Bacteria (eubacteria) consists of many layers of peptidoglycan, forming a thick, rigid structure, about 20-80nm in thickness and comprising approximately 50-90% of the dry weight of the cell wall depending upon the species. There is a small space observed between the plasma membrane and the wall in some Gram-positive Bacteria. This space is called the periplasmic space or periplasm.

In addition to peptidoglycan, few other substances may occur in the cell wall. For example the walls of streptococcus pyogenes contain polysaccharides that are covalently linked to the peptidoglycan. The cell walls of most Gram-positive eubacteria also have teichoic acids, which are acidic polymers of ribetol phosphate or glycerol phosphate. The teichoic acids are connected to either the peptidoglycan itself or to plasma membrane lipids, making them an integral part of the Gram-positive cell wall structure. In the later case, they are called membrane teichoic acids or lipoteichoic acids. Because of their negative charge (from the phosphate groups), teichoic acids may bind and regulate the movement of cations (positive ions) into and out of the cell. Teichoi acids bind magnesium ions (Mg2+), and there is some evidence that they help to protect the bacteria from thermal injury. They may also have a role in cell growth, preventing extensive wall breakdown and possible cell lysis. Finally, teichoic acids provide much of the wall' s antigenic specificity and thus make it possible to identify bacteria by certain laboratory tests. Teichoic acids are not persent in the wall of Gram-negative Bacteria.

The walls of most of the Gram-positive bacteria contain very little lipid. But there are some exceptions; for example cell walls of Mycobacterium and few other acid-fast genera are rich in lipids calls mycolic acids. The ability of these bacteria to exhibit acid-fast staining (i.e., when stained, the cells cannot be decolourised easily despite treatment with dilute acids) is correlated with the presence of cell wall mycolic acid. The walls of Mycobacterium consist of as much as 60% mycolic acid, whereas the rest is peptidoglycan.

NUTRITIONAL REQUIREMENTS

Microorganisms must have a supply of nutrients (substances used for the synthesis of cell materials and for the generation of energy) from the environmental for proper growth. Water accounts for about 80-90% of the total weight of microbial cells and is, therefore, the major essential requirement in quantitative terms. It acts as a solvent for nutrients and carries them across the cytoplasmic membrane in a hydrated form. Water, in addition to its inert activity as a carrier, is also required for the various hydrolytic reactions carried out by an cell. Because of the polar nature and capacity to bind other polar molecules, water also influences the shape and architecture of many biological polymers, especially proteins. In addition, the specific heat of water provides resistance to sudden temperature changes in the environment. The dry weight microbial cells contains, in addition to hydrogen and oxygen (derivable metabolically from water),carbon, nitrogen , phosphorus, sulphur, potassium, calcium, magnesium and iron in order of

decreasing abundance. These are called as macro elements are macro nutrients because they are required by microoraganisms in relatively large amounts. The first six elements (C,O,H,N,Sand P) account for about 95% of the cellular dry weight and are essential components of carbohydrates, lipids, proteins and nucleic acids. They arec required by microorganisms in gram quantities in an liter of culture medium and are therefore, called as, major elements are major nutrients. These six elements are described below as the components of the growth medium.

CARBON

Carbon exists in many different chemical forms in nature. It may appear as inorganic carbon, such as carbonate are gaseous carbon dioxide, are as a component of simple or complex organic compounds. In every microbial system, it is the most abundant element and is required as the backbone of functional biological molecules. Usually, the requirements for carbon, hydrogen and oxygen are often met together as the molecules serving as carbon sources also contribute both oxygen and hydrogen atoms. The only carbon source for which this is not true is carbon dioxide because it is oxidies and lacks.
Glucose is the most common carbon sources in microbiological media.

OXYGEN

All microorganisms require elemental oxygen to build their biochemical components. Most of the heterotrophic microorganisms obtain oxygen from the same organic compounds that provide carbon source. They also get some oxygen from the major nutrients, water. However, many microorganisms required atmospheric oxygen (O2). Such microorganisms which requires molecular oxygen are termed obligately aerobic as they are dependent on aerobic respiration for their metabolic energy.

NITROGEN

All the microorganisms requires nitrogen in some form for the synthesis of cell components like amino acids purines, pyrimidines, some carbohydrates and lipids enzyme co- factors and others substance. It makes up about 14% of the dry weight of most of the microorganisms. Unlike eukaryotes, a number of eubacteria and archaeobacteria have the ebility to use atmospheric nitrogen, a process called nitrogen fixation.

HYDROGEN

The requirement of carbon, hydrogen and oxygen are often satisfied together. Hydrogen plays a number of roles in the life if a microbes. It is a structural atom in organic molecules and it is a participant in the complex process of energy generations. In autotrophic microorganisms, source of hydrogen is essential to reduce CO2 to cell material (CH2O). In the cell membrane of most of the microorganisms, protons (H)are involved in the production of ATP synthase system.

SULPHUR

It is needed for synthesis of certain amino acids (cysteine, cystine and methionine), carbohydrates and B-vitamins (biotin and thiamine). Some microorganisms require organic sulphur compounds, some are capable of utilising inorganic sulphur compounds, and some can even use elemental sulphur. In a growth medium, sulphur is usually added as a sulphate salt, for example, magnesium sulphate (MgSO4) can be added as a source of both sulphur and magnesium.

PHOSPHORUS

Phosphorus usually supplied in the form of phospholate, is an essential component of nucleic acids, pholipids, nucleotides like ATP, several cofactors, some proteins and other cell components. Almost all microorganisms use free inorganic phosphate as their phosphorus source and incorporate it directly.
The three types of growth factors most often required in microbial nutrition are:

1. **Amino acids:** These are required in the medium for growth of such microbes, which cannot synthesise more than one or few of the 21 alpha-amino acids that are required for protein synthesis.
2. **Purines and pyrimidines:** these are required for nucleic acid synthesis. The need for adding nucleic acid bases to the growth medium is rare for free-living soil microorganisms, but is most often observed in lactic acid bacteria and other fastidious organisms with many growth factor requirements.
3. **Vitamins:** These are small organic molecules that function either as co-enzymes for several enzymes or as the building blocks for co-enzymes. Very small amounts of vitamins are required to sustain the growth of microorganisms are those that are less susceptible to destruction by light, for example, thiamine, biotin and nicotinic acid.

   Microbes do not require the fat-soluble vitamins such as vitamin A and D

1.3. CHARACTERSTICS .OF MYCOBACTERIUM SPP

Many mycobacterium species adapt readily to growth on very simple substrates using ammonia or amino acids as nitrogen sources and glycerol as a carbon source in the presence of mineral salts. Optimum growth temperature very widely according to the species and range from 25 ºC to over 50 ºC.
Some species can be very difficult to culture (i.e.they are fastidious), sometimes taking over two years to develop in culture. Further, some species also have extremely long reproductive cycle – M.leprae, may take more then 20 days to proceed through one division cycle (for comparison, some E. coli strains take only 20 minutes), making laboratory culture a slow process in addition the availability of genetic manipulating techniques still lags far behind that of other bacteria species.
A natural division occurs between slowly – and rapidly – growing species. Mycobacteria that from colonies clearly visible to the naked eye within seven days on subculture are termed rapid growers, while those requiring longer periods are termed slow growers. Mycobacteria cells are straight or slightly curved rods between 0.2 and 0.6 µm wide by 1.0 and 10 µm long.
Some mycobacteria produce carotenoid pigments without light. Oters require photoactivation for pigment production.

a) Photochromogen (group 1)

Produce non pigmented colonies when grown in the dark andt pigmented colonies only after exposure to light and reincubation. Eg. M.kansasii, M.marinum,

b) Scotochromogens (group 2)

Produce deep yellow to orange colours colonies when grown in the presence of either the light or the dark Ex.M. scrofulaceun, M.gordonae, M. xenopi,

c) Non-chromogens (group 3 and 4)

Non pigmented in the light and dark or have only a pale yellow, buff or tan pigment that does not intensify after light exposure. Ex.M.tuberculosis, M.avium, intra-culture eg.M. bovis, M.ulcerans.
Mycobacteria are classical acid fast organisms strains used in evaluation of tissue specimens of microbiological specimens includes Fites stain Ziehl-Neelsen strain, and kinyoun stain.
Mycobacteria appear phenotypically most closely related to members of Nocardia, Rhodococcus and corynebacterium.
Mycobacteria are widespread organisms, thpically living in water (including tap water treated with chlorine) and food sources some, however, including the tubercluses and the leprosy organisms, appear to be obligate parasites and are not found as free living members of the genus.
Mycobacteria can colonize their hosts without the hosts showing any adverse signs.
For example, billions of people around the world have asymptomatic infections of M.tuberculoses.
Mycobacterial can colonize their hosts without the hosts showing any adverse signs.
For example, billions of people around the world have asymptomatic infections of M tubercolosis.
Mycobacterial infections are notoriously difficult to treat. The organisms are hardy due to their cell wall, which is neither truly Gram negative nor positive. In addition, they are naturally resistant to a number of antibiotics that descript cell-wall biosynthesis, such as penicillin. Due to their unique cell wall, they can survive long exposure to acids, akalis, detergents, oxidative bursts, lysis by complement, and many antibiotics. Most mycobacteria are susceptible to the antibiotics clarithromycin and rifamycin, but antibiotic-resistant strains have emerged. As with other bacterial pathogens, surface and secreted proteins of M tubercolosis contribute significantly to
Phenotypic tests can be used to identity and distinguish different mycobacteria species and strains. In older systems mycobacteria are grouped based upon their appearance and rate of growth. However these are symplesiomorphies and more recent classification is based upon ciadistics. Over 100 species are currently recognised.

2. REVIEW OF LITERATURE

Mycobacterium revealed that the micro-organisms is Gram-positive, non-motile and acid-fast rod; colonies varies in size 5-15 mm on different agar media and appear as non-pigmented, rough with opaque aspect.
The isolation of mycobacteria from specimens heavily contaminated with other microbes, such as soil, requires decontaminating procedures and selective media to overcome the multiplication of the other bacteria or fungi. Various methods have been described for the isolation of mycobacteria from environmental sample (Songer, 1981). None of them are ideal, mostly due to the loss of cultures through growth of contaminants when the decontamination methods are too mild, or to the loss of mycobacteria when these methods are too drastic.Moreover, little information available on the number of cultures which are lost due to contamination by other environmental micro-organisms. During extensive studies of mycobacteria from the emvironment in Zaire (Portaels, 1973, 1978, 1980) and in the southeastern United States (F. Portaels unpublished results), a large number of mycobacterial strains were isolated from soil samples. Different decontamination methods were used (Petroff, 1915; Corper & Stoner, 1946; kubica et al; 1963) wolinsky & Rynearson, 1968; beerwerth & Schurmann, 1969); were less effective than the other methods, mostly due to the loss of cultures by contamination (portaels, 1978). For the present study, the choice of decontamination methods was guided by those previous findings, and different media have been compared in order to find a procedure which will permit the isolation of high yields of mycobacteria from heavily presented in detail since it enabled us to determine the optimium conditions for the isolation of mycobacteria from soil.
At Biochemical level, microbal cells can be characterized for nearly 1000 cellular phenotypes which are preconfigured into 10 microplates with 96 well formats. Each well represents an independent test center. Cells were cultured and added to the wells (independent test centre) of different plates. Cells metabolize a substrate present in a well, respire and release energy which reduces colourless tetrazolium redox dye. The reduced dye which is purple in colour can be detected by ELISA reader at 595nm.
Molecular biology is defined as " The study of the structure and function of large molecules associated with living organisms, in particular proteins and the nucleic acids DNA and RNA " (Oxford dictionary of biology, 4th ed, 2000). Molecular biology is among the most rapidly growing fields within biology with recent success stories such as the sequencing of the entire human genome (reported in Science and Nature).

Molecular biology is unusual in that the major information source for molecular biologists is not journals articles, but public detabases such as Gene Bank, PDB, and DDBJ. This is one reason why there are relatively fewer handbooks and treatises for molecular biology than there are for other biological subjects. There is a natural affinity between this chapter and the ones discussing references material for biochemistry and genetics. Given this very substantial overlap, it is essential to review the sources annotated in these chapter for a more complete understanding of the literature of molecular biology.

PCR chemistries, while having practical impacts in terms of specificity, detection limits and ease of analysis, and sensitivity are most likely not a key parameter in determining the ultimate quality of environmental qPCR. Accurate quantitative results can be obtained with all of the available chemistries. Despite the common view that specific oligoprobes necessarily increase the sensitivity of qPCR versus non-specific both approches have the potential to detect amplicous with equal efficiency (Mackay, 2007). Nevertheless, nested Scorpion PCR (De Bellis et al, 2007) has been reported to exhibit increased sensitivity (Bustin, 2004) and may be worth examining in more detail for environmental applications should lower detection limits be a high priority. In general, the use of specific oligoprobesmethods will be required for performance of multiplex qPCR.

The role of ribosomal RNA in translation has been in a state of rapid evolution in recent years. No longer considered a mere scaffold for the positioning of ribosomal proteins, rRNA currently appears instead to play an active and probably crucial part in proteins synthesid (reviewed inNoller, 1984). Intrest in its structure thus originates to a large degree from a desire to understand how it works. Perhaps this understanding will, in turn, shed light on how ribosomes (and life itself) oeiginated. Since that first 16s rRNA sequence appeared in 1978(Brosiuset al,1978, Carbon et al 1979) over thirty additional sequences have been completed (reviewed in Gutellet al , 1985).

A given helix is considered proven when two or more independent sets of compensating base changes are found between different 16S (or 18S)rRNAs. On this basis, the secondary structure was established (Gutellet al. 1984, Noller and Woeseet al.1983).

Many researcher ideas in ecology cannot be realised because identification of taxa poses a difficult problem. Moreover, drawing the wrong conclusions due to misinformation in basic identification of species could have serious consequences. Fernandez et al. (2006) emphasize the need to make molecular characterization of species a standard part of ecological analysis of population and communities. Molecular tools allow for the unambiguous identification of individuals (Lucentini et al. 2007) and create a new opportunities in a board spectrum of ecological studies (Bickford et al. 2007). A wide range of species-identification problems, form cryptic life stages (Chow et al. 2006) to prey species in gut contents (Zaidi et al, 1999), can be estimated by molecular markers. There is presently general agreement between secondary structures models for 16S rRNA derived independently by three groups of investigators (Brimacombeet al, 1988, Ebeletal, 1983).

Therefore tests based on the PCR-RFLP (Restriction Fragment Length Polymorphism) method have recently been developed for species identification in many texa, (Ce spedes et al ,2000, Cocolin et al. 2000, Aranishi,2005) As an alternative to laborious and costly DNA sequencing, RFLP is a highly realiable and easy method for identifying polymorphism in DNA sequences using restriction enzymes and gel electrophoresis.

The applicability of the PCR-RFLP method was not tested on digested or fixed material. However we believe that the tool is promising for these types of samples as mtDNA is present athign copy numbers in cells and is more likely to preserve for a longer periods than nuclear DNA. On the other hand the length of the PCR – fragment might be too large to amplify when the DNA is highly degraded. It is then necessary to develop additional internal primers to amplify shorter fragments that include the species – species restriction sites (jehaes et al, 2001, Deagle et al.2006) For the efficient identification of bacteria. Molecular techniques such as polymerase chain reaction (PCR) and amplified ribosomal DNA restriction analysis were recently proposed (Krizova et al, 2006, Young et al,2008).

3. MATERIALS AND METHODS

3.1 Culture details:

A mixed bacterial culture plate was provided by Ipca laboratories ltd. Mumbai.

3.2 Isolation of Mycobacterium spp.

3.2.1 Preparation of Medium

Bacteria display a widevariety of nutritional and physical requirements for their Growth. This includes water, a source of energy, sources of carbon, sulfur, nitrogen Phosphorus, minerals such as Ca2+ , Mg2+, Na+, and other vitamins and growth Factors. Nutrient agar, soya agar is act as general purpose media. In advance Middlebrook 7H10, LJ are the selective media for Mycobacterium spp. medium were Prapared with exact quantity of components and additional reagents necessary for bacterial strain revival and growth and the proper incubation conditions were ready.

3.2.2 Sterilization of media

Above prepared media were sterilized at 121°C temperature and 15 psi pressure for 15 minutes. And all the required glass wares were autoclave in advance.

3.3 Isolation methods:

Bacterial strains were isolated on a sterile different medium plate by streak-plate method and dilution methods:

3.3.1 Streaking:

The streak plate method is a rapid qualitative isolation method. The techniques commonly used for isolation of discrete colonies initially require that the number of oganisms in the inoculums be reduced. It is essentially a dilution technique that involves spreading a loopful of culture over the surface of an agar plate. This process was done in a laminar air flow cabinet aseptically.

3.3.2. Serial dilution:

A serial dilution is a series of dilution used to reduce a dense culture of cells to more usable concentration. Each dilution reduces the concentration of mycobacterium by the specific amount dilution is the methods to get single colonies of culture in individual form. It was done with normal saline. Normal saline (0.85% NaCl) was used because it has the same osmolarity as most intra cellular fluid. So when we add normal saline to suspend micro organisms in a culture, these micro organisms would not rupture due to either intracellular fluid coming out of the saline going in due to osmosis through the cell membrane.

3.4. Microscopic Identification of isolated bacterial strains:

Isolated bacterial strains were confirmed on the basis of gram staining, colony morphology, shape and size, by acid fast staining which were performed as per describe in Dubey & Maheshwari and K.R. Aneja, some of them are as follows.

3.4.1. Gram Staining

The gram staining method is named after the Danish Bacteriologist Hans Christian Gram (1853-1938) who originally devised it in 1882 (but published in 1884), to discriminate between pneumococci and klebsiella pneumonia bacteria in lung tissue. It is a differential staining method of differentiating Bacterial species into two large groups (gram positive and gram negative) based on the chemical and physical properties of their cell walls. This reaction divides the eubacteria into two fundamental groups according to their stain ability and is one of the basis foundation on which Bacterial identification is built. The differences in staining responses to the gram stain can be related to chemical and physical differences in their cell wall. The gram negative bacterial cell wall is thin, complex, multilayered structure and contains relatively a high lipid contents in addition to protien and mucopeptides. The bacteria which retain the primary stain appair dark blue or violet which are not decolourised called gram positive, where as those that loss the Cristal violet and counter stain by safranin appair pink and are reffered to as gram negative.

3.4.2 Acid Fast staining (Ziehl Neelsen Staining)

Spirochaetes such as the Mycobacteria that cause tuberculosis and leprosy do not stain well using the Gram Stain. Other stains that do not wash away with dilute acid are used instead. The bacteria are deeply stained, either bright red against a blue background or red against a green background. Because the stain cannot be removed by washing with acid, organisms stained by these methods are termed acid-fast bacteria.

Procedure:

- Flooded a fixed slide with strong carbol fuchsin.
- Heated the slide until it steams and keep steaming for 5 minutes
- Washed slide with water (preferably filtered water, tap water may contain mycobacteria leading to false positive results)
- Treated with 3% acid alcohol for 10 minutes or until only a suggestion of pink remains on the film.
- Washed film with water
- Counterstain for 15-30 seconds with methylene blue.
- Wash and blot dried.
- Acid-fast bacilli appeared bright red while tissue cells and other bacteria stain Blue.

3.5 Bacterial characterization (Nutrient utilization pattern):

Biolog microplates were used for characterization of microbial cells. A microbial cell can be characterized for nearly1000 cellular phenotypes which are preconfigured into10 microplates with 96 well formats. Each well represents an independent test centre. Cells metabolize a substrate present in a well, respire and release energy which reduces colorless tetrazolium redox dye. The reduced dye which is purple in color can be detected by ELISA reader at 595nm.
Performed the PM procedure for Mycobacteriums species.

3.5.1 Materials Required:

1. Equipments:
Omnilog PM, Turbidimeter, Multichannel Pipetter

2. Chemicals and Materials for Inoculation Procedure:

PM panels 1-10, IF0a GNGP Base (1.2x), Middlebrook 7H9 Broth, Middlebrook 7H10 Agar, Biolog Redox Dye mix G (100x), Biolog Redox Dye mix H (100x), sterile cotton swabs, sterile pipet tips, sterile reservoirs, sterile 20 x 150 test tubes, sterile 120 ml plastic vials, sealing tape for microplates (optional), magnesium chloride (MgCI2, 6H20), calcium chloride (CaCI2, 2H20), tween 80, D-glucose.

3.Composition and Preparation of 12x PM Additive Solutions

All 120x stock solutions were prepared, filter sterilized, and stored at 4ºC. Combine ingredients and Q.S. to 100 ml. Stored at 4° C.

Table 1: Chemical Composition and preparation of 12x PM Additive Solutions

Ingredient	120x Conc.	Formula Weight	Grams/ 100 ml	PM 1,2	PM 3,6,7,8	PM 4	PM 5	PM 9+
MgCl2,6H2O CaCl2,2H2O	240mM 120mM	203.3 147.0	4.88 1.76	10ml	10ml	10ml	10ml	10ml
Tween 80	1.2%	-	1.2	10ml	10ml	10ml	10ml	10ml
D-glucose	600mM	180.2	10.8	-	10ml	10ml	10ml	10ml
Sterile water	-	-	-	80ml	70ml	70ml	70ml	70ml
Total	-	-	-	100ml	100ml	100ml	100ml	100ml

5. CONCLUSION AND FUTURE WORK :

The objective of this study was to isolate the Mycobacterium spp. and it's characterization through different aspects like morphogical, physiochemical as well as molecular. The bacteria were grown mold like fashion on culture medium. Mycobacterium were nonmotile, slender rods with branching or Y shaped, that resembles to the actinobacterium group Hence, seems like Mycobacterium spp. Biochochemical characterization determined mycobacterial physiology, utilized different energy sources like carbon, nitrogen, amino acids, vitamins etc. At Molecular level mycobacterial DNA Isolation, 16s rRna gene analysis, phylogenetic tree and comparison between different mycobacterial spp. has been confirmed. I'm unable to perform detailed account of molecular techniques like 16s rRna gene amplification and it's sequencing. phylogenrtic analysis and comparision between different Mycobacterial spp. due to short training session.

Mycobaterium spp. is very beneficial at industrial level as well as in disease prevention. Mycobaterium spp steroid biotransformation reactions which are very useful for manufacture different industrially important products. Hence, much more work require on Mycobacterial spp.

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