Chapter 1

General Introduction
1.1.1 Microorganisms

Microorganisms are essential components in the biosphere of our planet. Microorganisms are ubiquitous and show their presence in all corners of life and can sustain at varied climatic conditions from hot springs to freezing conditions in Antarctica and highly acidic environment to alkaline conditions of different soda lakes and oceans. They also present in human body as normal micro flora and some pathogenic bacteria can induce infections by altering the normal body metabolisms by their act of pathogenesis. Due to their adaptive nature, high flexibility in tolerance with reference to environmental conditions, they can sustain life at all types of climatic and environmental conditions. Some microbes are pathogenic and some are non pathogenic and also play major role in routine metabolic activities, e.g. microflora of ruminants helps in digestion. Some specific bacteria i.e. Bacillus anthracis, Bacillus tuberculosis, Streptococcus pneumoniae etc acts as pathogens and some are industrially applicable such as Azotobacter as biofertilizer. A very common Lactobacillus species which grow in acidic conditions is useful in conversion of milk into dairy product i.e. curd and sporogensis species of same genus is useful for treating pediatric diarrhea by oral dose of Lactobacillus spores under brand name “Sporlac”.

Bacteria can degrade herbicides. Also pesticides can be degraded by bacteria and thus can groundwater that is contaminated be cleaned. How can ammonia, a component of dung and fertilizer, be beneficial to plants? Only when nitrifying bacteria convert it to nitrite and others change that to nitrate, which is a component that plants can use directly. The Nitrogen Cycle is explained here- Bacteria eat oil and a whole range of organic chemicals, like gasoline, diesel, benzene, toluene, acetone, and even PCB’s. Most of these are toxic to humans and higher organisms, can they be degraded into safe compounds by bacteria. This application of bacteria is called bioremediation. An even more spectacular property is that some bacterial species might be used for cleaning up our nuclear waste. The radioactivity cannot be destroyed by the bacteria, but they can 'eat' all chemical toxic solvents in which these radioactive wastes are often present, and thus slow down or prevent corrosion. Bacteria can also make plastic! And yet other bacteria can eat plastic. Some bacteria (e.g. Lysinibacillus sphaericus) are currently investigated for their power...
to kill mosquito larvae and in that way we could fight malaria with bacteria. In fact, the ways how bacteria can be applied are nearly as diverse as their life styles.\(^1\)

In a single day people come into contact with millions of bacteria that are present on surfaces we touch, in the food we eat, and in the air we breath. Bacteria can start to cause harm to our bodies when they enter our system. Infections caused by bacteria start out as small colonies of bacteria which grow and multiply. The bacteria start causing bigger problems when they get into the blood stream. In the year 2000, 5 million people died from diseases that were caused by bacteria. Diseases can be caused by bacteria that are known or there could be new unknown bacteria causing problems. The unknown bacteria might not be treated because there are no known treatments for it. Very little information is known about newly discovered bacteria.\(^2\)

Robert Koch correlated a causal relationship between a causative microbe and a disease. Some of the highly pathogenic bacteria are *Bacillus anthracis*, *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Streptococcus*, *Pseudomonas*, *Shigella*, *Campylobacter*, *Salmonella*, *Rickettsia*, *Brucella*, *Francisella*, *Legionella*, *Listeria*, *Haemophilus influenzae*, *Leptospira interrogans* and so on. Pathogenic bacteria are bacteria that cause bacterial infection. Although the vast majority of bacteria are harmless or beneficial, quite a few bacteria are pathogenic. One of the bacterial diseases with highest disease burden is tuberculosis, caused by the bacterium *Mycobacterium tuberculosis*, which kills about 2 million people a year, mostly in sub-Saharan Africa. Pathogenic bacteria contribute to other globally important diseases, such as pneumonia, which can be caused by bacteria such as *Streptococcus* and *Pseudomonas*, and food borne illnesses, which can be caused by bacteria such as *Shigella*, *Campylobacter*, and *Salmonella*. Pathogenic bacteria also cause infections such as tetanus, typhoid fever, diphtheria, syphilis, and leprosy.\(^3\) *Staphylococcus* or *Streptococcus* are also part of the normal human flora and usually exist on the skin or in the nose without causing disease, but can potentially cause skin infections, pneumonia, meningitis, and even overwhelming sepsis, a systemic inflammatory response producing shock, massive vasodilation and death.\(^4\)

Some species of bacteria, such as *Pseudomonas aeruginosa*, *Burkholderia cenocepacia* and *Mycobacterium avium* are opportunistic pathogens and cause disease
mainly in people suffering from immunosuppression or cystic fibrosis. Anthrax, as a fulminating infectious disease, threatens human’s health seriously. *Bacillus anthracis*, the agent of anthrax, was classified into the second kinds of pathogenic microorganisms (one kind of the highly pathogenic microorganism) in the List of Human Pathogenic Microorganisms issued by the Chinese government. The spores formed by *B. anthracis* are potential material for biological warfare agent and biological terror. Therefore, it is very important and pressing to develop sensitive, efficient detection methods for the bacteria.

1.1.2 Applications of microorganisms

Fermentation is a natural process and by using this process people applied fermentation to make products such as wine, mead, cheese and beer long before the biochemical process was understood, which was demonstrated by Louis Pasteur in the 1850s and 1860s. Biochemically, fermentation is a metabolic process in which an organism converts a carbohydrate, such as starch or a sugar, into an alcohol or an acid. Yeast performs fermentation to obtain energy by converting sugar into alcohol. Bacteria perform fermentation, converting carbohydrates into lactic acid. Wine, beer, yoghurt and many other products are fermented products.

Antibiotic production: In recent years there has been an intensive search for microorganisms which produce antibiotics during growth on artificial media. Many known antibiotics are produced by actinomycetes (particularly by representatives of the genus *Streptomyces*), which are filamentous soil bacteria. The antibiotics are produced via secondary metabolic pathways, and originate from a small number of simple precursors, including amino acids, small fatty acids, sugars, and nucleic acids. Secondary metabolism is more specialized than primary metabolism and varies widely even among members of the same genera. Several new antibiotics have been found by targeted or random inactivation of particular genes, leading to utilization of alternate biochemical pathways. An alarming number of microorganisms that occur in hospitals have become resistant against virtually all antibiotics. Several strains of pathogenic bacteria have been discovered that are resistant to essentially all known antibiotics. Resistance against so many different antibiotics has been induced by the indiscriminate use of heavy doses of
antibiotics in health care. Strains that have developed resistance survive and can propagate productively as all competitors have been killed off. The discovery of bacterial strains with so many resistances has put development of new antibiotics into high gear. One needs to have effective back-up antibiotics before bacterial strains with resistance to all currently known antibiotics have developed. The search for new antibiotics in many cases involves the combination of brute-force screening and targeted genetic modification of microorganisms that produce antibiotics: by selected modifications antibiotics with slightly different structure and properties may be produced by these microbes. Nonetheless, the search for new antibiotics probably will need to be a continuing one.

Bacteria have many properties that are useful to industry. The diversity of the Bacterial kingdom is reflected by the diverse applications of bacteria as a cheap labour force. Bacteria can be used to mine gold! well, not quite, but the discovery that *Thiobacillus ferrooxidans* and some species of *Lysinibacillus* can concentrate gold trapped in rock minerals drew the attention of mining companies, and they are now developing a method of applying these bacteria in the gold mining industry. Bio mining may be the way of mining in the future, and researchers are now trying to modify the bacteria so that they collect the ores of interest. Certain bacteria are used to clean our waste: be it pollution, compost heaps, or sewage: bacteria can get rid of things. There are microbes that clean sewage and generate electricity at the same time! Or let them clean up metal waste and serve as catalysts later on, to assist in chemical processes. Bacteria have a taste for mining wastewaters no matter how toxic the contaminants are for animals and humans. Specialized bacteria metabolise these toxic chemicals into non-toxic, or less toxic compounds.

Strong consumer demand for natural products has prompted many researchers to look for alternatives to synthetic pigments which are widely used. Synthetic pigments are not only undesirable or harmful, but can cause adverse effects to the environment. There are many sources of natural pigments, namely from microorganisms and plants. Among plants, the orange/yellow colour obtained from saffron is one of the most expensive natural pigments sold with a price range between USD 1,100 and 11,000 per kilogram. The ascomycetous fungi, Monascus on the other hand has been reported to produce a variety of red, yellow, orange, green and blue hues which are mainly used in food.
industries. This brief serves as a quick guide on the isolation, characterization and applications of pigments extracted from red, yellow and violet bacteria namely *Serratia marcescens*, *Chryseobacterium* sp. and *Chromobacterium violaceum*, respectively. For large scale production this may be highly significant to reduce the cost of the pigments. Considering the short life cycle of the bacteria, pigment isolation can be work out at a large scale.

### 1.1.3 Microbial identification

For application of bacteria in industry or just diagnosis of any bacterial originated disease, detection method is highly important to apply its properties for mankind or to treat infections by antibiotic therapy. In a broad way bacteria can be identify by three primary methods: Morphological, Biochemical and Molecular. Morphological identification includes gram staining, motility by hanging drop technique, endospore staining, colony characteristic etc. Biochemical identification includes panel of 12 tests for identification of gram negative rods. Ready to do kit consists of sterile media for Citrate utilization test, Lysine utilization, Ornithine utilization, Urease detection, Phenylalanine deamination (TDA), Nitrate reduction, H₂S production test, and 5 different carbohydrates utilization test- Glucose, Adonitol, Lactose, Arabinose, and Sorbitol. This kit is also supplied with other reagents such as TDA reagent (R036) and Nitrate reagents: α Naphthalene solution (R009) and Sulphanilic acid 0.8% (R015).

### 1.2.1 Molecular identification by 16S rRNA

Ribosomes are molecular machines that make protein out of amino acids. The central tenet of biology is that DNA makes RNA, which then makes protein. The DNA sequence in genes is copied into a messenger RNA (mRNA). Ribosomes then read the information in this RNA and use it to produce proteins. Ribosomes do this by binding to mRNA and using it as a template for the correct sequence of amino acids in a particular protein.
The amino acids are attached to transfer RNA (tRNA) molecules, which enter one part of the ribosome and bind to the messenger RNA sequence. The attached amino acids are then joined together by another part of the ribosome. The ribosome moves along the mRNA, "reading" its sequence and producing a chain of amino acids. In prokaryotes, 70S ribosomes are made from complexes of RNA and proteins. Ribosomes are divided into two subunits, one larger subunit (50S) and other smaller subunit (30S). The smaller subunit (30S) binds to the mRNA, while the larger subunit (70S) binds to the tRNA and

![Fig. 1.1: Four domains in 16S rRNA and its hypervariable regions](image-url)
the amino acids. The 30S ribosomal subunit has a 1540 nucleotide. RNA subunit 16S bound to 21 proteins.

The primary structure of 16S rRNA is highly conserved. 16S rRNA makes up the bulk of the 30S subunit. It consists of 1542 bases and contains the substrate binding A, P, and E sites. The P site is occupied by peptidyl-tRNA and is located in the major groove in an upper portion of the rRNA. The A site is the attachment site for an incoming aminoacyl-tRNA, and is wide and shallow, which gives it a lower affinity for tRNA so it may relocate to the P-site. The E site, occupied by deacylated tRNAs when they exit, is associated with ribosomal proteins more than the A- or P-site, perhaps facilitating the dissociation of the codon-anticodon pair during translation. The arrangement of the 16S rRNA creates a 5’ domain, central domain, 3’ major domain, and a 3’ minor domain. As mentioned in the figure 1.1 the 5’ domain consists of 19 double helices that make up the bulk of the body. The central domain of the rRNA generates the platform and is an elongated, curved structure of nine helices, with the junction of helices 20, 21, and 22 being at the heart of it. The 3’ major domain contains 15 helical elements and composes the head. Sequence analysis of the 16S ribosomal RNA (rRNA) gene has been widely used to identify bacterial species and perform taxonomic studies. Bacterial 16S rRNA genes generally contain nine “Hypervariable regions” that demonstrate considerable sequence diversity among different bacterial species and can be used for species identification. Hypervariable regions are flanked by conserved stretches in most bacteria, enabling PCR amplification of target sequence using universal primers.

1.2.2 Advantages and limitations of 16S rRNA gene analysis

The 16S rRNA gene is a commonly used tool for identifying bacteria for several reasons. Traditional characterization depended upon phenotypic traits like gram positive or gram negative, bacillus or coccus, etc. Today, Taxonomists consider analysis of an organism's DNA more reliable than classification based solely on phenotypes. Ribosomes (and correspondingly the DNA that codes for them) have been mostly conserved over time. The 16S rRNA gene is relatively short i.e. 1.5 kb, making it faster and cheaper to sequence than many other unique bacterial genes.
Apart from these advantages, there are limitations of 16S rRNA gene analysis. In diagnosis, there is a need to distinguish between pathogenic and non-pathogenic microorganisms. This cannot be carried only with the whole 16S rRNA sequence. For this more specificity must be present and this can be achieved with study of hypervariable region. Hypervariable regions are flanked by conserved stretches in most bacteria, enabling PCR amplification of target sequences using universal primers. Numerous studies have identified 16S rRNA hypervariable region sequences that identify a single bacterial species or differentiate among a limited number of different species or genera. Rapid approaches that detect certain species-specific sequences within a single hypervariable region are also in common use.

1.2.3 Hypervariable regions of 16S rRNA

Concept of highly variable or hypervariable regions derived from the regions present on light and the heavy chains of human immunoglobulins or antibodies where most of the variation in amino acid sequences occurs and were named as hypervariable regions. These are also sites of antigen binding. Present study is focused on bacterial hyper variable regions of 16S rRNA. As shown in figure 1.2, there are total nine hypervariable regions present in 16S rRNA and are flanked by conserved regions. Conserved regions are significant for primer designing for PCR and hypervariable regions are mostly important for differentiation of bacteria to species and subspecies level by comparative nucleotide studies.

V1 Region: - V1 region could be used to distinguish common pathogenic bacterial species. It’s a shorter 20 to 30 nucleotide long sequences. Staphylococcus species showed that the S. aureus 16S rRNA gene sequence contains four unique single nucleotide polymorphism (SNP’s) in this short region. This short sequence is ideal for designing S. aureus specific probe.

V2 Region: - This region could distinguish all Common pathogenic bacterial species like Escherichia sp, Shigella.sp and E. aerogenes. This V2 region is also the best target to
distinguish among mycobacterial species. V2 has an average length of 100 bp, which is a relatively large target region for DNA probe.

**Fig 1.2:** hypervariable regions of 16S rRNA (Adapted from Chakravorty et. al. 2007)\textsuperscript{14}

**V3 Region:** - V3 is shorter than V2 (65 nucleotides versus 106 nucleotides) and PCR amplification of V3 with universal primers specific to flanking sequences would result in a smaller amplicon compared to V2. The smaller amplicon size may be preferred in some real time PCR assays.

**V6 Region:**-V6 region was only 58 bp long.V6 appeared to be the best target region for assays designed. SNP was the only 16S rRNA polymorphism detected between two closely related bacteria that were present in all the copies of their 16S rRNA genes (in *B. anthracis* 11 copies and in *B. cereus* 12-13 copies).
V4, V5, V7, V8 and V9 Region: These four hyper variable regions were less suitable for species identification. But V7 may be useful for designing probes to detect most select agents. *B. anthracis* and *B. cereus* differed by two SNPs in this region.

### 1.2.4 Advantages of Hypervariable regions

16S rRNA hyper variable regions (HVR) exhibit different degrees of sequence diversity and this attribute of HVR can be utilized to species identification. By utilizing information coded in specific hypervariable region of 16S rRNA, species specific diagnosis can be accomplished. Largest study carried out by one scientific research on molecular diagnosis of pathogenic bacteria of 113 dataset of 16S rRNA gene sequences from all the 110 bacterial species used in that study to confirm species specific identification and to define the borders of each hypervariable region for the bacterial species of interest. Alignment confirmed presence of all the nine hypervariable regions in the bacterial species for which the complete 16S rRNA gene sequence was available covering maximum hypervariable regions.\(^\text{14}\)

Complete sequence data were available for the regions V1 – V8 for all 113 sequences included in one of the prominent study.\(^\text{14}\) Sequence data for V9 was either incomplete or not available for 15 of the 113 sequences analyzed. The bacteria *Pseudomonas aeruginosa*, *Rhodococcus equi* and *Salmonella paratyphi* contained ambiguous nucleotide residues in V1 and *Arcanobacterium pyogenes* and *Clostridium septicum* contained ambiguous residues in V8. In this entire study, they analyzed each hypervariable region for its potential to distinguish among the 110 bacterial species in this study. V2 & V3 were most suitable for distinguishing all bacterial species to the genus level except for closely related enterobacteriaceae. Bacterial species with identical DNA sequences in a given region were identified. This first reported comprehensive study forms the basis of 16S rRNA hypervariable region based species specific identification of bacteria.\(^\text{14}\)

### 1.3.1 Lonar Lake

Lonar lake is situated in the Buldhana district (Maharashtra, India (19°58'N, 76°31'E) in the formerly volcanic Deccan-Trap geological region.
It is the third largest crater in the world and the only known crater formed by meteoritic impact in basaltic rock. Based on geological studies, it is postulated that the lake originated as a meteorite impact crater around 52 thousand years ago. As shown in figure 1.3, it is almost circular, with its longest and shortest diameters being 1875 meters and 1787 meters respectively with a raised rim of about 30 meters and a depth of 135 meters. It also has high alkaline condition with the pH of the water 10.5. The high alkalinity is attributed to the abundance of carbonate, which is 2680 mg/ml. Salinity in terms of NaCl of sediment is 45375 - 46777.5 mg/L while that of water sample 4900 - 5197.5 mg/L.

1.3.2 Alkaliphiles in Lonar Lake

Alkaliphiles are microbes classified as extremophiles that prosper in alkaline environments with a pH of 9 to 11 such as lakes and carbonate rich soils. To survive, alkaliphiles maintain a relatively low alkaline level of about 8 pH inside their cells by
constantly pumping hydrogen ions (H\textsuperscript{+}) in the form of hydronium ions (H\textsubscript{3}O\textsuperscript{+}) across their cell membranes into their cytoplasm.

1.3.3 Types of alkaliphiles

There are no precise definitions of what characterizes an alkaliphilic or alkali tolerant organisms. The term “alkaliphile” is used for microorganisms that grow optimally or at pH values about 7 to 9, frequently between 8 and 9, but cannot grow or grow only slowly at the near-neutral value of pH 6.5.\textsuperscript{16} Industrial applications of these microorganisms have also been investigated extensively, and some enzymes, such as alkaline proteases, Azoreductase, alkaline amylases, alkaline cellulases, alkaline phosphatases and lipases have been put to use on an industrial scale.\textsuperscript{15,17} Alkaliphiles have clearly gained large amounts of genetic information by evolutionary processes and exhibit an ability in their genes to cope with particular environments; therefore their genes are a potentially valuable source of information waiting to be explored and exploited by the biotechnologists.\textsuperscript{18}

There are three major types of alkaliphiles. a. **Alkaliphiles**: Bacteria which optimally grow below pH 9.0 and can tolerate pH 9.0 to pH 10.0; b. **Facultative alkaliphiles**: Bacteria which grow between neutral pH to pH 10.00; c. **Obligate alkaliphiles**: Bacteria which grow at pH around 10.0 and not below pH 9.0.

The water is alkaline (pH 10.0 to pH 10.5) in Lonar lake; this high alkalinity is due to the high concentration of sodium carbonate. The Lake does not receive any industrial seepage. Although bacterial diversity of this lake was investigated based on identification by culture method supported by molecular method i.e. partial amplification of 16S rRNA gene, studies related to composition of alkaliphiles under different categories such as alkaliphiles, facultative alkaliphiles and obligate alkaliphiles are not completely understood. Although there is a chance of presence of different kinds of alkaliphiles including obligate alkaliphiles, there is lack of systematic data and it is incompletely understood.\textsuperscript{19}
1.4.0 Structure and function of F1FoATP synthase

1.4.1.1 Mitchells model of Chemiosmotic synthesis of ATP

Mitchell studied the mitochondrion, the organelle that produces energy for the cell. ATP is made within the mitochondrion by adding a phosphate group to ADP in a process known as oxidative phosphorylation. Mitchell was able to determine how the different enzymes involved in the conversion of ADP to ATP are distributed within the membranes that partition the interior of the mitochondrion as shown in figure 1.4.

Mitchell showed how these enzymes' arrangement facilitates their use of hydrogen ions as an energy source in the conversion of ADP to ATP. As electrons are passed down the chain, protons are pumped across the membrane (between the inner membrane and outer membrane of the cristae or thylakoids). This results in a pH and electrical gradient. The protons move back into the matrix through a pore created by ATP synthase allowing the enzyme to make ATP.

Fig. 1.4: F1Fo ATP synthase of Mitochondria (Adapted from Weber J. et al 2003)
1.4.1.2 Mechanism of cytoplasmic pH regulation

The central challenge for extremely alkaliphilic Bacillus species is the need to establish and sustain a cytoplasmic pH that is over two units lower than the highly alkaline medium. Its centrality is suggested by the strong correlation between the growth rate in the upper range of pH for growth, i.e., at values above pH 10.5, and the cytoplasmic pH. Diverse mechanisms that maintain active pH homeostasis greatly supplement the contribution of passive cytoplasmic buffering. Major categories of active pH homeostasis mechanisms include: coupling transmembrane proton movements to an energetically favorable exchange with cations (K⁺, Na⁺) or anions (Cl⁻), a strategy that is the central active component of alkaline pH homeostasis; metabolic switching to generate acidic or neutral end-products; acid-induced amino acid decarboxylases and base-induced amino acid deaminases; use of urease activity, sometimes working together with carbonic anhydrase activity, to regulate cytoplasmic and periplasmic pH; synthesis of acid-resistant membrane structures such as cyclopropane fatty acids and tetraether lipids; or increased synthesis of anionic phospholipids or specific neutral lipids at high pH and chaperone protection from temporary damage due to pH shift.

Some mechanisms are regulated as components of larger regulons, such as the RpoS-dependent acid resistance Gad regulon. Others respond to pH with a combination of transcriptional responses together with a substantial component of activity control by pH. For example, the major Na⁺/H⁺ antiporter of *E. coli*, NhaA, is transcriptionally regulated by both sodium and by an RpoS-mediated response to the growth phase but antiport activity is also dramatically and directly enhanced by alkaline pH activation. High resolution structural information recently obtained for NhaA has made it possible to integrate extensive biochemical and genetic data into a detailed model for the mechanism of activity control of the antiporter by pH. Structural biological studies will similarly be central to understanding the specific adaptations in the proton-translocating complexes of the proton cycles that support pH homeostasis in acidophiles and alkaliphiles. Some extremophile adaptations were found in functional assays, but additional examples are emerging as more extremophile genome data becomes available. Examples of adaptations in proton pumps include: the sequence-based proposal that the cytochrome oxidase of two iron-oxidizing acidophiles lacks one of the proton channels found in homologues
from neutralophiles adaptations of extremely alkaliphilic Bacillus species in cytochromes, including cytochrome c, that greatly reduce the mid-point potentials of the alkaliphile proteins relative to neutralophile homologues\textsuperscript{34} alkaliphile-specific sequence motifs in both the caa3-type cytochrome oxidases and alkaliphile-specific motifs in the proton translocating a and c subunits of the ATP synthase that are important in proton capture and retention during ATP synthesis at high pH\textsuperscript{35,36}.

The multiplicity of mechanisms and adaptations observed for bacterial pH homeostasis is perhaps to be expected, given that in principle, every macromolecule with pH-titratable residues is a potential “pH sensor.” Thus, evolution has generated numerous pH-detecting devices that operate independently. Some categories of pH protection mechanisms are ubiquitous, such as cytoplasmic buffering or almost ubiquitous, such as transmembrane proton transport of some type that is observed in all but a few bacteria that confine themselves to a narrow range of pH for example, \textit{Clostridium fervidus}. Other mechanisms are associated with groups of microorganisms that are adapted to particular ranges of environmental pH\textsuperscript{23}.

Acidophiles include iron and sulfur bacteria such as \textit{A. ferrooxidans}, as well as archaea such as \textit{Ferroplasma acidiphilum} that grow under the most extreme acid conditions (close to pH 2.0). Archaeal acidophiles possess tetraether membranes that are highly impermeable to protons. Neutralophiles include the majority of organisms that grow in association with human bodies as well as a majority of those that inhabit soil and most freshwater habitats. Neutralophiles show a wide range of pH-regulating mechanisms that involve heterotrophic metabolism and inorganic ion exchange. Alkaliphilic bacteria that grow well at pH values up to 10.5, for example, the extensively studied \textit{B. pseudofirmus OF4} or \textit{Bacillus halodurans C-125}, were isolated from soil or marine environments that are not consistently extremely alkaline\textsuperscript{37}. Even more extreme alkaliphiles have been isolated from natural enrichments such as soda lakes that typically have a pH above 10 or industrial enrichments, for example, indigo dye plants. In the alkaliphiles studied to date, a Na\textsuperscript{+} transport cycle that is coupled to cytoplasmic proton accumulation plays a major and indispensable role in pH homeostasis.\textsuperscript{22,38} However, extreme alkaliphiles have been isolated from non saline groundwater with a pH of 11.4. Some of these bacteria, for example, \textit{Bacillus foraminis} CV53T, grow better in the
absence than in the presence of sodium. Therefore, net proton uptake cycles that are based on coupling ions other than sodium are likely to be employed in some environments.

1.4.1.3 Cytoplasmic pH measurement

The study of cytoplasmic pH requires careful means of controlling and measuring pH, both inside and outside the cell. The pH of the medium must be maintained either in batch culture, through use of buffers that are pH-appropriate and non-metabolized, or through continuous culture (chemostat). Cytoplasmic pH is measured either by membrane permeant radiolabeled probes of $\Delta \text{pH}$, or by indicators of cytoplasmic pH that are independent of external pH, such as fluorimetry and phosphorus NMR.$^{25}$

![FoF1 ATP synthase proton translocation through c ring rotor of c chain Fo subunit (Adapted from Dittrich M et al 2004)](image)

**Fig. 1.5:** FoF1 ATP synthase proton translocation through c ring rotor of c chain Fo subunit (Adapted from Dittrich M et al 2004)$^{39}$
1.4.2.0 Genes and proteins involved in pH regulation

For targeted identification of specific genes to be practical, a clue is needed as to which gene/protein might be involved in alkaline pH homeostasis. The global proteomic and genomic assays can provide such clues, but critical insights are often obtained from physiological studies. When Na\(^+\)/H\(^+\) antiporters were described by West and Mitchell\(^{40}\) in intact *E. coli* cells, their potential for a role in adaptation to alkaline pH was noted, initiating research on the Na\(^+\)/H\(^+\) antiporters. The absolute requirement for Na\(^+\) for pH homeostasis in alkaliphilic bacteria and diminished Na\(^+\)/H\(^+\) antiporter activity in non-alkaliphilic mutants then provided strong support for an antiporter based pH homeostasis mechanism. Study of a Li\(^+\) resistant mutant led to the first cloning of an antiporter gene, EcnhaA of *E. coli* and its regulator EcnhaR. EcnhaA is the main antiporter of *E. coli* and many enterobacteria. The genome project provided a flood of eukaryotic and prokaryotic putative antiporter genes of which products were grouped by Saier and colleagues\(^{41}\) in the monovalent cation proton antiporter superfamily (CPA).

A recent phylogenetic analysis of database information shows that NhaA belongs to CPA2, a sub family of the monovalent cation/H\(^+\) antiporters, and its orthologs exist in many prokaryotic genomes as well as eukaryotic genomes including that of humans. The CPA1 family includes the main eukaryotic NHE Na\(^+\)/H\(^+\) antiporters and has many prokaryotic orthologs such as MjNhaP1. The Mrp-type antiporters, classified as the CPA3 family, are the main antiporters of alkaliphilic bacilli. They were discovered when a partial mrp/sha operon was found to complement a pH homeostasis negative and Na\(^+\) sensitive phenotypes of an alkaliphilic *Bacillus halodurans* C-125 mutant. Transpositional insertion libraries of *B. subtilis* for Na\(^+\)- and alkali sensitive mutants led to the identification of Bs-Tet (L) as a multifunctional (tetracycline-metal+) (Na\(^+\)) (K\(^+\))/H\(^+\) antiporter with a role in pH homeostasis.\(^{42}\) Once antiporter deficient strains of *E. coli* were constructed, functional complementation screens have become a common route to clone new antiporters genes and study the encoded antiporters.\(^{42}\)
1.4.3.1 F1Fo ATP synthase

ATPases (or ATP synthases) are membrane-bound enzyme complexes/ion transporters that combine ATP synthesis and/or hydrolysis with the transport of protons across a membrane as shown in figure 1.5. ATPases can harness the energy from a proton gradient, using the flux of ions across the membrane via the ATPase proton channel to drive the synthesis of ATP. There are different types of ATPases, which can differ in function (ATP synthesis and/or hydrolysis), structure (F, V and A ATPases contain rotary motors) and in the type of ions they transport. FATPases (F1F0 ATPases) in mitochondria, chloroplasts and bacterial plasma membranes are the prime producers of ATP, using the proton gradient generated by oxidative phosphorylation (mitochondria) or photosynthesis (chloroplasts). VATPase (V1V0 ATPases) primarily found in eukaryotic vacuoles, catalysing ATP hydrolysis to transport solutes and lower pH in organelles. An ATPases (A1A0 ATPases) are found in Archaea and function like F-ATPases. P ATPases (E1E2 ATPases) are found in bacteria and in eukaryotic plasma membranes and organelles, and function to transport a variety of different ions across membranes. E ATPases are cell-surface enzymes that hydrolyze a range of NTPs, including extracellular ATP.42

The membrane-bound ATP synthase (F1Fo) from mitochondria, chloroplasts and bacteria plays a crucial role in energy-transducing reactions. In the case of *Escherichia coli*, the reversible, proton-translocating ATP synthase complex consists of two different entities, F1 and F0, as shown in figure 1.6. *Escherichia coli* is a well known model organism with respect to ATP synthase structure and has a quaternary structure composed of eight subunits. The F1 domain has five subunits, αβγδε, and ε, in the proportion αβγδε. The F0 portion of ATP synthase consists of three subunits, a, b, and c, and has one a component, two b components, and between 10 and 14 c components. It is associated with the membrane embedded Fo complex, which functions as a proton channel. The circular “head” portion of the ATP synthase complex, which encompasses the three sites for ATP catalysis, is a hexamer that consists of an alternating pattern of three α and three β subunits. The α and β subunits have an N terminal β-barrel, a nucleotide binding site, and a C-terminal α-helix. Connected to the center of this “head” complex is the central stalk, which is comprised of one γ and one ε subunit. The γ subunit consists of two α-
helices arranged in a coiled-coil configuration. Attached to the side of the “head” for stabilization is the peripheral stalk, which is one $\delta$ subunit on top of two $b$ subunits. The membrane-embedded portion of the F0 domain contains one $a$ subunit right next to a circular ring of $c$ subunits that surrounds and interacts with the central stalk. The proton channel in the F0 domain is in between the $a$ and $c$ subunits.\textsuperscript{43} The water soluble F1 part carries the catalytic sites for ATP synthesis and hydrolysis. Subunit $b$ was isolated by preparative gel electrophoresis, acetone-precipitated and renatured in a cholate-containing buffer.\textsuperscript{44} Reconstituted subunit $b$ together with purified $ac$ subcomplex is active in proton translocation and F1 binding, thereby demonstrating that subunit $b$ had recovered its native conformation.\textsuperscript{44}
Fig. 1.6: F1Fo ATP synthase reconstructed image based on structural data available produced by different groups. The intersubunit arrangement is fitted into the electron microscopy density map and is therefore speculative. Subunits or subunit domains are shown in different colors (adapted from Wilkens et al 1998).

Circular dichroism spectroscopy of subunit $b$ reconstituted into liposomes revealed a rather high degree of α-helical conformation of 80%. After addition of a His6-tag to the N terminus of subunit $a$, a stable $ab2$ subcomplex was purified instead of a single subunit $a$, arguing in favor of a direct interaction between these subunits. After addition of subunit $c$ and reconstitution into phospholipid vesicles, a Fo complex was obtained exhibiting rates of proton translocation and F1 binding comparable with those of
wild-type Fo. The epitopes of monoclonal antibodies against subunit \(c\) are located in the hydrophilic loop region (cL31–Q42) as mapped by enzyme-linked immunosorbent assay using overlapping synthetic heptapeptides.\(^{45}\)

Binding studies revealed that all monoclonal antibodies (mAbs) bind to everted membrane vesicles irrespective of the presence or absence of F1. Although the hydrophilic region of subunit \(c\), and especially the highly conserved residues cA40, cR41, cQ42 and cP43, are known to interact with subunits g and e of the F1 part, the mAb molecules have no effect on the function of Fo, either in proton translocation or in F1 binding. However, the F1 part and the mAb molecule(s) are bound simultaneously to the Fo complex, suggesting that not all \(c\) subunits are involved in the interaction with F1.\(^{45}\)

1.4.3.2 ATP synthase Fo subunit a subunit: structure and function

The structure of subunit \(a\) of ATP synthase has been probed by construction of more than one hundred monocysteine substitutions.\(^{46}\) Surface labeling with 3-N-maleimidyl-propionyl biocytin (MPB) has defined five transmembrane helices, the orientation of the protein in the membrane, and information about the relative exposure of the loops connecting these helices. Cross-linking studies using TFPAM-3 (N-(4-azido-2,3,5,6-tetrafluorobenzyl)-3-maleimido-propionamide) and benzophenone-4-maleimide have revealed which elements of subunit \(a\) are near subunits \(b\) and \(c\).\(^{46}\) Use of a chemical protease reagent, 5-(-bromoacetamido)-1,10-phenanthroline-copper, has indicated that the periplasmic end of transmembrane helix 5 is near that of transmembrane helix 2.\(^{47}\)

Major function of \(a\) subunit is in the proton uptake pathway. Highly conserved Arginine of \(a\) subunit uptake proton in the form of hydronium ion and transport it to the outer helix of \(c\) chain of ATP synthase Fo subunit. This \(a\) subunit is membrane embedded.

1.4.3.3 ATP synthase Fo subunit c subunit

Subunit \(c\) (also called subunit 9 or \(c\) chain of ATP synthase Fo subunit, or proteolipid in F-ATPases, or the 16 kDa proteolipid in V ATPases) was found in the F0
or V0 complex of F and V ATPases, respectively. In F ATPases, ten c subunits form an oligomeric ring that makes up the F0 rotor.

The flux of protons through the ATPase channel drives the rotation of the C subunit ring, which in turn is coupled to the rotation of the F1 complex gamma subunit rotor due to the permanent binding between the gamma and epsilon subunits of F1 and the C subunit ring of F0. The sequential protonation and deprotonation of Asp61 of subunit C is coupled to the stepwise movement of the rotor. Atomic force microscopy and cryoelectron microscopy studies conducted on bacterial Na\textsuperscript{+} –ATP synthase confirmed that the rotor is undecameric. According to structural analyses, c subunit rotor comprises 10 c- subunits in yeast and 14 in chloroplasts ATP synthase.\footnote{48}

In V ATPases, there are three proteolipid subunits (c, c and c) that form part of the proton-conducting pore, each containing a buried glutamic acid residue that is essential for proton transport, and together they form a hexameric ring spanning the membrane.
As shown in figure 1.7, most important residues in the center of the bilayer are D\textsuperscript{61} (Aspartic acid) of c subunit and R\textsuperscript{210} (Arginine) of a subunit. Their concerted interaction is required for proton movement. Putative access channels for ingree/egress of protons are shown. “The rotor” c ring carries protons around on protonated Aspartic acid of c subunit as it rotates.\textsuperscript{20}

1.4.4.0 Bioenergetics in Alkaliphiles

Alkaliphilic microorganisms are widely distributed in nature. Among them, several aerobic alkaliphilic Bacillus species have been studied in terms of their
mechanisms of physiological adaptation under an extremely alkaline condition. On the basis of chemiosmotic theories, neutrophiles produce H\(^+\) electrochemical potential (\(\Delta p\)), which is the sum of transmembrane pH gradient (\(\Delta p\)H) (alkaline, inside) and membrane potential (\(\Delta \psi\)) (negative, inside), for active transport of solutes, motility, and ATP synthesis. In the case of alkaliophiles, it seems that Mitchell's chemiosmotic theories alone cannot explain clearly their positive H\(^+\) electrochemical potential (\(\Delta p\)) across the membrane because these bacteria exhibit \(\Delta p\)H in a direction opposite to that in neutrophiles, which seems to be causing extensively negative to produce energy, theoretically. Nevertheless, it is reported that ATP synthesis is more rapid at high alkaline pH than at near neutral pH in the facultative alkaliophile *Bacillus pseudofirmus OF4*. The respiratory system of alkaliophilic microorganisms might have an important role in compensating the reversed transmembrane pH gradient by means of ATP synthesis. To understand the function of the respiratory system in alkaliophiles, several respiratory components in alkaliophilic *Bacillus* species were isolated and characterized\(^{38, 52, 115}\). In these studies, respiratory components of alkaliophiles exhibiting several unique characteristics are identified. Extreme alkaliophiles do not use the much larger electrochemical gradient of sodium via a sodium coupled synthase, probably because outward sodium movements must be coupled to proton accumulation for purposes of pH homeostasis, and a primary sodium cycle would subvert that major process.

1.4.4.1 Transmembrane proton gradient in alkaliophiles

Alkaliophilic bacteria isolated from soda lakes, such as Lake Natron in Tanzania, grow at pH values as high as pH 14 while maintaining internal pH at 9–10. Thus, their pH is inverted, with 10,000 fold higher H\(^+\) concentration inside the cell. How do these alkaliophiles maintain a proton motive force driving protons inward? Maintaining internal pH several units lower than outside the cell seems incompatible with the generation of a proton potential that runs ATP synthase. So how do these organisms make ATP?\(^{49}\)

One model to explain ATP synthase coupling at high pH was proposed by in 2010\(^{49}\) (Fig. 1.8). They proposed that protons pumped by the electron transport system are sequestered just outside the membrane and directed into the ATP synthase (Fig. 1.9)\(^{49}\)
This model (as yet unproven) would require a cell compartment, or trap, for protons outside the cell membrane or else some kind of direct connection between the ETS proton pumps and the ATP synthase.

1.4.4.2 Mechanism of cytoplasmic pH regulation in alkaliphiles

Bacterial pH homeostasis is important for physiology, ecology and pathogenesis. Unravelling the phenomenology of bacterial pH homeostasis has depended on the continued refinement of techniques that accurately measure internal and external pH.

The structure of the hypothetical proton coupling connection is unclear, but one prediction from the model is that the membrane embedded Fo component of ATP synthase, which transduces proton flow, would have a form specialized for alkaliphilic conditions. Krulwich and colleagues compared the peptide sequences of Fo from an alkaliphilic species, *Bacillus pseudofirmus* OF4, with that of a neutralophilic species of the same genus, *B. megaterium*. Six amino acid differences were observed in the sequences of the membrane embedded a and c subunits, which mediate the proton flux.

Mutant strains of the alkaliphile *B. pseudofirmus* were obtained, each of which had an amino acid substitution at one of sites 1 to 6 (Fig 1.8). Four of the respective mutants failed to synthesize ATP when grown at high pH (mutation sites 1, 2, 4, and 6). This observation shows that the alkaliphile has evolved a form of ATP synthase specifically adapted to function at high external pH. It is consistent with a hypothesis that the alkaliphile’s cell membrane contains a special proton compartment or coupling device to drive ATP synthesis, although other explanations are possible.

As shown in figures, 1.8 and 1.9, left panel indicates ATP synthase structure in the alkaliphile *B. pseudofirmus* OF4, showing the membrane location of subunits a and c. Right panel indicates location of mutant residues in subunits a and c chains that inactivate the ATP synthase at high pH. Sites on a and c chains have special amino acids, as per reported studies. Mutations that inactivate ATP synthase are shown as position numbers 1, 2, 4 and 6. 49
**Fig. 1.8:** Proposed Alkaliphile specific FoF1 ATP synthase subunit a and c chains by Krulwich TA.

**Fig. 1.9:** Unknown mechanism of ETS generated protons sequestration outside the cell membrane (Adapted from Krulwich TA).
Alkaliphiles maintain an inverted pH gradient. At external pH several units higher than internal pH, how do alkalophilic bacteria make ATP? As per the proposed mechanism\(^{49}\), protons pumped by the electron transport system might be sequestered just outside the membrane and directed into the ATP synthase (Fig. 1.9).

### 1.4.4.3 ATP synthesis in alkaliphiles

ATP synthases of alkalophilic bacteria and, in particular, those that successfully overcome the bioenergetic challenges of achieving robust H\(^+\) coupled ATP synthesis at external pH values >10. At such pH values the protonmotive force, which provides the energetic driving force for ATP synthesis, is too low to account for the ATP synthesis observed. The protonmotive force is lowered at a very high pH by the need to maintain a cytoplasmic pH well below the pH outside, which results in an energetically adverse pH gradient. Several anticipated solutions to this bioenergetic conundrum have been ruled out. Although the transmembrane sodium motive force is high under alkaline conditions, respiratory alkalophilic bacteria do not use Na\(^+\) instead of H\(^+\) coupled ATP synthases. Nor do they offset the adverse pH gradient with a compensatory increase in the transmembrane electrical potential component of the protonmotive force. Moreover, studies of ATP synthase rotors indicate that alkaliphiles cannot fully resolve the energetic problem by using an ATP synthase with a large number of c subunits in the synthase rotor ring. Increased attention now focuses on delocalized gradients near the membrane surface and H\(^+\) transfers to ATP synthases via membrane associated microcircuits between the H\(^+\) pumping complexes and synthases. Microcircuits likely depend upon proximity of pumps and synthases, specific membrane properties and specific adaptations of the participating enzyme complexes. ATP synthesis in alkaliphiles depends upon alkaliphile specific adaptations of the ATP synthase and there is also evidence for alkaliphile specific adaptations of respiratory chain components.

### 1.4.4.4 ATP synthase F\(_o\) subunit a chain in alkaliphiles

Most of the studies were reported from one of the prominent facultative alkaliphile i.e. \textit{B. pseudofirmus} OF4. A topological representation, (as indicated in figure 1.10) of the putative five transmembrane helix structure of the \textit{B. pseudofirmus} OF4 \textit{a} subunit with
the location of the residues in TMH4 and TMH5 that were mutated as well as selected alkaliphile specific residues of interest in other regions that are not conserved in the α subunit from thermoalkaliphile C. thermarum; the replacements made in each location are indicated. Residues mutated to the residue found in C. thermarum at that position have a white background while other mutations have a black background.

Fig. 1.10: A topological representation of the putative five transmembrane helix structure of the B. pseudofirmus OF4 α subunit (adapted from Fujisawa 2010)\textsuperscript{132}.

The final finding of interest was the phenotype of the mutant, K180G/G212K, that, switched the position of the Lys\textsuperscript{180} with that of alkaliphile specific Gly\textsuperscript{212}. The retention of synthetic function supported earlier evidence that the two residues are part of the proton uptake pathway and function interactively in cytoplasmic proton retention.\textsuperscript{130} The enzyme also retains native ATPase and DCCD sensitivity profiles that are roughly comparable to wild type. Thus the observed proton leakiness, which probably accounts both for the malate-growth deficit and for the somewhat low and highly variable synthetic capacity at pH 10.5, is not the result of a completely disrupted structure. Rather, the re-positioned Lys\textsuperscript{180} may be compromised in a direct role in preventing proton leaks or may be compromised in a role in enhancing the ability of Arg\textsuperscript{172} to prevent proton loss through short circuits during rotary function of the enzyme. In alkaliphiles, specific
adaptations of both the $c$ and $a$ subunits of the alkaliphile ATP synthase are critical to both the ATP synthesis that achieves this proton capture and to prevention of loss of cytoplasmic protons through the synthase.

1.4.4.5 ATP synthase $F_0$ subunit $c$ chain in alkaliphiles

Various groups characterized ATP synthase $c$ subunit. Liu et.al.\textsuperscript{113} reported systematic comparative sequence studies of these bacteria. Panel A of figure 1.11 shows an alignment of the $c$ subunit of the ATP synthase from several alkaliphiles and neutralophiles. The organisms shaded are the alkaliphilic Bacillus species. Residue numbers are given for the $B.\ pseudofirmus$ $OF4$ (top) and $Ilyobacter$ $tartaricus$ $c$ subunits (bottom). The alanines of the alkaliphile $AxAxAxA$ motif are shaded with colors corresponding to those in panel B, and $PxxExxP$ motifs and the $RQPE$ periplasmic loop region are shaded in gray. The two serines of a distinct thermoalkaliphile motif are shaded in gray, as they play roles in packing of the $Alkalibacillus$ $thermarum$ ring. The open boxes show the conserved $GxGxGxG$ motif in the inner helix of selected neutralophiles. Asterisks denote that residues were deleted from the N-terminal end of the longest $c$ subunits to maximize the size of the motif areas, 5, 6, and 10 residues from the subunits of $Escherichia$ $coli$, $Spirulina$ $platensis$, and $I.\ tartaricus$, respectively. Panel B of figure 1.11 shows location of the $AxAxAxA$ motif in the $c$-ring structure of $B.\ pseudofirmus$ $OF4$. The four alanines are shown in different colors. The red spheres represent the bound $H_2O$ molecules. The close-up shows the $c$-ring on the level of the ion binding site perpendicular to the membrane as viewed from the cytoplasm and the alanines displayed with the same color coding.\textsuperscript{113}

In one of the major site directed mutagenesis studies it was suggested that the specific amino acids are required for facultative alkaliphile to sustain life at non fermentative oxidative phosphorylation at high alkaline pH. They found that the alkaliphile specific features of $a$ and $c$ subunits of ATP synthase were critically involved in OXPHOS under non fermentative growth. The experimental design involved was specific mutagenic changes to the consensus sequence for non alkaliphiles that was likely to allow most of the mutants to assemble an active ATP synthase. The current
observation of diverse pH 10.5 specific deficits in fermentative growth and OXPHOS features found in these mutants demonstrate that the alkaliphile specific features of the ATP synthase were important for OXPHOS at pH 10.5.35

Fig. 1.11: Comparison of ATP synthase c subunit of various alkaliphiles and neutrophiles (Aapted from Liu J et al 2011)113

The cartoon model to the right of the alignment (fig. 1.12) shows a putative topological arrangement of five TMH of the B. pseudofirmus OF4 a subunit with the C terminus emerging on the N side of the membrane. The Lys180 and Gly212 found in alkaliphilic Bacillus species are highlighted in blue, the circled conserved Arg172 is highlighted in red, and four other residues that are found in alkaliphilic Bacillus species, but not generally in non alkaliphiles, are circled.30
Fig. 1.12: ATP synthase a subunit alignment of alkaliphiles and neutrophiles. The names of the alkaliphiles are in blue, neutrophiles in black and bacteria with Na+ translocating ATP synthases in green. (Adapted from Hicks DB 2010) 

Fig. 1.13: ATP synthase c subunit alignment of alkaliphiles and neutrophiles. Alkaliphile specific protein sequence in B. pseudofirmus and other alkaliphiles compared with non alkaliphiles GxGxGxG in N terminal and G/AxxExxP in C terminal region (Adapted from Krulwich TA et al 2011).
Recently, Janto et al.\textsuperscript{52} reported involvement of various transporter proteins including secondary transporter proteins. The ABC type MDRs (multi drug transporters) included in the 36 total MDRs are also counted in the ABC transporter total of 49. The selected groups of secondary transporters that are shown have potential or established roles in (as indicated in figure 1.14): cytoplasmic pH homeostasis (yellow); sodium dependent efflux of toxins and uptake of nutrients, ions or compatible solutes (gray); and the TRAP-T transporters, with DCA (dicarboxylic acids as a substrate), which are highlighted in purple because the 24 loci significantly exceeds the 16 loci found in the \textit{B. halodurans} C-125 genome (Adapted from Janto B et al 2011).\textsuperscript{52}

\textbf{Fig. 1.14:} Recently reported protein including secondary transporter proteins involved in pH homeostasis.\textsuperscript{52}
As the direction of transmembrane proton gradient (∆pH) in alkaliphile is exactly opposite to that in neutrophiles, explanation based on Mitchell’s theories of proton translocation pathway for ATP synthesis could not satisfactorily explain mechanism of pH homeostasis in alkaliphiles. Various groups have studied mechanism of proton binding, retention and transportation for ATP synthesis in alkaliphiles. Some groups suggested presence of alkaliphile specific ATP synthase a and c subunits and Na⁺/H⁺ antiporter dependent pH homeostasis as the major strategy for pH homeostasis of extremely alkaliphilic Bacillus species. Recent development in molecular studies of facultative alkaliphile indicates presence of highly conserved AxAxAxA motif in the inner amino terminal helix and PxxExxP motif in the outer carboxy terminal helix of ATP synthase c chain and highly conserved lysine residue at 180 (Lys⁽¹⁸⁰⁾) position in a chain in widely studied Bacillus pseudofirmus OF4 strain, an established facultative alkaliphile. It will be interesting to find out experimental evidence based on actual alkaliphile from environmental samples other than Bacillus pseudofirmus OF4.

As per review of published literature, there are no precise definitions of what characterizes an alkaliphilic or alkalitolerant organisms. The term “alkaliphile” is used for microorganisms that grow optimally or very well at pH values about 7 to 9, often between 8 and 9, but cannot grow or grow only slowly at the near neutral value of pH 7.0. Industrial applications of these microorganisms have also been investigated extensively, and some enzymes, such as alkaline proteases, alkaline amylases, alkaline cellulases, alkaline phosphatases, azoreductase and lipases have been put to use on an industrial scale. Alkaliphiles have clearly gained large amounts of genetic information by evolutionary processes and exhibit an ability in their genes to cope with particular environments; therefore, their genes are a potentially valuable source of information waiting to be explored and exploited by the biotechnologists. Various groups from Indian subcontinent isolated different valuable bacterial strains but most of the molecular studies were limited to bacterial diversity based on 16S ribosomal RNA and basic understanding of adaptation of ATPase a and c subunits at molecular level is not completely understood.
1.5.0 Applications of alkaliphile in Bioenergy generation

Although, due to their unique alkaliphilic properties, bacteria isolated from Prestine Crater Lake Lonar, were explored for various applications including biotransformation and bioremediation, alkaline phosphates and lipases etc., specific information of their uses in bioenergy generation is not completely understood. Out of various applications mentioned in the introductory part of this chapter, this section provides detail information on applications of alkaliphiles microbial fuel cell for bioenergy generation. Most of the information on MFC technology is based on physical part of the MFC but biological aspects i.e. type of alkaliphilic bacteria was least covered.

1.5.1 Microbial Fuel Cell (MFC) basic principle and design

An MFC is a device that converts chemical energy to electrical energy by the catalytic reaction of microorganisms. In a Microbial Fuel Cell, bacteria catalyze the oxidization of organic substrates and transfer the resulting electrons to an anode. These electrons then move through an electrical circuit to the cathode where they are transferred to an electron acceptor like oxygen. Extensive research has been performed throughout the world on bioelectricity production from a range of fermentation products and waste organic materials. Studies of MFCs have been focused on the attachment and growth of the biofilm of anode respiring bacteria (ARB) and the means of transferring electrons to the surface of anode.

A fuel cell is an electrochemical conversion device. It produces electricity from fuel on the anode side and an oxidant on the cathode side, which react in the presence of an electrolyte. The reactants flow into the cell, and the reaction products flow out of it, while the electrolyte remains within it. Fuel cells can operate virtually continuously as long as the necessary flows are maintained. Fuel cells are different from electrochemical cell batteries in that they consume reactant, which must be replenished, whereas batteries store electrical energy chemically in a closed system. Additionally, while the electrodes within a battery react and change as a battery is charged or discharged, a fuel cell's electrodes are catalytic and relatively stable. Many combinations of fuel and oxidant are possible. A hydrogen cell uses hydrogen as fuel and oxygen (usually from air) as oxidant. Other fuels include hydrocarbons and alcohols. Other oxidants include chlorine and
chlorine dioxide. The disadvantages of fuel cells are Fuel cells are in general slightly bigger than comparable batteries or engines. However, the size of the units is decreasing. Fuel cells are currently expensive to produce, since most units are hand-made.

**Fig. 1.15:** Schematic diagram of typical two-chamber microbial fuel cell (adapted from Du Z 2007)

Microbial fuel cells (MFCs) are devices which convert organic matter to energy (electricity or hydrogen) using microorganisms as catalysts. Generally bacteria are used in MFCs to generate electricity while accomplishing the biodegradation of organic matters or wastes. Schematic diagram (Fig. 1.15) shows typical two-chamber microbial fuel cell for producing electricity. It consists of anodic and cathodic chambers partitioned by a proton exchange membrane (PEM). The anode compartment is typically maintained under anoxic conditions, whereas the cathode can be suspended in aerobic solutions or exposed to air. Electrons flow from the anode to the cathode through an external electrical connection that typically includes a resistor, a battery to be charged or some other electrical device. Microbes in the anodic chamber of an MFC oxidize added substrates and generate electrons and protons in the process. Carbon dioxide is produced as an oxidation product. However, there is no net carbon emission because the carbon dioxide in the renewable biomass originally comes from the atmosphere in the
photosynthesis process. Unlike in a direct combustion process, the electrons are absorbed by the anode and are transported to the cathode through an external circuit. After crossing a PEM or a salt bridge, the protons enter the cathodic chamber where they combine with oxygen to form water. Microbes in the anodic chamber extract electrons and protons in the dissimilative process of oxidizing organic substrates.  

Microbial fuel cell (MFC) employing low-cost materials Non-coated plain graphite electrodes) without any toxic mediators (aerated cathode and mediator less anode) was evaluated under alkaliphilic (anode pH of 9) conditions using anaerobic media to enumerate the influence of substrate loading rate on bioelectricity generation from anaerobic wastewater treatment at ambient temperature (28.2 °C).

1.5.2 Microorganisms used in earlier studies

Bacteria capable of transferring electrons to an electrode are referred to as anode respiring bacteria (ARB) or exoelectrogens. This electron transfer is performed by the bacteria either through a direct transfer or by using an electron shuttling compound or mediator. Obligate and facultative anaerobic bacteria use certain metals or inorganic oxidized compounds as their terminal electron acceptors. Iron (III) is one of the most common metals used by bacteria under anaerobic conditions. In an MFC, this iron (III) is replaced with the anode electrode (usually a carbon material). In many investigations, microbes were initially enriched for iron (III) reduction activity before being employed in an MFC. Some of the first reported ARB includes Clostridium, Geobacter, Aeromonas, Rhodoferax, Desulfobulbus, and Shewanella. Gamma proteobacteria, Beta proteobacteria, Rhizobiales, and Clostridia are predominantly found on the anode surface.

It was suggested that a synergistic interaction exists in an MFC that would account for the higher power densities obtained by using a mixed rather than a pure culture. For example, some species in a mixed culture may act fermentative, degrading the more complex organic material and providing the breakdown products to the ARB, which would then oxidize the organic compound completely. Several modifications in the design of MFCs have been adopted to improve current and power densities. A dual chambered configuration is the most common one applied in research.
Fig. 1.16: Proteins involved in mechanism of microbial fuel cell technology. Involvement of inner and outer membrane bound proteins in MFC. (Adapted from Lovely DR 2008)\textsuperscript{147}

The anode and the cathode materials for the reactor were being selected based on several properties, including high surface area, improved chemical stability, biocompatibility (anode), and good conductivity. The choice of materials and conditions, and the understanding of the mechanism by which the bacteria attach and grow on these electrodes are important to obtain better yields of current. By augmenting the electrochemical potential achieved by bacteria in this MFC with an additional voltage of 250 mV or more, it was possible to produce hydrogen at the cathode directly from the oxidized organic matter. More than 90\% of the protons and electrons produced by the bacteria from the oxidation of acetate were recovered as hydrogen gas, with an overall Coulombic efficiency (total recovery of electrons from acetate) of 60–78\%. This is equivalent to an overall yield of 2.9 mol \( \text{H}_2/\text{mol acetate} \) (assuming 78\% Coulombic efficiency and 92\% recovery of electrons as hydrogen).\textsuperscript{59}
The discovery and usage of new anodophilic microbes that vastly enhance the electron transport rate from the biofilm covering an anode to the anode are much needed to improve the power density output in MFCs. Mutagenesis and rDNA technology can conceivably be used in the future to obtain some “super bugs” for MFCs. Microbes may be used as a pure culture or a mixed culture forming a synergistic microbial consortium to offer better performance. One type of bacterium in a consortium may provide electron mediators that are used by another type of bacterium to transport electrons more efficiently to an anode. Furthermore, there are many microorganisms yet to be discovered that might be beneficial for electricity production.

The well coordination efforts of different scientific fields like electrochemists, materials scientists, engineers and microbiologists is well require in the development of the several potential practical applications of microbial fuel cells. Even if the generation of high levels of electricity from microbial fuel cells is a long way off, an understanding of the coupling of organic matter oxidation to electron transfer to electrodes is likely to yield important insights into the diversity of microbial respiratory capabilities and might lead to as-yet-unforeseen applications in nano-electronics. Extremophilic microorganisms in MFCs have been applied extensively, particularly thermophiles and acidophiles. However, the application of halophilic or alkaliophile bacteria in MFCs has not been investigated extensively.

### 1.5.3 Mechanism of generation of electric potential in Microbial Fuel Cell:

In normal microbial catabolism, a substrate such as a carbohydrate is initially oxidized anaerobically when its electrons are released by enzymatic reactions. The electrons are stored as intermediates (e.g., Nicotinamide adenine dinucleotide -NADH, quinones) which become reduced and are then used to provide the living cell with energy. The ending location for the electrons is molecular oxygen or dioxygen at the end of the respiratory chain. A MFC uses bacteria to catalyze the conversion of organic matter into electricity by transferring electrons to a developed circuit. Microorganisms can transfer electrons to the anode electrode in three ways: exogeneous mediators (ones external to the cell) such as potassium ferricyanide, thionine, or neutral red; using mediators produced by the bacteria; or by direct transfer of electrons from the respiratory enzymes.
(i.e., cytochromes) to the electrode as shown in figure 1.16. These mediators can divert electrons from the respiratory chain by entering the outer cell membrane, becoming reduced, and then leaving in a reduced state to shuttle the electron to the electrode. The basic reactions are presented below; when micro-organisms consume a substrate such as sugar in aerobic conditions they produce carbon dioxide and water. However when oxygen is not present they produce carbon dioxide, protons and electrons as described below:

**Anodic reactions :**

\[
12\text{CO}_2 + 48\text{H}^+ + 48\text{e}^- \rightarrow 2\text{C}_12\text{H}_{22}\text{O}_{11} + 13\text{H}_2\text{O}
\]

**Cathodic reaction :**

\[
2\text{H}_2\text{O} \rightarrow \text{O}_2 + 4\text{e}^- + 4\text{H}^+ 
\]

A microbial fuel cell (MFC) is a device that converts chemical energy to electrical energy with the aid of the catalytic reaction of microorganisms. An MFC consists of anode and cathode separated by a cation-specific membrane i.e. salt bridge. In the anode compartment of an MFC microorganisms oxidize fuel (substrate) generating electrons and protons. The protons and electrons are transferred to the cathode compartment, the protons through the membrane and the electrons through the external circuit. They consume reducing oxidant at the cathode, usually oxygen supplied by aeration;

\[
4\text{H}^+ + 4\text{e}^- + \text{O}_2 \rightarrow 2\text{H}_2\text{O} \text{ or } 4\text{H}^+ + 4\text{e}^- + 2\text{O}_2 \rightarrow 2\text{H}_2\text{O}_2
\]

The bacterial cells in the anode of MFC consume substrate as fuel and transfers electrons directly to the electrode. In the subsequent studies it has been shown that electrochemically active microbes can be enriched using an electrochemical fuel cell type. Several steps have been identified as the limiting steps in a mediator-less MFC. They are (1) fuel oxidation at the anode, (2) electron transfer from microbial cells to anode, (3) resistance of the circuit, (4) proton transfer through the membrane, and (5) oxygen reduction at the cathode. In a mediator-less MFC, as in other MFCs, the membrane separates the anode from the cathode and the membrane functions as an electrolyte that plays the role of an electronic insulator and allows protons to move through. These functions of the membrane are believed to be indispensable in the operation of an MFC.
Many microorganisms can contribute to electricity production in microbial fuel cell. Recently researchers have discovered a new metabolic type of electricity-producing microorganisms that has indicated that a wide diversity of organic compounds can be effectively converted to electricity in self-sustaining microbial fuel cells. These organisms, known as electricigens, can completely oxidize organic compounds to carbon dioxide, with an electrode serving as the sole electron acceptor and conserve energy to support growth from this electron transfer.

1.5.4 Mediator Microbial Fuel Cell

Most of the microbial cells are electrochemically inactive. The electron transfer from microbial cells to the electrode is facilitated by mediators such as thionine, methyl viologen, methyl blue, humic acid, neutral red and so on. Most of the mediators available are expensive and toxic.

1.5.5 Mediator-less Microbial Fuel Cell

Mediator-less microbial fuel cells does not require a mediator but uses electrochemically active bacteria to transfer electrons to the electrode (electrons are carried directly from the bacterial respiratory enzyme to the electrode). Some bacteria, which have pili on their external membrane, are able to transfer their electron via these pili. Mediator-less MFCs are a much more recent development and this affects the optimum operation, such as the bacteria used in the system, the type of ion membrane, and the system conditions such as temperature, are not particularly well understood. Bacteria in mediatorless MFCs typically have electrochemically-active redox enzymes such as cytochromes on their outer membrane that can transfer electrons to external materials.

Experimental data showed the feasibility of power generation from waste water treatment. However, the overall voltage generation, power yield and substrate degradation was found to depend on the substrate/organic loading rate (OLR). Maximum potential difference of 650 mV was recorded at stable operating conditions. Apart from power generation, the fuel cell also demonstrated substrate removal (62.5%). Applied OLR documented marked influence on both power yield and substrate degradation rate.
Voltage and current started decreasing due to substrate exhaustion (COD reduction) in the anode chamber. It is evident from the experimental data that the anodic chamber of MFC mimicked anaerobic suspended growth reactor normally used for wastewater treatment with respect to COD removal. The study documented the advantage of both wastewater treatment and electricity production in a single system.

Application of MFCs for wastewater treatment is very attractive due to energy recovery from waste as well as reducing production of excess sludge, disposal of which is very expensive. It is expected that this process would generate much less excess sludge than a conventional activated sludge process, since the major part of energy available from the oxidation of the organic contaminants is converted to electricity, and the remaining energy is used for microbial growth.

1.5.6 Factors affecting the MFCs efficiency

**Electrode Material:** Type of material used in electrode preparation will show vital effect on MFCs efficiency. Better performing electrode materials usage will always improve the performance of MFC because different anode materials result in different activation polarization losses. Platinum and platinum black electrodes are superior to graphite and carbon-cloth electrodes for both anode and cathode constructions, but their costs are much higher. Schroder et al. reported that a current of 2–4 mA could be achieved with platinum zed carbon-cloth anode in an agitated anaerobic culture of *E. coli* using a standard glucose medium at 0.55 mmol/L. Platinum also has a higher catalytic activity with regard to oxygen than graphite materials. MFCs with Platinum or platinum coated cathodes yielded higher power densities than those with graphite or graphite felt cathodes.

**pH Buffer and Electrolyte:** If no buffer solution is used in a working MFC, there will be an obvious pH difference between the anodic and cathodic chambers, though theoretically there will be no pH shift when the reaction rate of protons, electrons and oxygen at the cathode equals the production rate of protons at the anode. The PEM causes transport barrier to the cross membrane diffusion of the protons, and proton transport through the membrane is slower than its production rate in the anode and its consumption rate in the cathode chambers at initial stage of MFC operation thus brings a pH...
difference. However, the pH difference increases the driving force of the proton diffusion from the anode to the cathode chamber and finally a dynamic equilibrium forms. Some protons generated with the biodegradation of the organic substrate transferred to the cathodic chamber are able to react with the dissolved oxygen while some protons are accumulated in the anodic chamber when they do not transfer across the PEM or salt bridge quickly enough to the cathodic chamber. It is possible that the buffer compensated the slow proton transport rate and improved the proton availability for the cathodic reaction. This again suggests that the proton availability to the cathode is a limiting factor in electricity generation. Increasing ionic strength by adding NaCl to MFCs also improved the power output possibly due to the fact that NaCl enhanced the conductivity of both by anolyte and the catholyte.

**Proton Exchange System:** Proton exchange system can affect an MFC system's internal resistance and concentration polarization loss and they in turn influence the power output of the MFC. The ratio of PEM surface area to system volume is important for the power output. The MFC internal resistance decreases with the increase of PEM surface area over a relatively large range. Membranes and Kaolin septum are prone to fouling if the fuel is something like municipal wastewater. Membrane-less MFCs are desired if fouling or cost of the membrane becomes a problem in such applications.

**Operating conditions in the anodic chamber:** Substrate type, concentration and feed rate are important factors that impact the performance of an MFC. Power density varies greatly with different substrates using same given microbe or microbial consortium. Electricity generation is dependent on substrate concentration both in batch and continuous-flow mode MFCs. Usually a higher substrate concentration yields a higher power output in a wide concentration range. It is reported that a higher current level with lactate (as substrate) concentration increased until it was in excess at 200 mM in a single-compartment MFC inoculated with *S. putrefaciens*. It is also found that the current increased with a wastewater concentration up to 50 mg/L in their MFC. It is noted that there is definite effect of substrate concentration on the performance of an MFC and it is showed that the power density was increased with the increase in substrate concentration.
Operating conditions in the cathodic chamber: Oxygen is the most commonly used electron acceptor in MFCs for the cathodic reaction. Power output of an MFC strongly depends on the concentration level of electron acceptors. Studies indicated that dissolved oxygen was a major limiting factor when it remained below the air-saturated level. Surprisingly, a catholyte sparged with pure oxygen that gave 38 mg/L dissolved oxygen did not further increase the power output compared to that of the air-saturated water. Rate of oxygen diffusion toward the anode chamber goes up with the dissolved oxygen concentration. Surely changing operating conditions can improve the power output level of the MFCs. The bottlenecks responsible for the low power output are: Low rate of metabolism of the microbes in the MFCs; the biotransformation rate of substrates to electrons has a fixed ceiling which is inherently slow.

To improve the MFCs efficiency one should be focused on how to break the inherent metabolic limitation of the microbes for the MFC application. As we know high temperature can accelerate nearly all kinds of reactions including chemical and biological ones. Use of thermophilic species might benefit for improving rates of electron production, however, to the best of our knowledge, no such investigation is reported in the literature. Therefore this is probably another scope of improvement for the MFC technology from the laboratory research to a real applicable energy source.

1.7.0 Aims and objectives
The aim of this research is to correlate existing hypothesis of adaptation of alkaliphiles at high alkaline pH conditions with reference to F1F0ATP synthase a and c subunit comparative studies. As most of the reported studies on Lonar Lake include molecular identification based on 16S ribosomal RNA, basic understanding of why these bacteria are highly adapted to such alkaline conditions is incompletely understood. With this aim, present study was planned to compare and analyze alkaliphile specific domains of ATP synthase gene of selected alkaliphiles from Lonar Lake with following objectives:

1. To isolate alkaliphiles from Lonar lake and to identify by 16S rRNA studies
2. To perform molecular studies on a and c chains of ATP synthase gene

1.8.0 Thesis outline

First introductory chapter is all about Lonar Lake including literature search on established studies with reference to alkaliphiles, facultative and obligate alkaliphiles and their properties studied by earlier worker and other institutions. Second part of this chapter was devoted to literature search on ATP synthase in bacteria in general, structure and function of the enzyme and current hypothesis on working model of ATP synthase of bacteria.

Second chapter is all about isolation and identification of bacteria from Lonar and non Lonar sources. This study deals with isolation, characterization and identification of two obligate and five facultative alkaliphile from Lonar Lake and seven bacterial strains isolated from sources other than Lonar Lake. Non Lonar Lake sources were selected as: four neutrophiles from Mula river Pune, two alkaliphiles from hen’s egg stool sample present on egg shell surface and one acidophile from Citrus limon peel i.e. canker. For bioinformatics based approach for differentiation of bacteria, 16 S rRNA sequences were selected from known and established acidophiles, neutrophiles and alkaliphiles. These comparative studies are based on growth conditions with reference to pH of the growth medium, molecular studies involving 16S rRNA sequence determination of all selected total bacterial isolates, sequence alignment and its phylogenetic relationship for initial identification of bacteria based on complete available 16S rRNA sequence.
Third chapter is devoted to detail analysis of 16S rRNA hypervariable regions of all selected bacterial isolates from Lonar Lake with earlier reported obligate and facultative alkaliphile isolated from Lonar lake as well as from non Lonar Lake sources.

The fourth chapter, which is a heart of the project, is devoted to studies on ATP synthase of two bacterial species originally isolated from Lonar Lake. Entire F_o subunit of both the bacteria were studied for analysis of a and c chains of ATP synthase of *Lysinibacillus DL15* and *Stenotrophomonas DL18*. To find out why alkaliphiles from Lonar Lake are adapted to alkaline pH, complete study on ATP synthase F_o subunit a and c chains of two aerobic facultative alkaliphiles were carried out to test existing hypothesis of proton transportation pathway for pH homeostasis in facultative alkaliphiles i.e. presence of alkaliphile specific amino acids in a and c chains of F_o subunit of ATP synthase. As both most of the reported studies are based on Bacillus pseudofirmus as a model organism which is aerobic, bacillus type gram positive facultative alkaliphile, originally isolated both strains in the present study were analyzed separately based on their similarity and difference with existing model organisms. *Lysinibacillus DL15* strain is gram positive, bacillus, facultative alkaliphile whereas, *Stenotrophomonas DL18* is gram negative, non bacillus, facultative alkaliphile.

Fifth and last chapter is based on how to apply isolated alkaliphile while keeping common goal of differentiation of obligate and facultative alkaliphiles. By using both alkaliphiles, electricity can be generated in one of the tested experiment of microbial fuel cell (MFC). MFC is not new for reporting use of bacteria, but for obligate and facultative alkalophilic bacterial differentiation, this may prove significant with reference to choice of microbe for application in MFC, and can expand this field whenever selection of bacteria is concerned for utilization in MFC.