CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

New Players in the Sulfur Cycle the Microbial Ecology of Anaerobic and Archaeal Sulfur Reduction; A Step Towards Efficient Microbial Enhanced Oil Recovery

by

Rabiya Shahid

A thesis submitted in partial fulfillment for the degree of Master of Science

in the

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Dedicated to **Allah Almighty, Hazrat Muhammad (SAW)** and my Parents whose utter support and sheer trust help me to achieve this.

CERTIFICATE OF APPROVAL

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Abstract

Crude oil is the world's largest source of energy being used as a means of economy. As the price of a barrel oil rises, crude oil reserves decline, development of oil resources in existing deposits is crucial to meet future energy needs. As the modern recovery technologies commonly used have become less effective for uses, there is a persistent need for the development of a new technology that helps to improve heavy crude oil. More than 2 trillion barrels of conventional oil remain reserves worldwide since traditional production techniques have been depleted. Oil reservoirs are extreme environments for microbial life characterized by high toxicity, hydrophobicity and low water activity, as well as high temperature, salinity, and pressure. Nevertheless, oil reservoirs offer a broad range of niches for a multitude of bacteria and archaea, such as sulfate-, nitrate-, and iron-reducers, fermenters, acetogens, and methanogens. Microbial enhanced oil recovery (MEOR) is a significant tertiary oil recovery approach that is cost-effective and sustainable technique to move residual oil embedded in reservoirs. The ability of microorganisms to dissolve crude oil to minimize viscosity in MEOR is known to be very successful. In this study physicochemical characteristics of micro and macro nutrients were measured by using AAS method. Six water samples from Nandpur, Panjpir and Bahu oil and gas field and four soil samples from Nourag and Rajian were taken. Metagenomics analysis was done at sequencing depth of 85000 to 89000. Results shows highest pH (8.5) in Rajian (RJ1) and lowest pH (6.3) in Bahu (BH1). Alpha and beta-diversity analysis was done that showed highest concentration of nitrates and sulphur found in Panjpir (PN1) and Nandpur (NP2). Archaeal rich diversity of phylum *Euryarchaeota* and *Thaumarchaeota* and bacterial phylum *Acidobacteria, Actinobacteria, Bacteriodete, Firmicutes, Planctomycetes, Protobacteria* and *Chloroflexi* were found dominating in reported samples.

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Abbreviations

BDL	Below Detection Limit
BH	Bahu
CN	Chak Nourang
EOR	Enhanced Oil Recovery
ER	Enhanced Oil Recovery
MEOR	Microbial Enhanced Oil Recovery
NP	Nanpur
PN	Penjpir
Rj	Rajion
SBR	Sulphate Reducing Bacteria
SRB	Sulphate Reducing Bacteria

Chapter 1

Introduction

1.1 Background

Population of world is increasing continuously, there is 45 percent increase in it within next 4 decades and there will be more than 9 billion people by the middle of the century. Per capita energy is a good indicator for quality life, suggesting energy requirements will keep rising with population of the world and the desire to improve standards of living [1]. State's economic development and stability will rely on how well people manage their energy requirements and resources. How we should satisfy future demand for more energy is an important issue [2]. New techniques and appliances just retrieve approximately one third to a half of the oil confined in a reservoir. Roughly 1 trillion barrels of oil (0.16 Tm^3) were recovered globally, but two to four trillion remains in reservoirs of the oil that are focus of the Enhanced oil recovery (EOR) technologies [3]. Over 300 billion barrels of oil (47.6 Gm^3) remain irrecoverable in the United States but after conventional technologies they attain their economic boundaries [4].

Pakistan has oil and gas resources that are distributed in whole country and include many prospective reservoirs. Approx., total oil reserves in Pakistan are twenty-seven billion barrels and other recoverable reserves are approx. 936 million barrels. The capacity of crude oil refining is 13 million tones. 18.6 million Tones oil is

imported in the country [5]. Presently, Pakistan is producing oil with primary and secondary recovery, 69,286 bbl per day approximately. According to EIA, USA 2012 statistics the verified oil reserves of Pakistan are 0.31 billion barrels [6].

Pakistan is blessed with massive resources and it also contains a vast amount of oil and gas wells. In Baluchistan, the Sui place and some areas of Sindh contain a greater amount of these resources. But unluckily, these recourses are not appropriately channelized by the government of Pakistan rather overseas companies are exploring and playing a great part in the production. The demand for oil and gas is increasing day by day and the local production is too low regardless of the fact that Pakistan has its own oil and gas resources and also has the potential to produce it by self [7]. Table 1 shows the Pakistan oil sector overview.

Table 1.1: Pakistan's oil sector overview

Total proved Reserves	Total oil Production	Total crude oil production	Total oil consumption
0.31 billion barrels	62.09 thousand barrels/day	59.08 thousand barrels/day	426.72 thousand barrels/day
Imports	Refinery capacity	Exploratory	Wells Up to 2012 Discovered
634.43 thousand barrels/day	286 thousand barrels/day	785	69 (OIL)

New technologies are needed for recovering trapped oil and slowing down production declines in the limited wells to upsurge oil reserves [8]. Recently, the MEOR technique has become prevalent in recovering trapped oil, therefore a considerable knowledge of the multi-phase flow characteristics of reservoir rock as well as the different strategies are important for the achievement of any MEOR project, including microbial ones [9]. Several classes of microbes were discovered from oil reservoirs with varying physiological and metabolic capacities and phylogenetic relationships. The presence of indigenous microbial communities in oil reservoirs can tolerate an underground deep biosphere, that is sovereign of primary

productivity above the surface [10], coupled with the proven ability of anaerobic micro-organisms to consume multiple oil components [11], It is currently a well-established scientific datum that retains and harbors numerous bacterial and archaeal species in oil reservoirs [12]. This research focuses on the occurrence of one of the various groups of microbes in oil fields that are sulfate-reducing SRB bacteria, their role in MEOR, and the potential to mediate various metabolic processes that occur in oil fields.

1.2 Sulfate Reducing Bacteria

The element Sulphur is present in most wide form on Earth. It is present in several forms such as pyrite, gypsum in rocks and sediments and sulphate in seawater. The complexity of Sulphur cycle is due to its wide-ranging oxidation states that are -2 to +6 and it can be altered biologically as well as chemically [13]. In transformation of sulfur into different forms, microorganisms play an important role (figure 1.1).

Sulphur from nutrients reduced to sulphide followed by sulphide assimilation and become a part of amino acids and proteins. Redox reactions include sulphide oxidation by chemo lithotrophic Sulphur bacteria and sulphate reducing bacteria SRB are found to be very important for the production or generation of metabolic energy [14].

A community of microbes using sulfate (SO_4^-) as the final acceptor of electrons rather than breathing oxygen is known as sulphate reducing bacteria (SRB) [16]. SRB use wide range of low molecular organic materials to grow, while reducing sulphate to hydrogen sulphide [17]. In oil fields SRB's are considered as dangerous for production processes because they cause problems in oilfield water system such as iron rusting and reduction of injection water due to precipitation of amorphous ferrous sulphide [18].

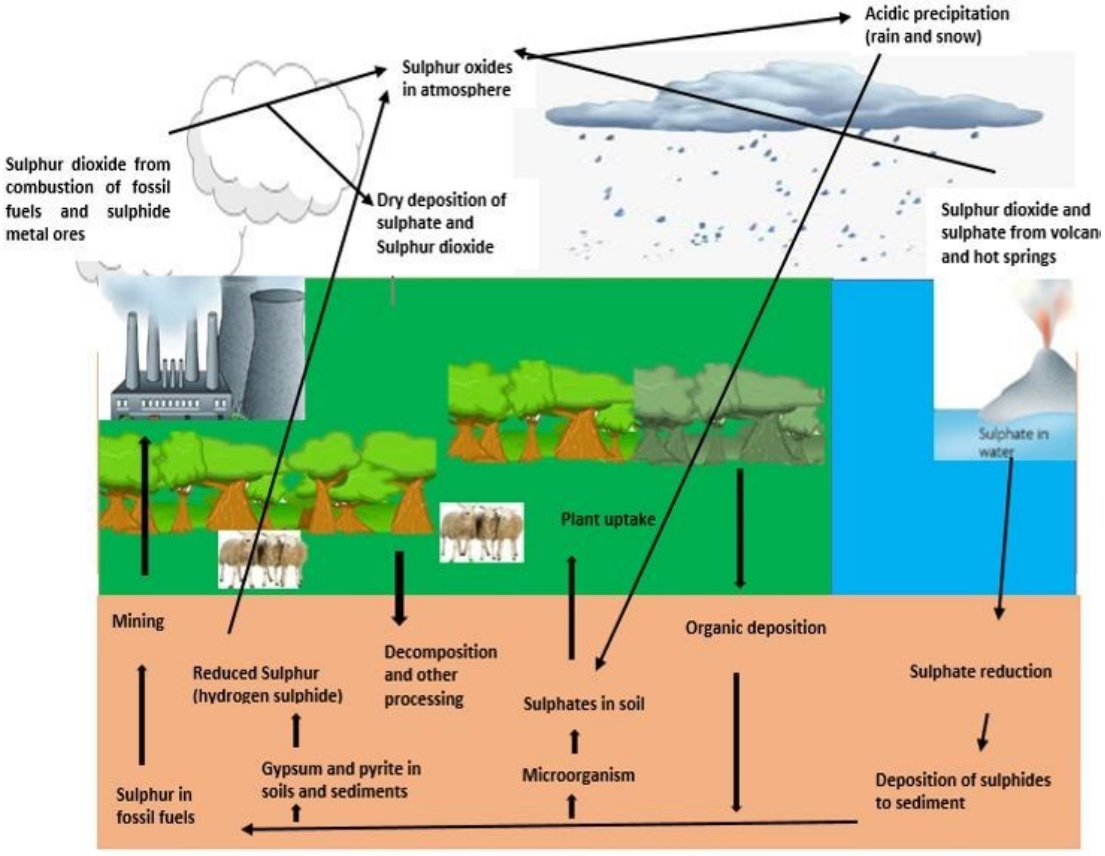

FIGURE 1.1: Shows Sulphur cycle. Sulfur, which is a vital element, is absorbed by microorganisms and plants, and ultimately by animals, as sulfate. Dead organism decomposition in the absence of oxygen releases the Sulphur as hydrogen sulphide again. The burning of fossil fuels and the disposal of volcanic gases releases sulfur dioxide into the atmosphere, where it reacts with water, thereby producing sulphuric acid and causing acid rain. Madigan, M. T. et al. (2006) Pearson Education.

Several studies have recently reported that sulphur reducing bacteria can play a significant role for Microbial Enhanced Oil Recovery [19] e.g sulphate reducing bacteria may minimize the oil's viscosity, replenish the reservoir's declining pressure. Several kinds of SRB's are widely present in global oil reservoirs, therefore can be considered as trademark to improve oil recovery [20].

SRB improved the surface activity of its metabolites including interfacial and surface tensions, oil recovery rate from saturated oil sand, and effectiveness of emulsification. Many previous studies demonstrated that to reduce interfacial tension among oil and water, metabolites act as biosurfactants generated by some kind of microbes, thus improves the oil mobility and solubility [21].

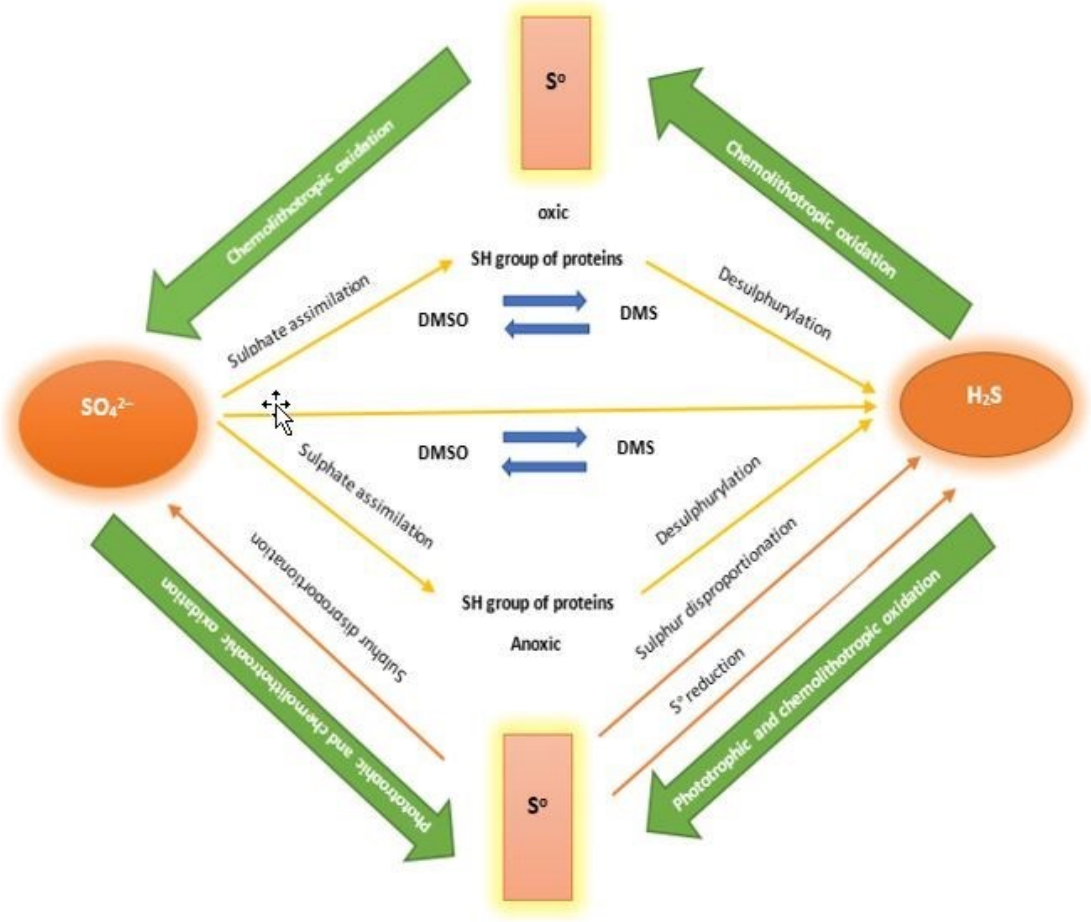

FIGURE 1.2: Transformations of sulfur. Bacteria that reduce sulphate have a major role throughout Sulphur cycle. sulphate (SO_4^{2-}) in the breakdown of organic matter as a terminal electron acceptor, that resulted in the development of hydrogen sulphide (H_2S). Subsequently, chemolithotrophic sulphur-oxidizing bacteria or phototrophic sulphur bacteria to elemental sulphur (S) and (SO_4^{2-})can aerobically oxidize the sulfide. Many processes performed out by different groups of micro-organisms lead to a decrease in Sulphur [15].

It has been stated that SRB could directly use alkanes, aromatics and other oil components as electron donors for SO^{4-} reduction [22], thereby changing oil compositions, thus involved in bio-degradation of total petroleum hydrocarbons (TPH). SRB metabolites could expressively diminish surface tension and IFT and were also able to recover oil from saturated oil sand with a recovery rate of 39.2%. Moreover, SRB metabolites showed remarkable emulsion effectiveness with crude oil. Microbial metabolites contain amphipathic molecules (both hydrophilic and hydrophobic) capable of reducing both ITF and surface tension [23].

1.3 Types of SRB (Sulfate Reducing Bacteria)

In many anaerobic environments, SRB create different prokaryotic groups which subsidize a number of vital functions. Sulphate reducing bacteria involve in many essential processes, so they are studied extensively in last few years. The SRB populations are systematized into 4 distinctive groups: Gram positive spore forming Sulphur reducing bacteria (include genus *Desulfotomaculum*), thermophilic bacterial (*Thermodesulfobacterium commune* and T*hermodesulfovibrio yellowstonii* specie), Gram negative mesophilic (include *Desulfurella, Desulfuromusa, Desulfuromonas,Myxobacteria, Bdellovibrio, Pelobacter, Geobacte*) and thermophilic archaeal sulphate reducing bacteria [24].

1.4 Isolated Strains of SRB from Oil Reservoirs

Sulfate-reducing bacteria (SRB) is the first species which were recovered from the oilfields. Sulphate reducing capacity is associated four bacterial phylae that are *Proteobacteria, Firmicutes, Nitrospir*a and *Thermodesulfobacterium* along with *Arechaea, Euryarchaeota* and *Crenarchaeota. Archaeal* domain isolates belong to three to four bacterial phyla as well as Euryarchaeota were identified. Namely *Desulfovibrio, Desulfomicrobium, Desulfovermiculus halophilus, Desulfobacter, Dsulfobulbus, Desulfotignum, Desulfobacterium, Desulfacanium, Thermosulforhabdus, Desulfotomaculum, Desulfurispora, Desulfovirgula, Desulfosporosinus, Thermodesulfobium, Thermodesulfobacterium, Thermodesulfatator, Archaeoglobus, Caldivirga* strains of SRB were isolated from oil [25]. Other microbes that are isolated from the oil reservoirs included Methanogens (Hydrogenotrophic methanogens, Methylotrophic methanogens, Aceticlastic methanogens), Fermentative microorganisms, Hyperthermophiles, Syntrophic microorganisms, Autotrophs, and Nitrate, iron, and manganese reducers.

Pakistan has significant resources of oil and gas that if used strategically can help to accelerate growth on the continent. To fulfill the energy requirements, Pakistan

primarily relies on oil and gas reserves. The domestic oil resources are not enough to fulfill the rising energy thirst. That's why Pakistan has to import massive quantities of oil and oil-based products from middle east countries [26].

The purpose of writing this research is to highlight the present position of petroleum reservoirs through the physiochemical analysis of samples for selected traits and concentration of Macro and Micronutrients. Furthermore, Oil reservoirs are the home of microbial species that are phylogenetically and metabolically diverse. our understanding of the phylogenetic diversity, metabolism, ecological roles and population dynamics of microbial oil reservoir species is not complete [28]. Absence of microbiological understanding of the oil reservoir also contributes to deleterious effects, like souring or plugging [27]. Nevertheless, microbiological knowledge can be used to improve the procedures, therefore, in this study prevalence of the Sulfur oxidizing gene in metagenomically analyzed samples and statistical analysis is carried out to obtain the knowledge necessary to make MEOR process efficient.

1.5 Problem Statement

Regardless of major efforts to limit our energy reliance on fossil fuels we still fulfill about 80% of our energy demand from fossil fuels, there is a lot to explore about microbial communities present in the oil field in order to increase oil production to fulfill the future energy demands and to reduce dependence on natural resources [28].

1.6 Aims and Objectives

1. Physicochemical analysis of samples for selected traits and concentration of Macro and Micro nutrients.

2. Prevalence of Sulphur oxidizing genes in samples metagenomically

3. Analysis of correlation between Sulphur reduction into sulfite and sulfide with relevant genes concentration.

Chapter 2

Literature Review

Until 1980 the SRB was recognized as the restricted community of microbes. They are able to partially oxidize some basic organic acids and alcohol's in anaerobic environment. In the same year Widdel (1980) published his study and characterized numerous latest genera and species of sulfate reducers belonging to Eubacterial group. These newly recognized species and genera include Thermophilic Archeal bacterium, which is tentatively referred as the Archeoglobus fulgidus" sequestered from an oceanic hydrothermal origin [1-3]. SRB are polymorphic and exist in many forms some are spherical (2mm in diameter), filamentous (6-8mm) and road shaped (9-13mm). The GC content of its DNA is 34 to 66, suggest significant heterogeneity of SRB [29]. A lot of SRB species can grow autotrophically and they have significant adaptability to use organic matter. The metabolic capabilities among individual SRB species vary a lot and are recognized as genetically diverse group with metabolic versatility [30-32].

2.1 Physiology, Diversity, Distribution of SRB

SRB are able to live in different substrate conditions and are versatile in use of many kind of electron donors and acceptors. They are found in various engineered and natural habitats in the presence of sulphate. They are present abundantly

at saturating oxygen concentrations in hypersaline microbial mats [40]., also detected in habitats like acid mine drainage sites with extreme pH value (low as 2 and high up to 10) [28]. Sulphate reducers have two major groups one includes incomplete degraders of organic compounds in to acetate, other group consist of complete degraders of organic compounds in to carbon dioxide [41]. SRB don't have substrate of polymeric organic compounds (for example DNA, RNA, starch, cellulose, protein), other microbes that degrade these substrates can be used by SRB (figure 2.1) [4-5].

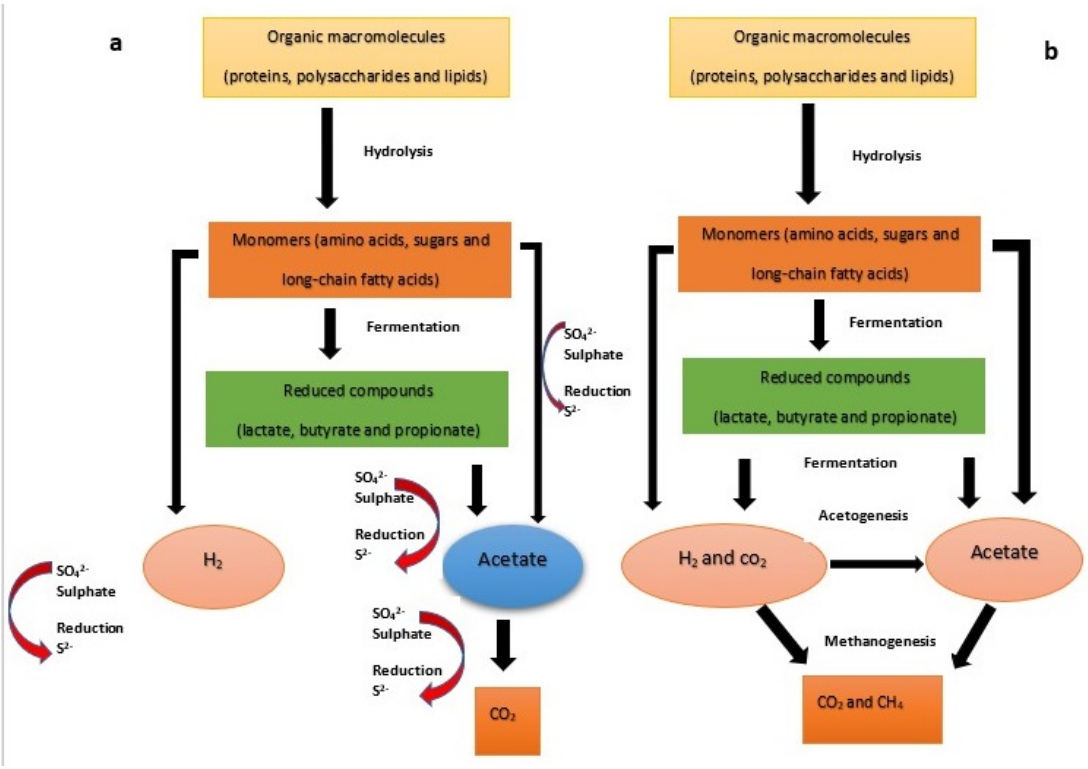

FIGURE 2.1: The sequence of microbial degradation in low oxygen substrate (in the presence and absence of sulfate). Hydrolytic bacteria hydrolyze macromolecules and fermentative bacteria produce many fermentation products. a. In the existence of sulfate SRB consume fermented products produced by fermentative bacteria in this process sulfate doesn't exist. b. Production of hydrogen and acetate (acetate is produced by fermentation or by acetogenesis). [42-43].

2.2 Biotechnology of SRB's

2.2.1 Bioremediation of Organic Compounds

The utilization of bacteria in various (remediation) applications is considered by scientists due to concern for a clean environment and SRB are appropriate candidates. Complex organic molecules utilized as either acceptor or donor of electron by specific isolates of SRB.

2.2.2 Oxidation of Monoaromatic Hydrocarbons

SRB are able to grow with different kind of organic compounds with the utilization of lactate and pyruvate as electron donor [44]. SRB are able to oxidize up to 100 different organic and aromatic compounds in fuel.

2.3 Central Metaboloic Pathway of SRB

SRB's members from genus Desulfoyibrio that are easily and rapidly cultured are sulphate reducers. They are being used for biochemical and physiological researches. Substrate level phosphorylation is inadequate for their growth to support, therefore dissimilatory sulphate reduction in Desulfoyibrio species is associated to electron transport coupled phosphorylation [90]. ATP requirements to minimize sulphate [45], the cytoplasmic localization of two enzymes adenylysulfate (APS) reductase and bisulphate reductase that are involved in pathway of respiratory sulphate reduction [89], periplasmic localization of few hydrogenases and the abundance of multihemic c-type cytochrome are major unique biochemical and physiological characteristics of SRB's of *Desulfoyibrio* [46].

2.3.1 Sulphur Metabolism

Sulphur and the organic compounds of Sulphur plays a major role in Sulphur cycle because of its various oxidation states from +6 in sulphate to -2 in sulphide. Both in an energy producing dissimilatory pathway and an energy consuming assimilatory pathway Sulphur reduction can occur. Several organisms have been identified that have pathway to produce reduced sulphur compounds for S-containing amino acids synthesis and does not proceed to direct sulphide excretion via assimilatory pathway [92]. Also, the path that is limited to bacterial and archaeal anaerobic lines, sulphur is terminal electron acceptor of respiratory chain that produce excess amounts of sulphide via dissimilatory pathway [102-109]. Both pathways begin by the activation of sulphate by reaction with ATP to form adenylyl sulphate (APS) [101]. APS is converted to 3-phosphoadenylyl sulphate and reduced to sulphide that is further reduced to sulphide in dissimilatory pathway.

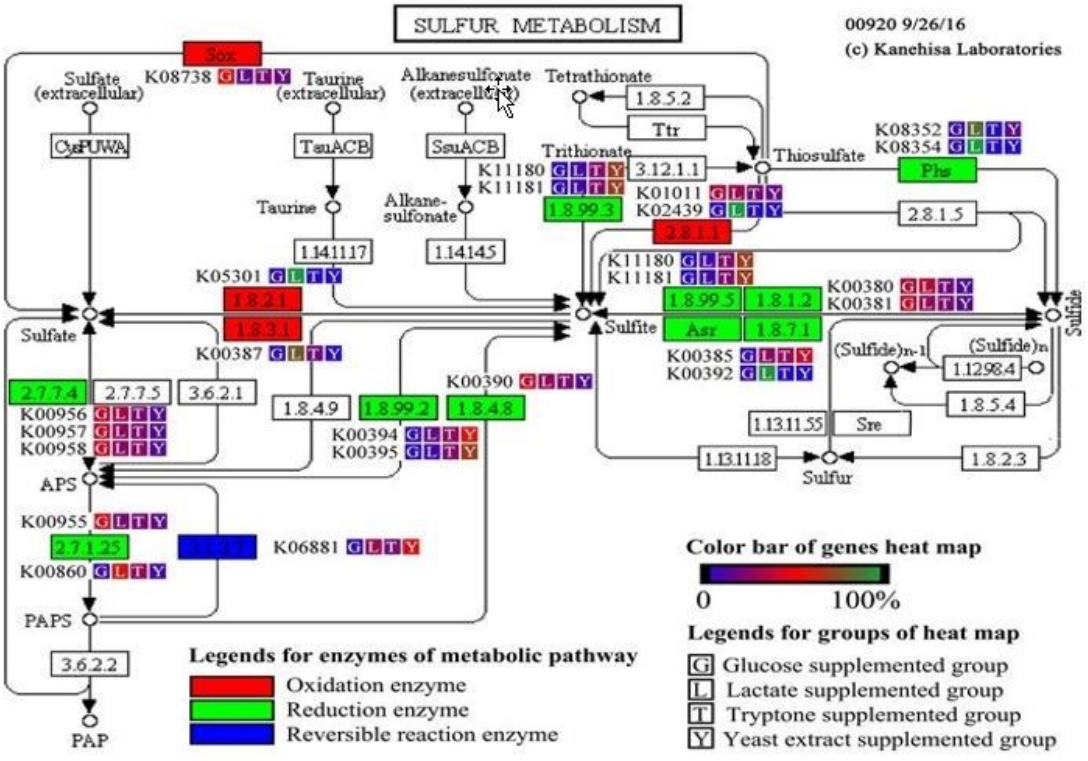

FIGURE 2.2: Differences in the sulfur metabolic pathway in the different groups, sulfur metabolic pathway is obtained by KEGG.). [42-43].

The oxidation ability of sulphur among bacteria and archaea is widespread including both phototrophs as well as chemiolithoautotrophs. The sulphur oxidation

system SOX is found in both groups photosynthetic and non-photosynthetic sulphur oxidizing bacteria with well-known sulphur oxidizing pathway [93-95]. Sulphur compounds such as sulphide, elemental sulphur, thiosulphates are used by Purple sulphur bacteria and green sulphur bacteria in anoxygenic photosynthesis, in the same way as the electron donor for photoautotrophic growth. In some chemiolithoautotrophic oxidizers such as *Thiobacillus denitrificans*, dissimilar sulphur reduction enzymes have been suggested that act in reverse direction creating a Sulphur oxidation pathway from sulfite to APS and then sulphate [96-99].

2.4 Nutritional Aspects of SRB's

SRB's are reported as microaerophilic [47] and they can be autotrophs, litho autotrophs, or heterotrophs. Autotrophs use CO_2 as carbon and obtain electron from the oxidation of H_2. Heterotrophs use organic compound as substrates [48]. Recently it is reported that wide variety of substrate can be used as electron acceptor and donor by SRB's [49] both organic as well as inorganic including sulphates, sulphides, thiosulfates etc [50]. SRB donors include hundreds of various compounds including sugars, amino acids, alcohols, monocarboxylic acids and aromatic compounds [52], [110], [111]. Low molecular weight organic compounds as carbon and energy source is preferred by SRB's [112-121].

2.5 Microbial Enhanced Oil Recovery

2.5.1 Background

Oil production is declining due to maturity of oil fields throughout the world. For example, all major oil fields in North Sea [51]. Increase in energy demand due to global population growth and unavailability of new resources are major factors for this downgrade. Therefore, it is necessary to search out new alternative technologies to increase oil recovery from existing reservoirs because fossil fuel still

remains the key source of energy besides other resources such as solar energy, wind energy etc.

Oil is required to produce fossil fuel energy, 30 to 40% oil is contributed by primary oil recovery while 15-20% is recovered by secondary method leaving behind 35-55% of oil as residual oil in reservoirs, during oil production process [54]. This residual oil is focus of several enhanced oil recovery technologies. This residual oil has production capacity of 2-4 trillion barrels approximately 67% of total oil reserves [88]. Oil companies are looking for cheap and efficient technologies that will raise the global oil production. Methods that are currently used in oil industries are Enhanced Oil Recovery (EOR) also called as tertiary recovery. EOR include Chemical flooding, miscible carbon dioxide injection and thermal enhanced oil recovery methods that uses heat as main source for additional oil recovery [55]. Several companies are focusing on residual oil via EOR technologies [56].

Microbial Enhanced Oil Recovery (MEOR) is based on microorganisms which pull remaining oil from reservoirs and proved to be potential EOR methods [5759]. Approximately 50% of residual oil can be extracted by this very low-cost technology [59].

2.6 Reason for Oil to Left Behind

Fundamental cause for leaving oil behind is Economics. Recovering method of oil form conventional reservoir includes [58].

1. A pathway connecting oil to the surface in the pore space of a reservoir
2. enough reservoir energy to drive the oil onto the surface

2.7 Microbial Enhanced Oil Recovery (MEOR)

MEOR is advanced tertiary oil recovery technique that use Microbes. Normally oil recovering is three stage process. Primary recovery stage recovers 12-15% of the oil without the introduction of any additional substance. In second stage water is flooded and additional substances are introduced. Tertiary phase is the last phase that include several methods including MEOR.

Benefits of MEOR include formation of oil-water emulsions, reduced interfacial tension and clogging the high permeable zones [59]. Microbes can produce useful products by fermentation of low-cost substrate or raw material therefore MEOR can be substitute for Chemical Enhanced Oil Recovery (CEOR) that is expensive. Furthermore, microbial products are biodegradable and have low toxicity [60].

2.8 SRB's Role in MEOR

Oldest microbes on Earth are SRB's Sulphate Reducing Bacteria of Proterozoic Period [61]. Dissimilative sulphate reduction cycle is one of the few metabolic pathways which have note undergone mutation or horizontal gene transfer [63-65]. Based on research a reduction in bacterial sulphate is thought to have evolved earlier than photosynthesis of oxygen [62].

The first study was conducted on metabolism and physiology in 1864 observed the production of biogenically produced hydrogen sulphide in marine sediments. Studies in 1950 and 1960 suggested the role of microbes in drilling corrosion.

SRB's are diverse group of bacteria that are heterotrophic, absolute anaerobic, Gram Negative with exception of Desulfonema. They utilize sulphate and other sulphur compounds [64]. Some SRB's have spore production capability such as *Desulfosporosinus Orientis* [87], *Desulfotomaculum halophilum sp. Desulfosporosinus meridiei sp.* [65]. SRB's are found in crude oil [66]. They produce hydrogen

sulphide and main cause of bio corrosion [67-69]. They have capability of biodegradation [68], [50].

TABLE 2.1: Outlines several species of microorganisms reducing sulfates that have been isolated from crude oil exploitation areas [70].

Species	Salt Content (wt%)	T (oC)	Occurrence
Desulfotomaculum nigrificans	0-4	40-70	Oil field
Desulfacinum infernum	0-5	40-65	
Thermodesulfobacterium mobile	Lack of data	45-85	
Thermodesulforhabdus norvegicus	0.5-6	44-74	
Desulfomicrobium apsheronum	0-8	4-40	Formation water
Desulfovibrio gabonensis	1-17	15-40	
Desulfovibrio longus	0-8	10-40	
Desulfovibrio vietnamensis	0-10	12-45	
Desulfobacterium cetonicum	0-5	20-37	Formation water
Desulphomaculum halophilum	1-14	30-40	Drill bit
Desulfobacter vibrioformis	1-5	5-38	Oil water separation
Desulfotomaculum thermocisternum	0-5	41-75	Marine sediments

The most common mesophilic SRB that cause adverse effects on drilling equipment's include *Desulfovibrio longus, D. Vietnamensis*, and *D. gabonensi*s [69]. As SRB's have several abilities to metabolize various organic compounds such as aliphatic, aromatic and polycyclic aromatic hydrocarbons sulphate reducing bacteria play a vital role in oil reservoirs.

2.9 Field Trial

Microbial enhanced technologies for oil recovery has been tested in laboratory in early 1980s and in 1990 it is shifted to field. Oil reservoirs are very complex biological structures and making laboratory simulations for microbial activities and testing are very difficult. There is competition between microbial consortia and indigenous microflora when introduced in oil reservoirs [71]. To evaluate the effectiveness of microbial processes and to check the validity of laboratory studies and models' fields trials are performed.

The introduction of MEOR in field experiments showed promising results for significant increase in production of oil from reservoirs in comparison of controls [72]. MEOR technology showed net oil production increase to 40% after 12-month treatment. Microbial enhanced water flooding technology has also been applied to field trials in United States [86]. Application of MEOR processes in the field in Asian, Malaysian and Indian oil fields have also been reported [80-85], three approaches through which oil production can be improved are Gas Injection, Chemical Injection and Thermal Method [73-75].

2.10 Enhance Oil Recovery in Pakistan

Oil and Gas resources in Pakistan are scattered in whole country. Pakistan have approximately 27 billion barrels of oil reserves and recoverable reserves are 936 Million barrels (Ministry of Petroleum and Natural resources, Government of Pakistan, "Petroleum Policy 2012"). Pakistan is producing 69286 barrels per day

using primary and secondary recovery (Pakistan petroleum information services, "Upstream petroleum Activities"). There is no Enhanced Oil Recovery EOR been implemented in Pakistan. Oil production can be increased using latest EOR methods. Recently EOR programs have been conducted as tertiary and secondary and they have enhanced main operations over low-pressure reservoirs [102-105].

Usage of the EOR process is inevitable in Pakistan. Implementation of the EOR processes in Pakistan requires extensive research, development, and expertise. The decision to initiate the EOR projects depends more on an evaluation of economics. Extensive laboratory and numerical simulation work are however necessary to check the feasibility of the EOR Project. The first step to evaluating the feasibility is analytical technical screening of the EOR process for a particular reservoir.

Chapter 3

Materials and Methods

3.1 Sample Collection /Sampling Site Description

The water and soil samples were collected from deep oil well cavities of Punjab platform namely Nandupur (NP) (Nandpur gas field is located about 60km north-east of Multan city in Punjab province) Panjpir (PN) (Panjpir gas field located approximately 70 kilometers north-east of Multan in Punjab province) Bahu (BH) (Bahu gas field is located approximately 220km from Multan Punjab Province) chak Nourag (CN) Rajian (RJ) (Chak Nourag and Rajian oil field is located in Chakwal in Punjab Province) There were about 6 water samples 2 collected from each site (Nandpur Panjpir Bahu) at the depth of about 1717m-1884m. 2 Soil samples in duplicate from (CN RJ) were collected at a depth of about 2515m A detailed description of the samples was provided in table 3.1.

Table 3.1: Detail of water and soil samples.

Sample	Well Name	GL (m)	Reservoir	Reservoir Depth (m)	Lithology	Reser. Tempe
BH	Bahu-02	146	Samanasuk	1717-1733	Carbonate	56

Table 3.1 continued from previous page

NP	Nandpur-05	141	Lumshiwal	1792-1797	Sandstone	60
PN	Pinjpir-10	143	Samanasuk	1878-1884	Carbonate	59
CN	Chak Nourag-5A	142	Lower Sakessar	1000-2515	sandstone	61
RJ	Rajian-8	143	Nara Mughlan	2000-3550	Sand stone	60

The samples were provided by Oil and Gas Development Company Ltd. Samples were collected in falcon tubes and were preserved in the refrigerator and then utilized for analysis.

3.2 Geographical Location of Sampling Site

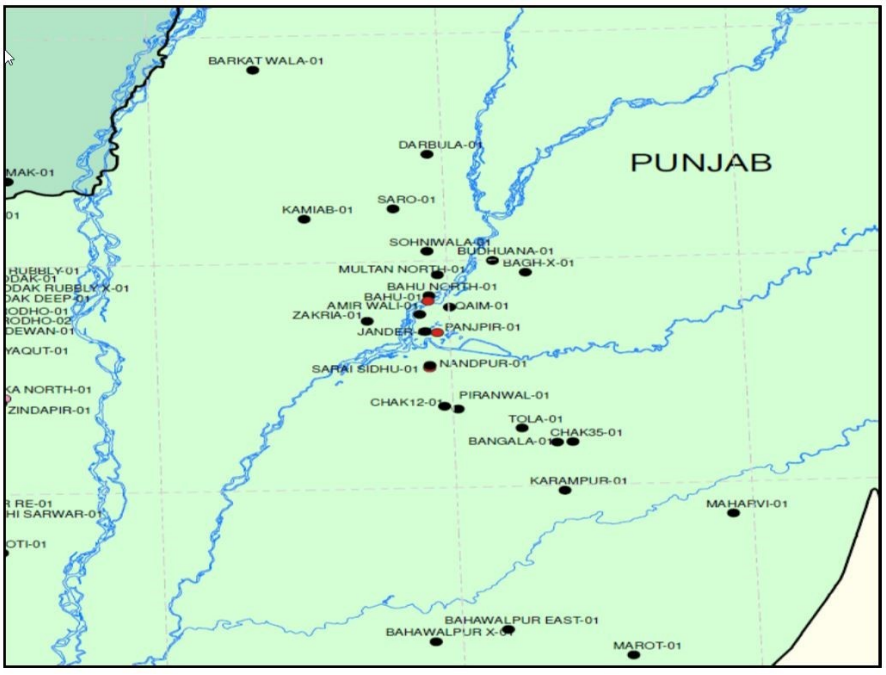

FIGURE 3.1: Site Map of Nandpur Penjpir and Bahu Gas Field

FIGURE 3.2: Site Map of Chak Naurang Oil Field

FIGURE 3.3: Site Map of Rajion Oil Field

3.3 Research Methodology

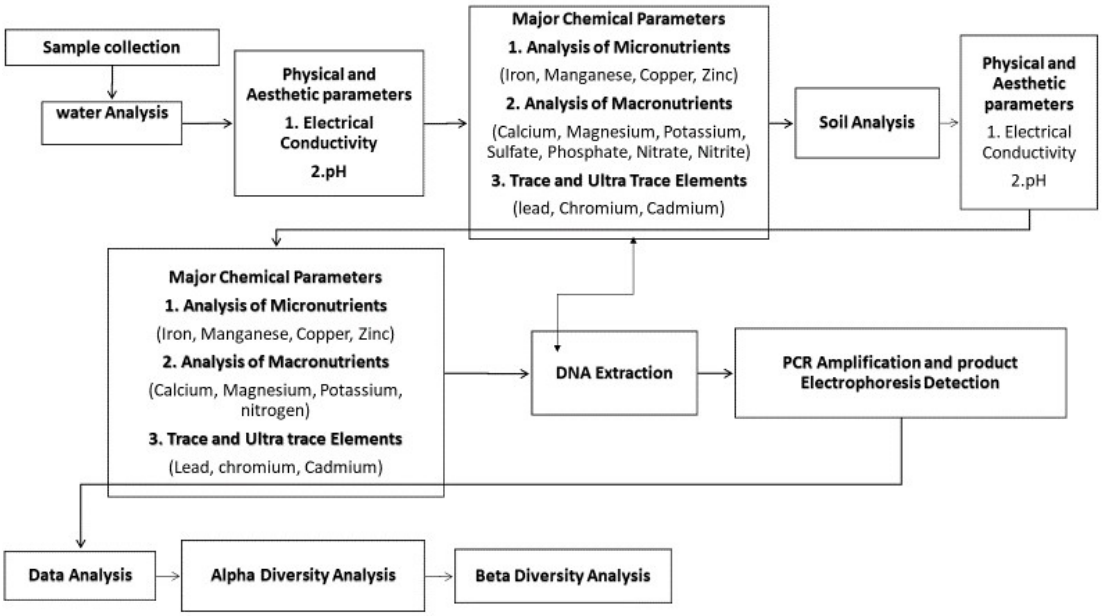

FIGURE 3.4: Methodology Overview

3.3.1 Soil Analysis

3.3.1.1 Soil Sample Drying

Soil samples were dried in wooden trays soil were dried in air. Soil can be dried by placing the trays in racks in hot air cabinet. The temperature for drying should be between below 35 degree Celsius and humanity content should be 30 to 60%. Excessive drying by oven should be avoided to ensure availability of the nutrients in sample [109]. Drying process had minimal effect on total Nitrogen content but NH_4 and NO_3 content may vary with time and temperature. Drying at high temperature leads to the death of microorganisms present in soil samples

3.3.1.2 Soil Sample Preparation

Soil samples were grounded to fine powder using wooden pestle and mortar samples were converted to fine state using 2-mm sieve so that the heterogeneity can be reduced and also to provide max. surface area for physiochemical reactions [99].

3.3.1.3 Soil Sample Digestion

1 gram of prepared dried soil sample was taken in 250 ml beaker and 15ml of aqua Regia was added. This mixture was then digested at 70 degree Celsius up to the transparency of sample solution. The digested solution was filtered using filter paper No 42. Filtered solution was then diluted to 50ml using deionized water [111]. Sample solution was analyzed for con. of Pb Cr Cd Ca Fe Na Cu Mn Mg and K via flame atomic absorption spectrophotometer (Perkin Elmer Analyst 7000).

3.3.2 Chemical Analysis of Soil

The content of calcium, magnesium, potassium sulphate, phosphate, nitrate, nitrite, copper, iron, manganese, lead, chromium, calcium, and zinc from soil samples was determined by AAS Analyst 7000. Compressor was turned ON after fixing nitrous oxide and acetylene gas. Extra trapped liquid was removed. AAS and Extractor controls were turned ON. Slender tube and nebulizer were cleaned, the acetylene gas pressure was set at 700 KPa that is equivalent to 100 psi and the valve was set to 11 psi for acetylene and 45 psi for air. The new sheet on Spectra AA software was opened, "Add Method" was used and desired element for analysis was selected. Type, Optical, SIPS parameters were selected accordingly.

Joined PC was used for the worksheet of ASS programming. Empty cathode was used for light embedded in light holder. The beam was adjusted to hit target zone of the arrangement cards for required light throughput. At that point the machine was turned off [61-63]. 10 ml graduated chamber containing deionized water was used to estimate the yearning rate. Calibration solutions were prepared along with analytical blank. Both were atomized and response was measured. Graph was plotted for each solution than sample solutions were atomized. The concentration of various elements from sample solution was determined [78-79].

3.3.3 Water Analysis

3.3.3.1 Apparatus

Apparatus required for water analysis was pH meter containing combined electrodes, TFE beakers, stirrer with plastic coating and plastic wash bottle.

3.3.3.2 Reagents

Reagents required were Potassium Chloride 0.01m, 0.745g KCL was dissolved in DI water and solution was brought to 1 liter, buffer solutions for pH 4 and 7, boiled and cooled distill water that had conductivity less than 2 micromhos/cm.

3.3.3.3 Procedure

PH meter was calibrated, 50ml water sample was taken in 100ml flask, combined electrodes were introduced in water sample about 3-cm deep. Readings were taken after 30 seconds. combined electrodes were removed from sample and rinsed with DI water; excess water was tried with tissue.

3.3.4 Electrical Conductivity

3.3.4.1 Apparatus

Conductivity meter, conductivity cell, thermometer, beakers Reagents Potassium Chloride Solution 0.01N was taken, 2 to 3 grams of KCL was dried at 110 Degree Celsius for 2 hours. 0.745g KCL was dissolved in DI water and solution was brought to 1 liter. The solution was transferred to plastic flask.

3.3.4.2 Reagents

Took Potassium Chloride Solution (KCl) 0.01N. Dried about 2-3 g KCl in an oven at 110℃ for 2 hours. Dissolved 0.7456 g KCl in DI water and brought to 1-L volume. The solution was transferred to a plastic flask.

3.3.4.3 Procedure

Conductivity meter was calibrated according to maker's instruction. Conductivity cells were rinsed thoroughly with distilled water. Excess water was dried carefully. Conductivity cells were rinsed with measured solution for few times. 75 ml of sample was taken and conductivity cells were inserted, readings were taken.

3.3.5 Water Chemical Analysis

The content of calcium, magnesium, potassium sulphate, phosphate, nitrate, nitrite, copper, iron, manganese, lead, chromium, calcium, and zinc from water samples was determined by AAS Analyst 7000. Compressor was turned ON after fixing nitrous oxide and acetylene gas. Extra trapped liquid was removed [88-68]. AAS and Extractor controls were turned ON. Slender tube and nebulizer were cleaned, the acetylene gas pressure was set at 700 KPa that is equivalent to 100 psi and the valve was set to 11 psi for acetylene and 45 psi for air [78]. The new sheet on Spectra AA software was opened, "Add Method" was used and desired element for analysis was selected. Type, Optical, SIPS parameters were selected accordingly.

Joined PC was used for the worksheet of ASS programming. Empty cathode was used for light embedded in light holder. The beam was adjusted to hit target zone of the arrangement cards for required light throughput. At that point the machine was turned off [61-63]. 10 ml graduated chamber containing deionized water was used to estimate the yearning rate. Calibration solutions were prepared along with analytical blank. Both were atomized and response was measured.

Graph was plotted for for each solution than sample solutions were atomized. The concentration of various elements from sample solution was determined [78-79].

3.4 DNA Extraction Amplification and Pyrosequencing

3.4.1 Experiment Process

3.4.1.1 DNA Extraction

The DNA extraction kit /CTAB method /SDS method was used for genomic DNA extraction. The integrity purity and conc. of DNA was checked by 1electrophoresis [50].

3.4.1.2 PCR Amplification and Product Electrophoresis Detection

Using genomic DNA as template according to the selection of sequencing region specific primers with barcode and Takara premier Taq version 2.0 (Takara Biotch. Co. Dalian China) were used for amplification by PCR.

3.4.1.3 Primer Corresponding Region

Primer corresponding regions include 16S V4 primers (515f and 806r) that identify bacterial diversity 18S V4 primers (528f and 706r): identify the diversity of eukaryotes; ITS1 primers (its5-1737f and its2-2043r): identify the diversity of fungi; In addition, the amplification region also includes: 16S v3-v4 / 16S v4-v5; Archaea 16S v4-v5; 18S V5 and ITS2 Region; functional gene corresponding primers etc.

3.4.1.4 PCR Reaction System

TABLE 3.2: Reagent name and dosage

Reagent Name	Dosage
2x Premix Taq	25 μl
Primer-F	10 mM
Primer-R	10 mM
DNA	60 ng
Nuclease-free water	Add to 50 μl

3.4.1.5 PCR Reaction Conditions

PCR reaction cycles were performed as follows:

1. After 5min of initial denaturation at 94℃ followed by 30 cycle of
   94℃ for 30sec
   52℃ for 30sec
   72℃for 30sec

2. Extension step at 72℃ for 10min

3. Hold at 4℃

Each sample was repeated three times and the PCR products were mixed PCR instrument: BioRad S1000 (CA).

3.4.2 Electrophoresis Detection of PCR Products

The concentration and length of PCR product were identified by 1% agarose gel electrophoresis. The length of the main band was within the normal range [60]. The length of the main band was within the normal range. (for example, 16S v4:290-310bp / 16S v4-v5:400-450bp etc.) can be used for further experiments.

3.4.3 Pooling and Gel Cutting Purification

By comparing the conc. of PCR products by gene tools analysis software the volume of each sample was calculated with respect to principle of equal quality and then the PCR products were mixed the E.Z.N.A PCR Gel Extraction Kit was used to recover PCR mixed products. TE buffer was used to eluate the target DNA fragment [49].

3.4.4 Database Building and Sequencing

3.4.4.1 Database Building

Build the database according to the standard process of nebnext ultra-DNA library prep kit for Illumina (New England Biolabs USA).

3.4.4.2 Sequencing

The amplified library was sequenced by PE250 using Illumina Nova 6000 platform (Guangdong Magigene Biotechnology Co. Ltd. Guangzhou China).

3.4.5 Analysis Process

3.4.6 Sequencing Data Processing

1. **Paired End Raw Reads Data Filtering**: use fastp (an ultra-fast all in one fastq preprocessor version 0.14.1 https://github.com/opengene/fastp) to cut the sliding window quality (- w4-m20) of two end raw reads data respectively and use cut adapt software (https://github.com/marcelm/cutadapt/) to remove the primer information at both ends of the sequence Primer obtained the paid end clean reads after quality control.ired end raw reads data filtering: use fastp (an ultra-fast all in one fastq preprocessor version 0.14.1

https://github.com/opengene/fastp) to cut the sliding window quality (- w4-m20) of two end raw reads data respectively and use cut adapt software (https://github.com/marcelm/cutadapt/) to remove the primer information at both ends of the sequence Primer obtained the paid end clean reads after quality control.

2. **Paired End Clean Reads Splicing:** for the data of two terminal sequencing according to the overlap relationship between PE reads usearch-fastq'mergepairs (V10 http://www.drive5.com/usearch/ preset parameters include the minimum overlap length set to 16bp the maximum mismatch allowed in the overlap area of splicing sequence 5bp etc.) should be used to filter the inconsistent tags and obtain the original ones Raw tags.

3. **Raw Tags Sequence Quality Filtering:** use fastp (an ultra-fast all in one fastq preprocessor version 0.14.1 https://github.com/opengene/fastp) to cut the raw tags data with sliding window quality (- w4-m20) and get effective splicing fragments (clean tags).

3.4.6.1 OTU Clustering and Species Annotation

1. **OTU Clustering:** OTU or operational taxonomic units is one of the most common terms in microbiology. The platform provides the following three methods and the default clustering method is uparse:

   (a) UPARSE (RC Edgar. highly accurate OTU sequences from microbial amplicon reads. Nature methods 2019 10(10): 996)

   (b) UNOISE3 (RC Edgar. UNOISE2: Improved error-correction for Illumina 16S and ITS amplicon read. bioRxiv 2016)

   (c) UCLUST (RC Edgar. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 2010 26(19):2460-2461)

2. **Representative Sequence Species Annotation:** use usarch - sinax to compare the representative sequence of each OTU with Silva (16S) RDP

(16S) greengenes (16S) Silva (18S) unite (ITS) database. Through the comparison the species annotation information (the default confidence threshold is 0.8 and the default database is Silva (16S) Silva (18S) and unite (ITS)) so as to understand the origin of all sequence species. The taxonomic results from species annotation can be divided into seven levels that are Kingdome (L1) Phylum (L2) Class (L3) Order (L4) Family(L5) Genus(L6) Species(L7).

3. **Contaminated OUT Removal:** the OTU and its tags annotated as chloroplast or mitochondria (16s amplicon) was removed that were unable to annotate to the boundary level the number of effective tags sequences were obtained for OUT table of taxonomy for each sample.

3.4.6.2 OTU Statistics

1. OTU table: Based on the above-mentioned OTU table after removing singleton OTU chimera and contaminated OTU count the number of reads and OTU contained in the sample or group.

2. Pan'u core species analysis: Use Qiime2 View to count the number of Union (Pan) and intersection (core) of the target classification level in different product numbers to evaluate whether the sample size is sufficient.

3.4.6.3 Species Community Analysis

1. Community structure of species: Use Qiime2 View software to make statistics of common and endemic species community composition analysis and species abundance cluster analysis.

2. Phylogenetic analysis Single sample - phylogenetic analysis of each classification level: Based on phylogenetic relationship and relative abundance of each OUT in sample Qiime2 view was used to visualize the species annotation results

3.4.6.4 Alpha Diversity Analysis

1. Alpha diversity index statistics (default parameter): Based on the OTU abundance table useQiime2View (https://view.qiime2.org) to calculate diversity indexes (richness Chao1 Shannon 2).

2. Dilution curve (default parameter): Based on OTU abundance table use Qiime2View (https://view.qiime2.org) to calculate the dilution curve of the above diversity indexes.

3. Rank independence curve (default parameter): Based on OTU abundance table Qiime2View (https://view.qiime2.org) is used.

3.4.6.5 Beta Diversity Analysis

1. PCA analysis (default parameter): Based on OTU abundance table use the Qiime2View (https://view.qiime2.org) for analysis.

2. PCoA analysis (default parameter): Based on OTU abundance table Qiime2View (https://view.qiime2.org) is used to analyze with the above nine distance algorithms.

3.4.6.6 Correlation Analysis of Environmental Factors

Based on OTU abundance table and environmental factor data the association between community structure and environmental factors will be studied.

Chapter 4

Results and Discussion

4.1 Physical and Aesthetic Parameters

Table 4.1: Physical and Aesthetic Parameters of soil and water samples

Sample	Parameter								
	EC	pH	Cu	Fe	Mn	Zn	Ca	Mg	K
NP1	28500	6.6	1.21	8.8	0.89	1.43	1041	826	808
NP2	28900	6.8	1.25	8.6	0.69	1.45	1001	866	800
PN1	47600	6.6	2.11	32.9	1.42	2.29	566	488	208
PN2	51600	6.5	2.01	33.1	1.36	2.14	561	456	200
BH1	68100	6.3	1.50	9.80	1.0	1.11	1411	1098	860
BH2	68800	6.6	1.44	9.88	1.2	1.01	1402	1084	852
CN1	44600	8.1	4.58	34.16	0.95	2.21	29.45	4.55	3.05
CN2	43100	8.1	4.51	34.27	0.92	2.03	29.75	4.63	3.60
RJ1	34000	8.5	5.40	35.70	1.00	1.30	30.43	5.60	4.03
RJ2	33800	8.3	5.02	35.00	1.50	1.03	30.00	5.30	4.00
C1	24000	7.5	0.47	4.31	0.20	3.21	20.10	4.00	0.08
C2	20000	7.5	0.45	4.26	0.29	3.02	20.64	4.01	0.80
Samples	SO_4^{2-}	H_2S	PO_4^{3-}	NO^{3-}	NO^{2-}	Pb	Cr	Cd	
NP1	500	466	10.6	1.77	BDL	0.01	0.12	0.021	

Table 4.1 continued from previous page

NP_2	505	520	11.1	1.87	BDL	0.05	0.14	0.023	
PN1	81	67	0.51	3.47	BDL	0.11	0.004	0.013	
PN2	79	71	0.62	3.39	BDL	0.84	0.003	0.019	
BH1	33	34	0.03	1.88	BDL	0.01	0.064	0.010	
BH2	32	36	0.06	1.18	BDL	0.53	0.061	0.011	
CN1	402	403	12.46	0.64	BDL	68.44	0.656	0.019	
CN2	406	433	12.46	0.60	BDL	68.49	0.658	0.012	
RJ1	249	244	11.05	1.32	BDL	70.33	1.508	1.018	
RJ2	255	250	11.05	134	BDL	70.00	1.502	1.013	
C1	19.1	6.3	0.0001	2.37	0.0001	0.64	0.152	0.001	
C2	19.4	6.5	0.0001	2.43	0.0001	0.61	0.159	0.002	

BDL: Below Detection Limit, NP: Nanpur, PN: Penjpir,

BH: Bahu, CN: Chak Nourang, Rj: Rajion

Table 4.1 shows physical and Aesthetic parameters of soil and water samples, these parameters shows effect on microbial flora. Electrical conductivity is maximum in BH2 sample (68800 micro mhos/cm). pH shows acidic to basic value among different samples, highly acidic value of pH is observed in NP2 (6.8) and highly basic in RJ1 (8.5).

Maximum value of copper (5.40) in RJ1, Iron (35.70) in RJ1, Mn (1.50) in RJ2, Zn (3.21) in C1, Ca ((1041) in NP1, Mg (1098) in BH1, K (860) in BH1, SO_4^{2-} (505) in NP_2, H_2S (12.46) in CN1 and CN2, PO_4^{3-} (12.46) in CN2, NO^{3-} (134) in RJ2, NO^{2-} value is below detection limit (BDL) in all samples except control this may be due to respiration in nitrite is unlikely in all samples, maximum value of Pb (70.33) in Rj1, Cr (0.658) in CN2, Cd (1.018) in RJ1.

4.2 Faith PD Values for Petroleum Reservoir Samples Against Control

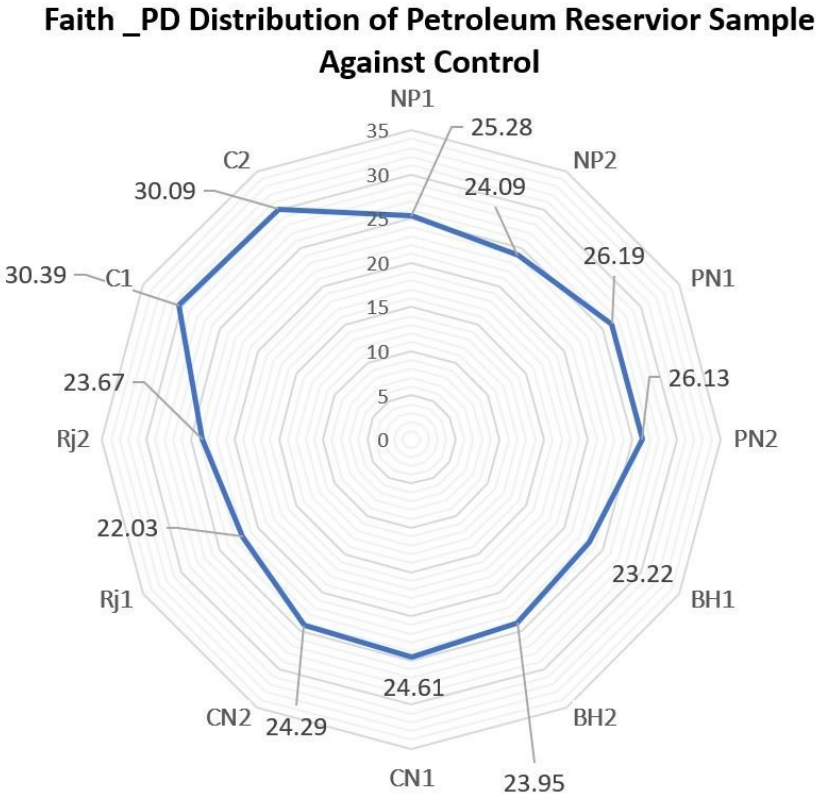

FIGURE 4.1: Faith PD values for petroleum reservoir samples and control. First of all, the observed PD values among different sites are varying but major difference is observed among samples and control samples, where PD higher for null distribution (control) is highest.

TABLE 4.2: Faith PD values for petroleum reservoir samples against control

Ser no.	Sample	Faith_PD
1	NP1	25.28
2	NP2	24.09
3	PN1	26.19
4	PN2	26.13
5	BH1	23.22
6	BH2	23.95
7	CN1	24.61
8	CN2	24.29
9	Rj1	22.03
10	Rj2	23.67

Table 4.2 continued from previous page

11	C1	30.39
12	C2	30.09

Faith PD (Table 4.2; Figure 4.1) explains the distance between two samples. Null hypothesis here presents that either there is no difference between different samples or samples do not have any difference with the control. The observed values differ from the null distribution. However, because the observed PD is lower than the null PD, this means that less phylogenetic diversity was present in our samples than were expected. In the literature, you can find different explanations of the deviations from the null distribution (e.g. lower than null may indicate phylogenetic conservatism or selection pressures because of stressed environment).

4.3 Distance Metrices Between Different Sites

Table 4.3: Distance Matrices among sample sites and control

	NP1	NP2	PN1	PN2	BH1	BH2
NP1	0	0.500312	0.564456	0.570251	0.780778	0.465565
NP2	0.500312	0	0.582419	0.625978	0.783509	0.519435
PN1	0.564456	0.582419	0	0.379011	0.773225	0.42371
PN2	0.570251	0.625978	0.379011	0	0.772739	0.451802
BH1	0.780778	0.783509	0.773225	0.772739	0	0.70498
BH2	0.465565	0.519435	0.42371	0.451802	0.70498	0
CN1	0.437933	0.523432	0.433123	0.472681	0.693927	0.357003
CN2	0.688905	0.718332	0.77051	0.763775	0.768959	0.712936
Rj1	0.500512	0.525978	0.779011	0.672739	0.437933	0.437933
Rj2	0.560056	0.483509	0.713225	0.651802	0.523432	0.523432
C1	0.170251	0.119435	0.22371	0.272681	0.433123	0.433123
C2	0.180778	0.123432	0.233123	0.263775	0.472681	0.472681
	CN1	CN2	RJ1	RJ2	C1	C2

Table 4.3 continued from previous page

NP1	0.437933	0.688905	0.500512	0.560056	0.170251	0.180778
NP2	0.523432	0.718332	0.525978	0.483509	0.119435	0.123432
PN1	0.433123	0.77051	0.779011	0.713225	0.22371	0.233123
PN2	0.472681	0.763775	0.672739	0.651802	0.272681	0.263775
BH1	0.693927	0.768959	0.437933	0.523432	0.433123	0.472681
BH2	0.357003	0.712936	0.437933	0.523432	0.433123	0.472681
CN1	0	0.704428	0.437933	0.523432	0.433123	0.472681
CN2	0.704428	0	0.693927	0.523432	0.433123	0.472681
Rj1	0.437933	0.437933	0	0.433123	0.472681	0.693927
Rj2	0.523432	0.523432	0.433123	0	0.433123	0.472681
C1	0.433123	0.433123	0.472681	0.433123	0	0.433123
C2	0.472681	0.472681	0.693927	0.472681	0.433123	0

Table 4.3 Shows distance metrices between different sites. This table is used to calculate the similarity between distribution of microflora among different sites.

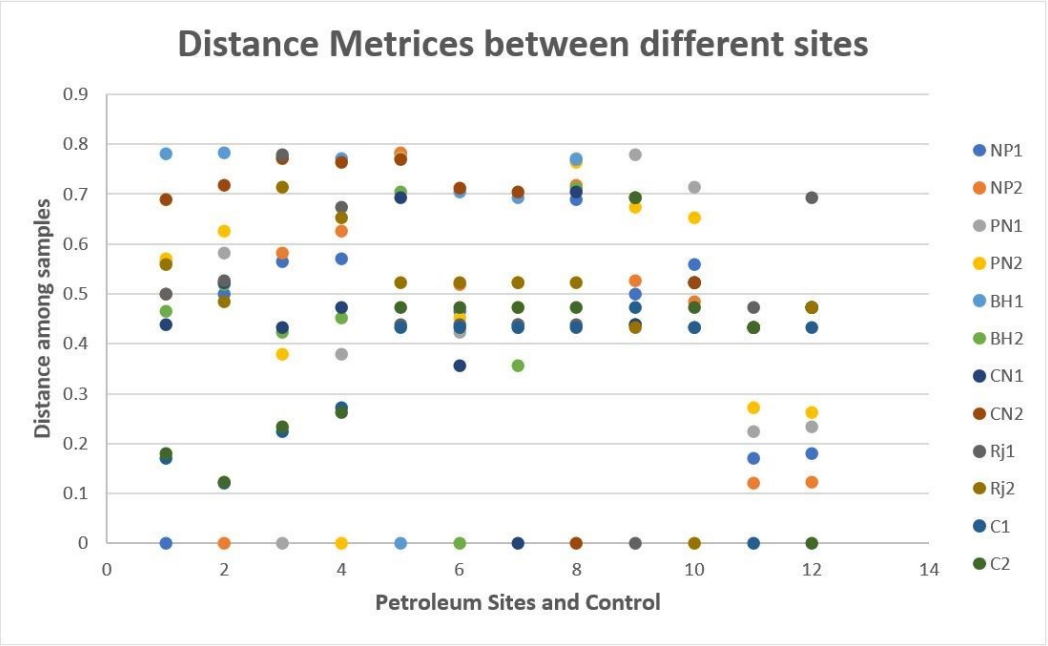

FIGURE 4.2: Distance metrices Xy Scatter chart showing distribution of OTUs in space. Color codes are presenting different samples and their overlapping OTUs among different sample sites. Overlapping positively showing homogeneity in the microflora of sites.

4.4 Species Community and Phylogenetic Analysis

4.4.1 Phylogenetic Abundance of Archaea

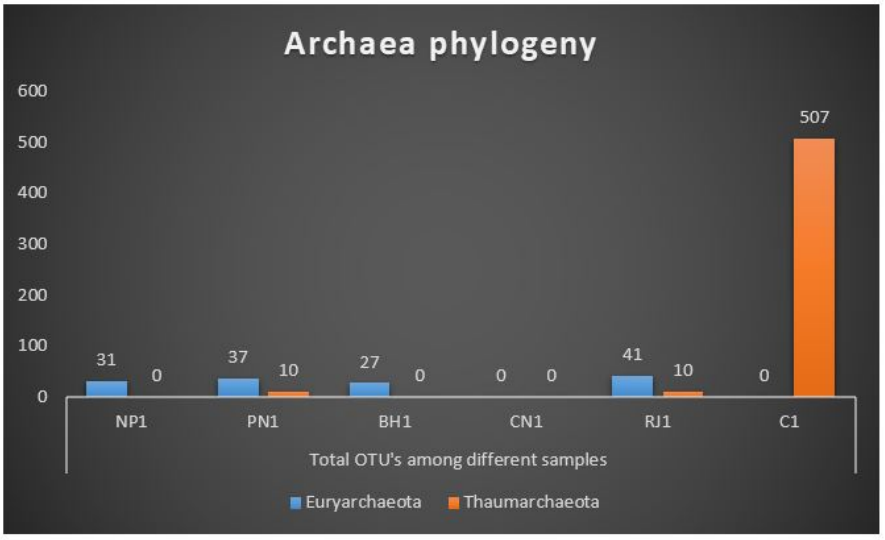

FIGURE 4.3: Showing taxonomic abundance of different archaeal OTU'S among the samples. Figure depicted that total number of OTU's are distributed among two archaeal phyla namely *Euryarchaeota* and *Thaumarchaeota*, phylum Euryarchaeota show higher number of OTU's in RJ1 sample, whereas phylum *Thaumarchaeota* class of Soil Crenarchaeotic Group (SCG) archaea shows their abundance in C1 (control sample). In literature we can found reason that soil Crenarchaeotic group dominated at intermediate salinities, also nitrogen content impart effect on relative abundance of *archaea*.

TABLE 4.4: Showing Phylogenetic Abundance of *Archaea* and Number of OTU's Present

Phylum	Total OTU's among different samples					
	NP1	PN1	BH1	CN1	RJ1	C1
Euryarchaeota	31	37	27	0	41	0
Thaumarchaeota	0	10	0	0	10	507

Table 4.4 shows Phylogenetic abundance of archaea and number of OTU's present in different samples. Two phyla of archaea namely *Euryarchaeota* and *Thaumarchaeota* were identified. Among phylum *Euryarchaeota genus Halorubrum,*

Methanobacterium, Methanosarcina, Methanomassiliicoccus were identified and among phylum *Thaumarchaeota* class of Soil Crenarchaeotic Group (SCG) archaea are present in different samples.

4.4.2 Phylogenetic Abundance of Bacteria

TABLE 4.5: Showing abundant bacterial phylum's OTU's

Phylum	Total OTU's among different samples					
	NP1	PN1	BH1	CN1	RJ1	C1
Acidobacteria	1280	13,343	2178	157	2404	763
Actinobacteria	3552	211	2866	3732	3808	3314
Bacteriodete	159	260	311	6938	480	448
Firmicutes	162	188	272	156	146	118
Planctomycetes	193	216	200	210	211	316
Protobacteria	17337	15552	20090	31296	31097	15573
Chloroflexi	4375	4212	373	17	4050	568

4.4.3 Graphs of Abundant Bacteria Phylum's

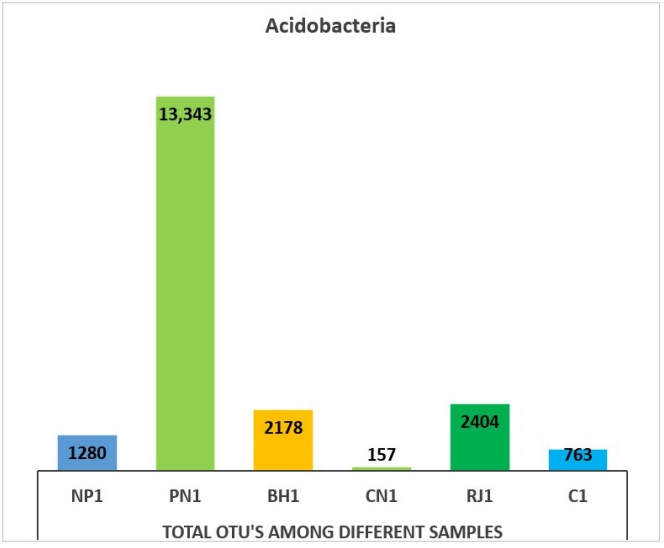

FIGURE 4.4: shows OTU's of *acidobacteria* among different samples, higher number of OTU's were observed in PN1 sample

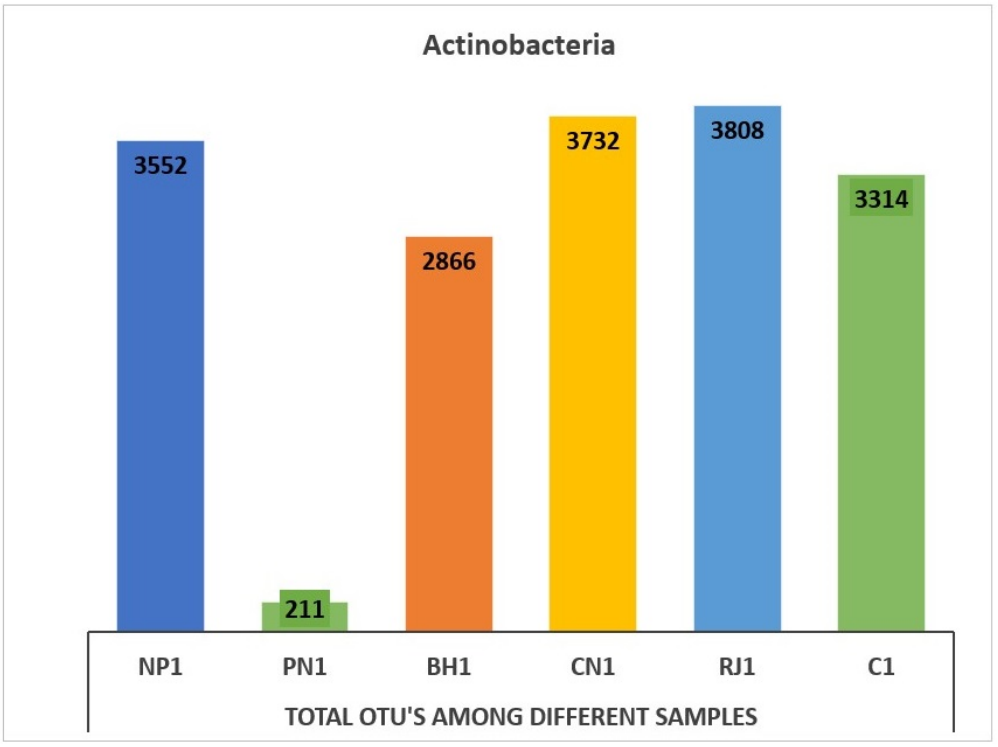

FIGURE 4.5: Shows OTU's of *acidobacteria* among different samples, higher number of OTU's were observed in PN1 sample

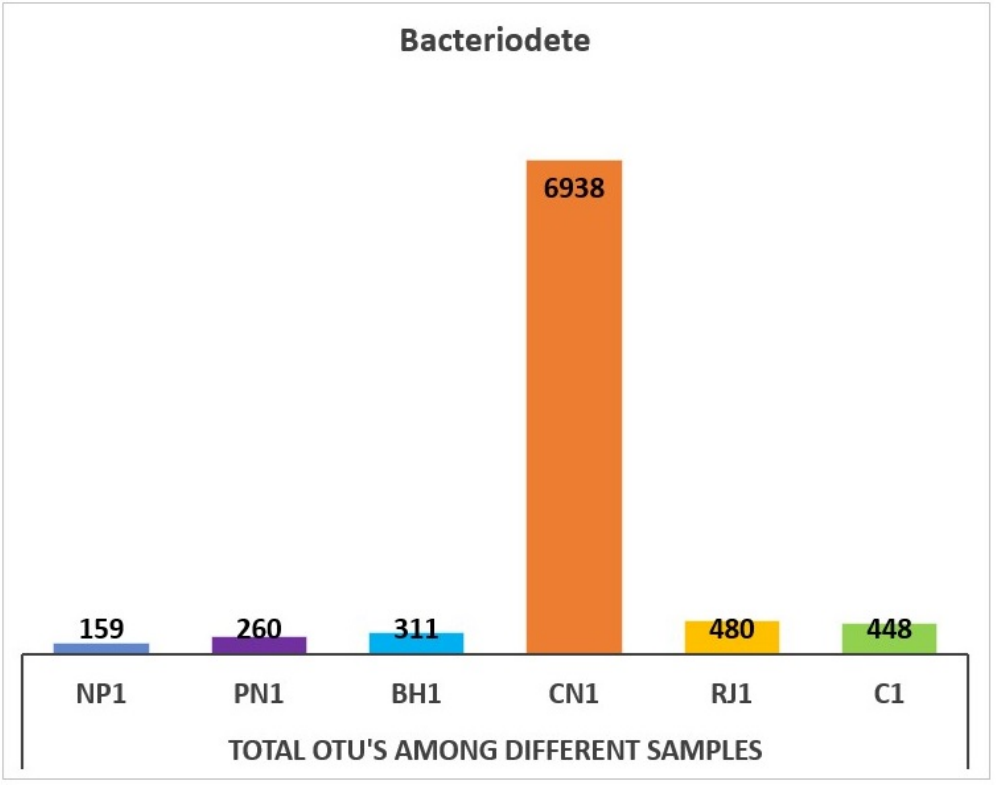

FIGURE 4.6: shows OTU's of *bacteriodetes* among different samples, higher number of OTU's were observed in CN1 sample

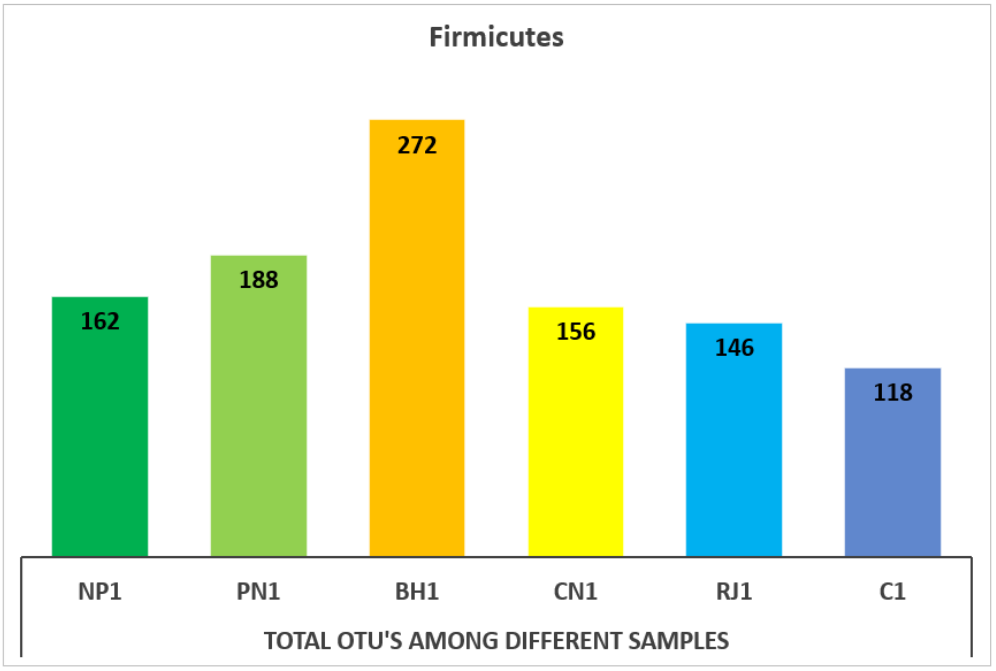

FIGURE 4.7: Shows OTU's of *firmicutes* among different samples, higher number of OTU's were observed in BH1 sample

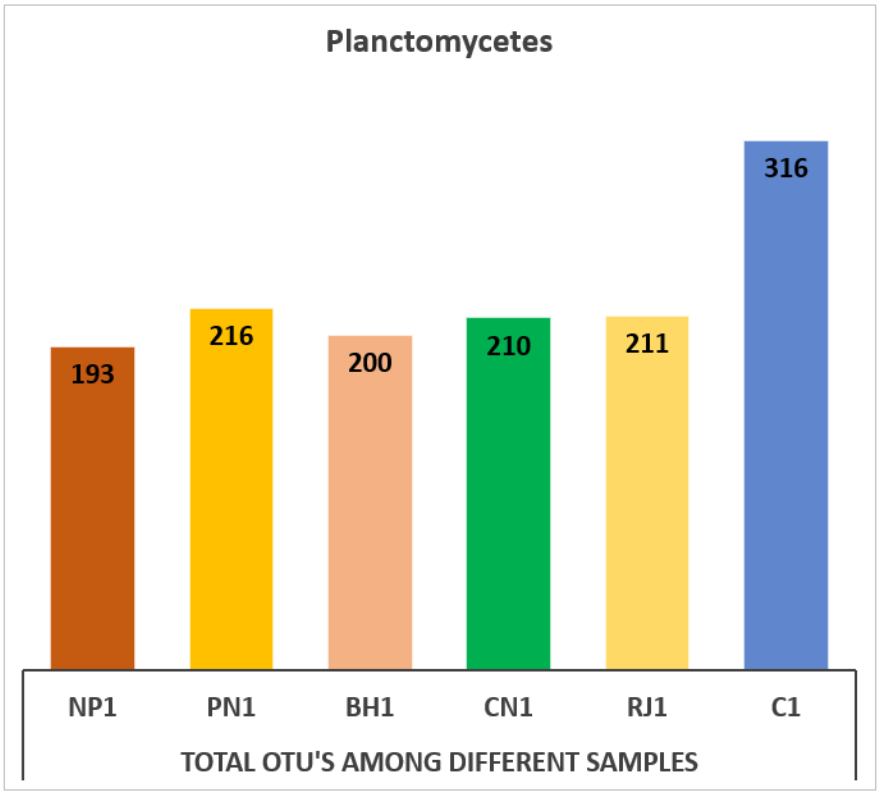

FIGURE 4.8: Shows OTU's of *planctomycetes* among different samples, higher number of OTU's were observed in C1 sample.

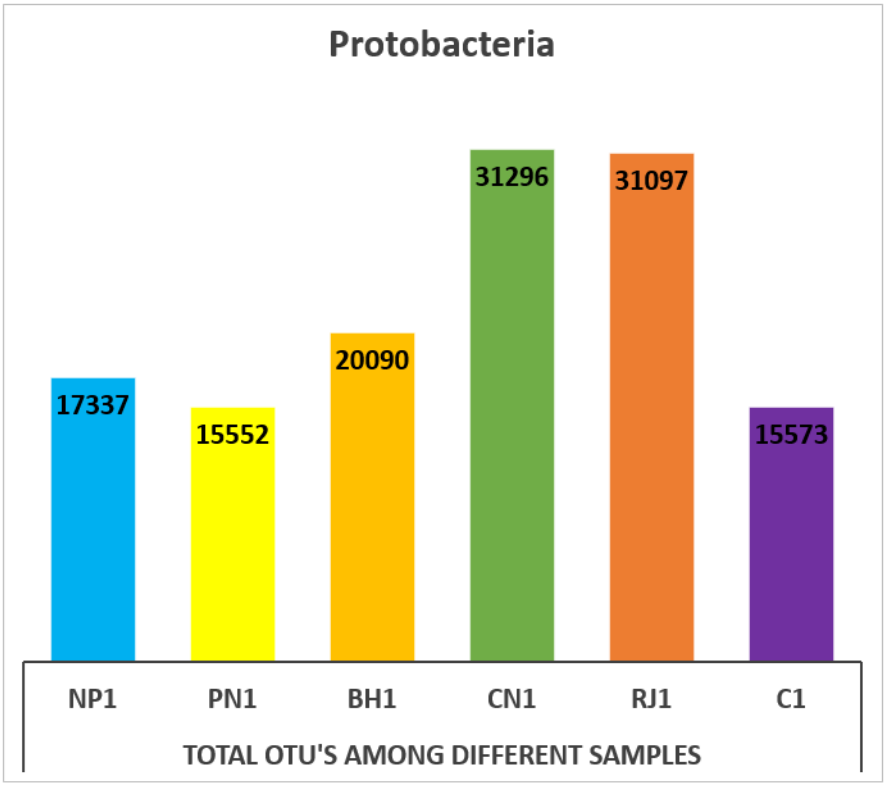

FIGURE 4.9: Shows OTU's of *Protobacteria* among different samples, higher number of OTU's were observed in CN1 and RJ1 sample.

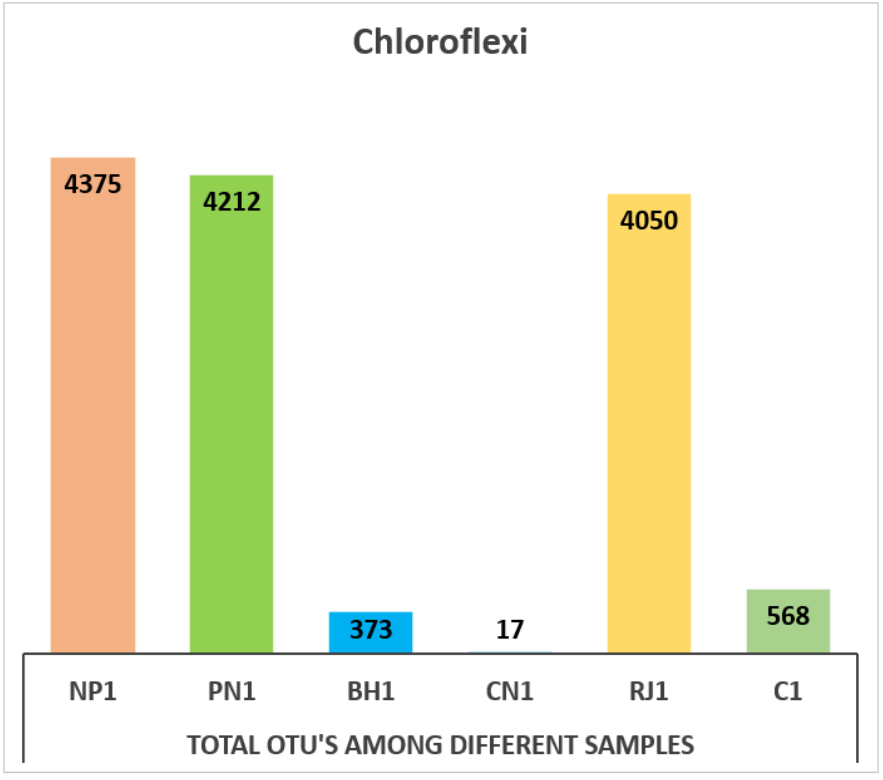

FIGURE 4.10: Shows OTU's of *Chloroflexi* among different samples, higher number of OTU's were observed in NP1 sample.

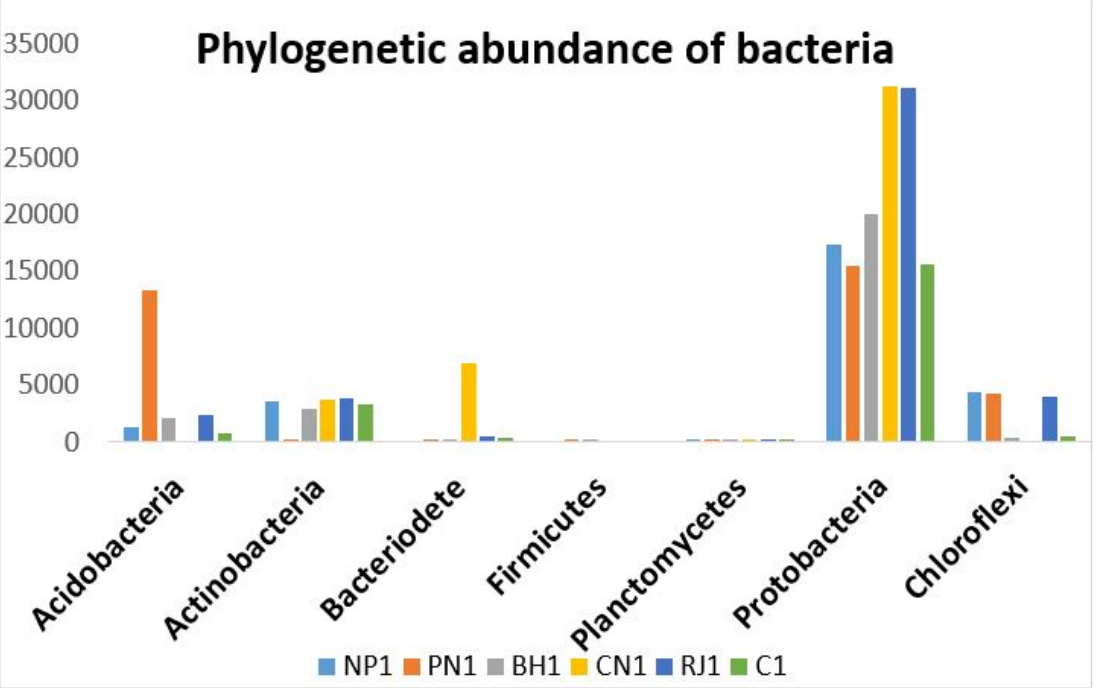

FIGURE 4.11: Shows all the seven abundant phyla of bacteria namely Acidobacteria, *Actinobacteria, Bacteriodete, Firmicutes, Plantomycetes, Protobacteria, Chloroflexi.* Higher number of OTU's (41,966) were observed in CN1 sample.

4.5 Phylogenetic Abundance of Sulphur Reducing Bacteria

TABLE 4.6: Shows phylogenetic abundance of Sulphur Reducing Bacteria

Phylum	Total OTU's among different samples					
	NP1	PN1	BH1	CN1	RJ1	C1
Acidobacteria	10	0	0	0	0	0
Firmicutes	19	0	0	0	15	0
Protobacteria	161	425	193	4	209	31

Table 4.6 shows the OTU's of three abundant Sulphur reducing bacteria phylum. Phylum *Acidobacteria* (genus uncultured *Desulfovirga*) is present only in NP1 sample. Phylum *Firmicutes* (genus *Desulfotomaculum*) shows abundance in NP1

sample and phylum *Protobacteria* (genus *Defluviicoccus*) shows abundance in RJ1 sample, genus *Desulfovibrio* in NP1 and PN1, genus H16 in BH1 sample, genus *Geoalkalibacte*r in PN1 sample, genus *Geobacter* in C1 sample, genus *Geothermobacter* in C1 sample, genus *Desulfovirga* shows abundance in PN1 sample.

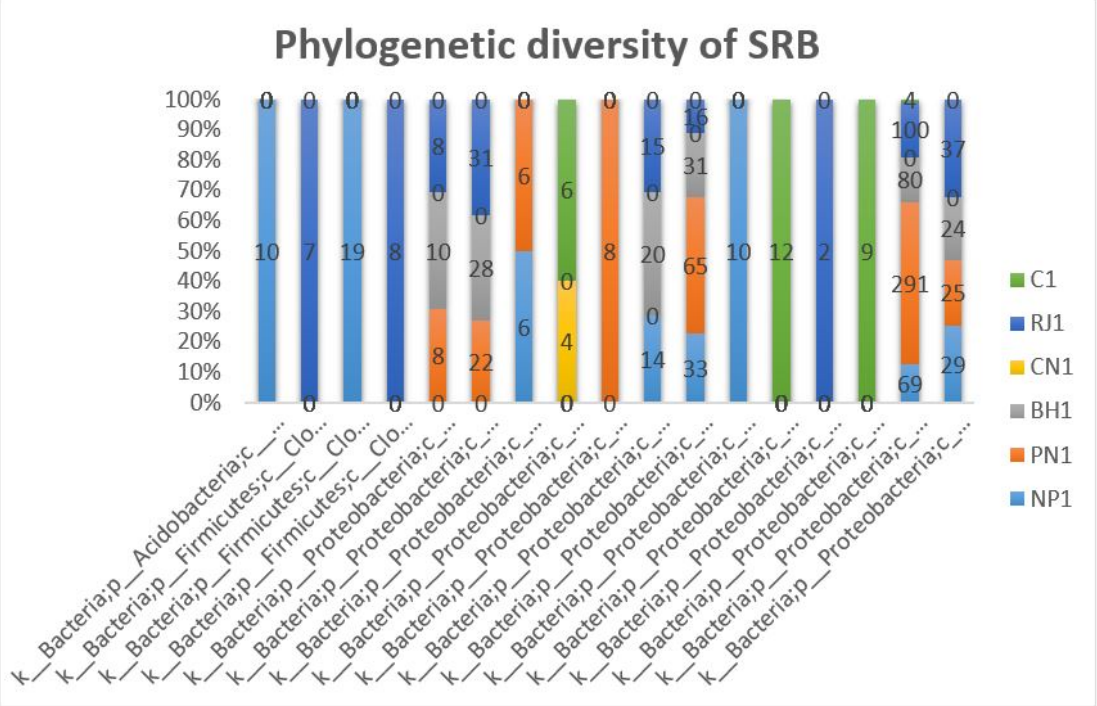

FIGURE 4.12: Shows all the three abundant phyla of Sulphur reducing bacteria namely *Acidobacteria, Firmicutes*, and *Protobacteria*. Higher number of OTU's (10) of phylum *Acidobacteria* and *Firmicutes* OTU's (19) are present in NP1 sample, Higher number of OTU's (425) of phylum *Proteobacteria* are present in PN1 sample. Overall higher abundance is found in PN1 sample.

4.5.1 Alpha Diversity Analysis

4.5.1.1 Alpha Box Plot of SO_4^{-2}

TABLE 4.7: Alpha Box Plot of SO_4^{-2}

Group 1	Group 2	Group 3
502.5(n=2)	19.25(n=2)	0.001
80(n=2)	19.25(n=2)	0.002
32.5(n=2)	19.25(n=2)	0.01

Table 4.7 continued from previous page

404(n=2)	19.25(n=2)	0.001
252(n=2)	19.25(n=2)	0.001

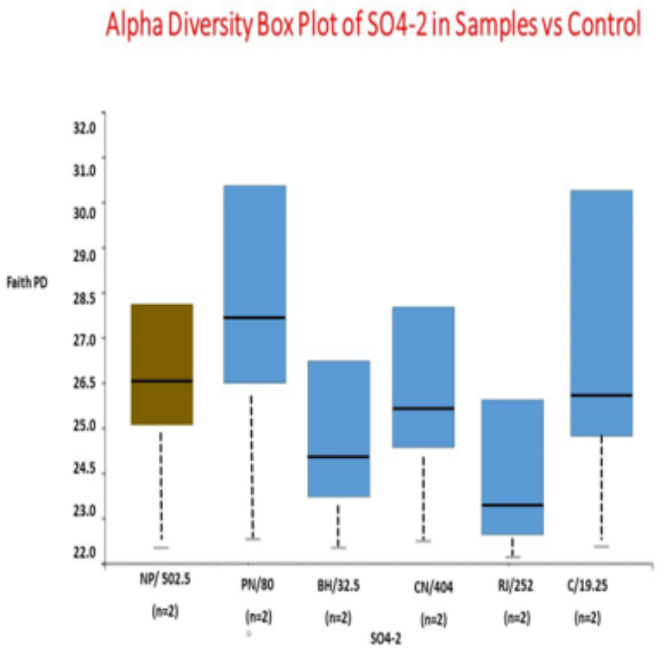

FIGURE 4.13: Alpha Box Plot of SO_4^{-2} sample vs control

Table 4.7 shows mean value of SO_4^{-2} concentration among all samples and control, the P-values between different groups were shown P value is below 0.05 for sites NP, PN, RJ, CN and BH when compared with Control.

TABLE 4.8: kruskal-Wall results for SO_4^{-2} Concentration

Sample	SO_4^{-2} Concentration	Faith PD
NP1	500	25.28
NP2	505	24.09
PN1	81	26.19
PN2	79	26.13
BH1	33	23.22
BH2	32	23.95
CN1	402	24.61

Table 4.8 continued from previous page

CN2	406	24.29
Rj1	249	22.03
Rj2	255	23.67
C1	19.1	30.39
C2	19.4	30.09

Table 4.8 shows SO_4^{-2} concentration and faith PD value of all samples, data of table 4.7 and 4.8 is used to construct Box Plot figure 4.13. Boxplot of phylogenetic diversity index of SO_4^{-2}in different groups is shown in figure 4.13, the boxes denote interquartile ranges (IQR) with the median as a black line and whiskers extending up to the most extreme points within 1.5-fold IQR. Box Plot between control and sampling sites is showing significant difference between OTUs distribution between individual samples as well as between targeted sites and control. Higher concentration of Sulphur (SO_4^{-2}) is found in NP sample.

4.5.2 Beta Diversity Analysis

4.5.2.1 PCO-A Electrical Conductivity pH, Cu, Fe

Figure 4.14(a) and 4.15(b) The X-axis of the graph represents the variable physicochemical characteristics of Cu, Fe, SO , H_2S, PO_4 , NO_2 for effect of Electrical conductivity and PH respectively. while y-axis shows the values of pieloue.

Black red dots of figure 4.14 a and 4.15 b are representing Sampling sites and size of dots is representing the concentration of OTUs. Graphs show positive effect of variables EC, pH, Cu, SO_4^{-2} , H_2S, respectively. But Nitrate and Fe and PO_4 showed clustering of sampling sites based on their effects, highly corelated samples form clusters so concentration of nitrate, iron and phosphorus is highly corelated among all samples. Spearman showed P-value 0.2551 for electrical conductivity shows significant effect on distribution of OTUs. P-value showed by spearman for pH depicted value of 0.4017 that has significant effect on distribution of OTUs.

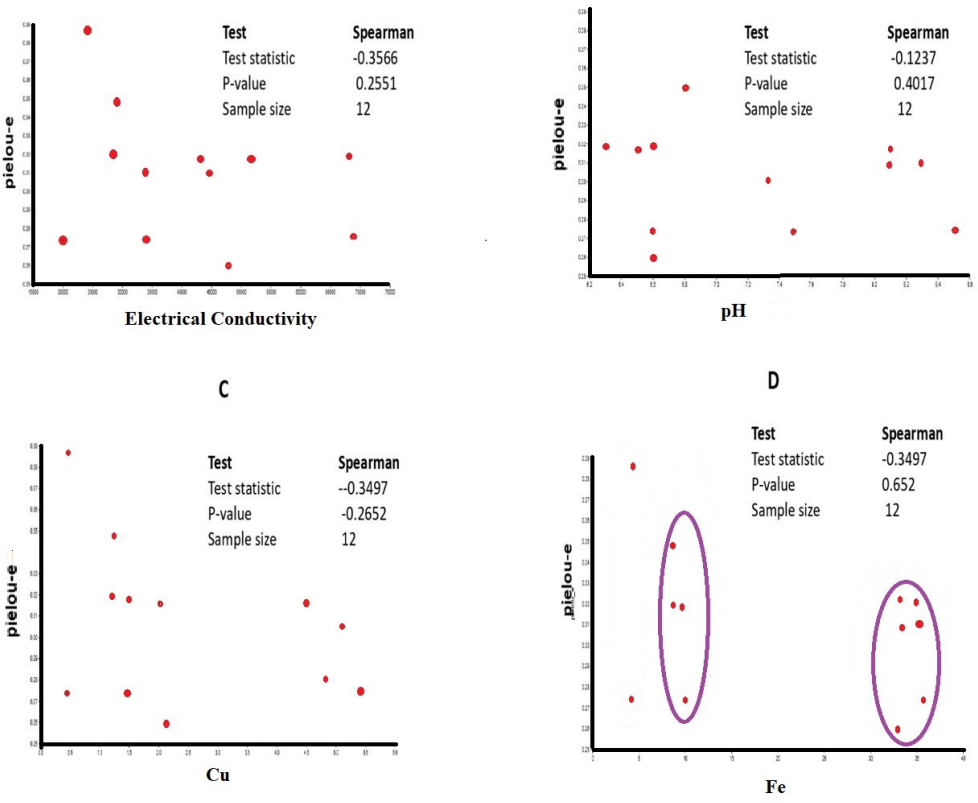

FIGURE 4.14: (a): PCO-A Electrical Conductivity, pH, Cu, Fe

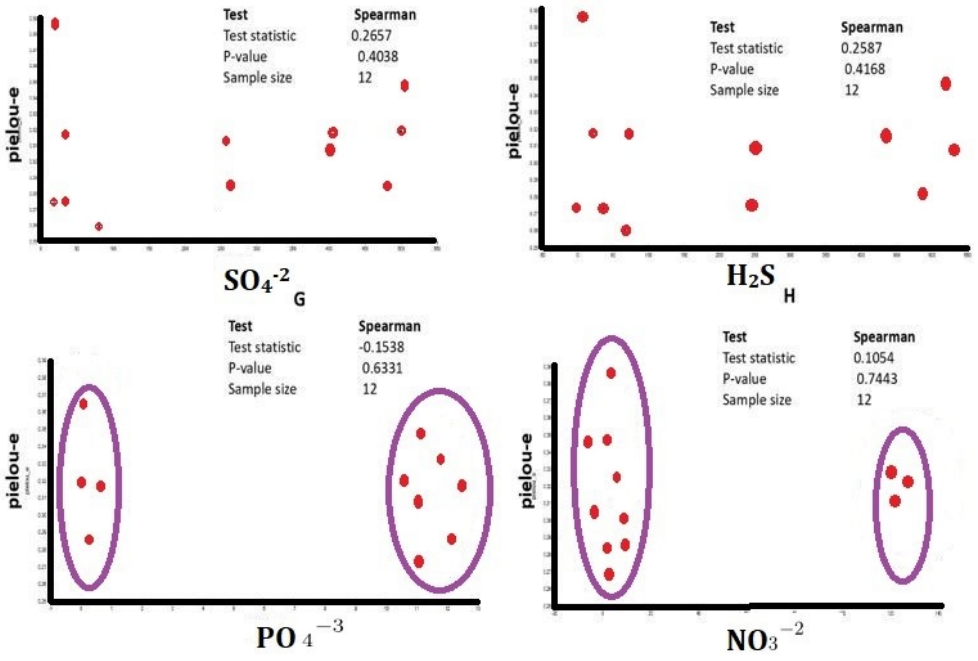

FIGURE 4.15: (b): PCO-A SO_4^{-2} , H_2S, PO_4 , NO_2

P-value for 12 sample size showed value of 0.2652 for Cu element that represents positive effect of Cu on distribution of OTUs. P value for Fe is 0.652 does not represent significance effect of Fe on distribution of OTUs. Spearman showed P-value of SO (0.4038) that represent significant effect of SO on distribution of OTUs. P-value of H_2S by spearman 0.4168 showed significant effect of H2S on distribution of OTUs. P-value of PO and NO by spearman 0.6331 and 0.7443 respectively showed no significant effect of PO and NO on distribution of OTUs.

4.5.2.2 CCA (Canonical Correlation Analysis

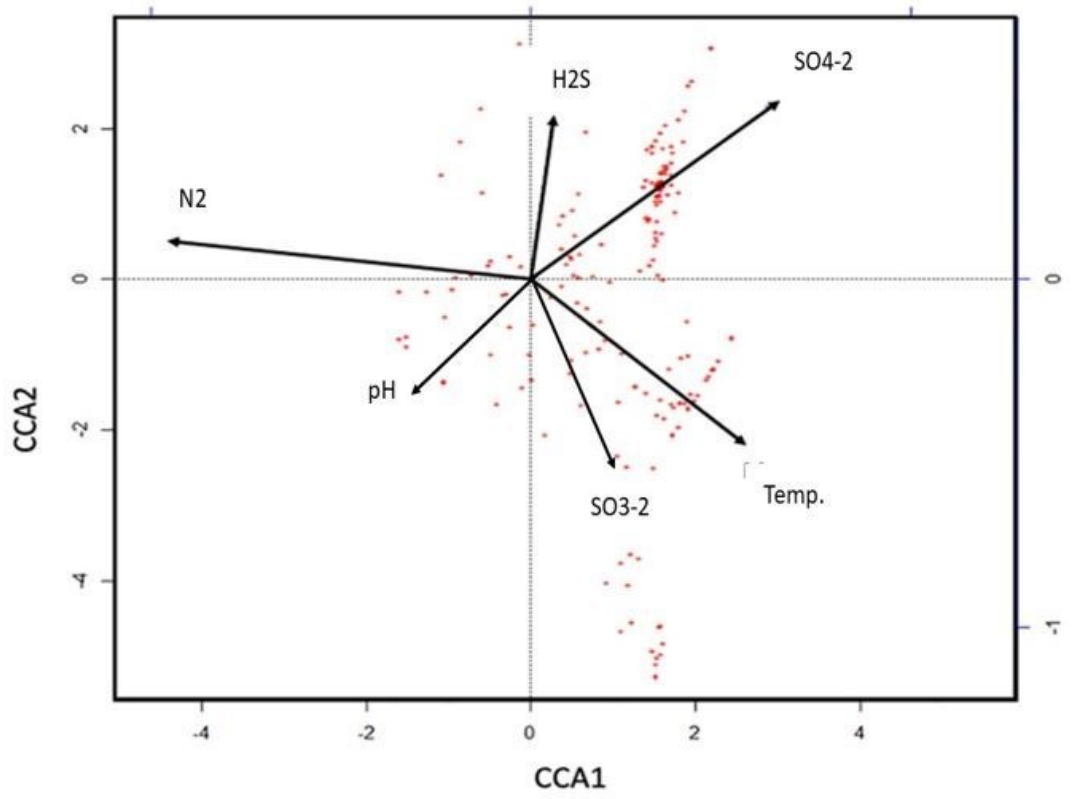

FIGURE 4.16: Canonical Correlation Analysis (CCA) was plotted at CC1 with 25.90% coordinates and at CCA2 with 18.49% coordinates.

CCA showing 3 dimensional coordinates with one controlled direction. CCA dimension 1 showing Sulphur percentage in PN1 and PN2. Red dots are presenting SO black are depicting control. Arrows shows presence of OTUs of a specific sampling site. The clustering at center indicated that large number of microbial taxa

were shared by samples. Tight clustering shows sharing of unique OTU's from close geographical locations.

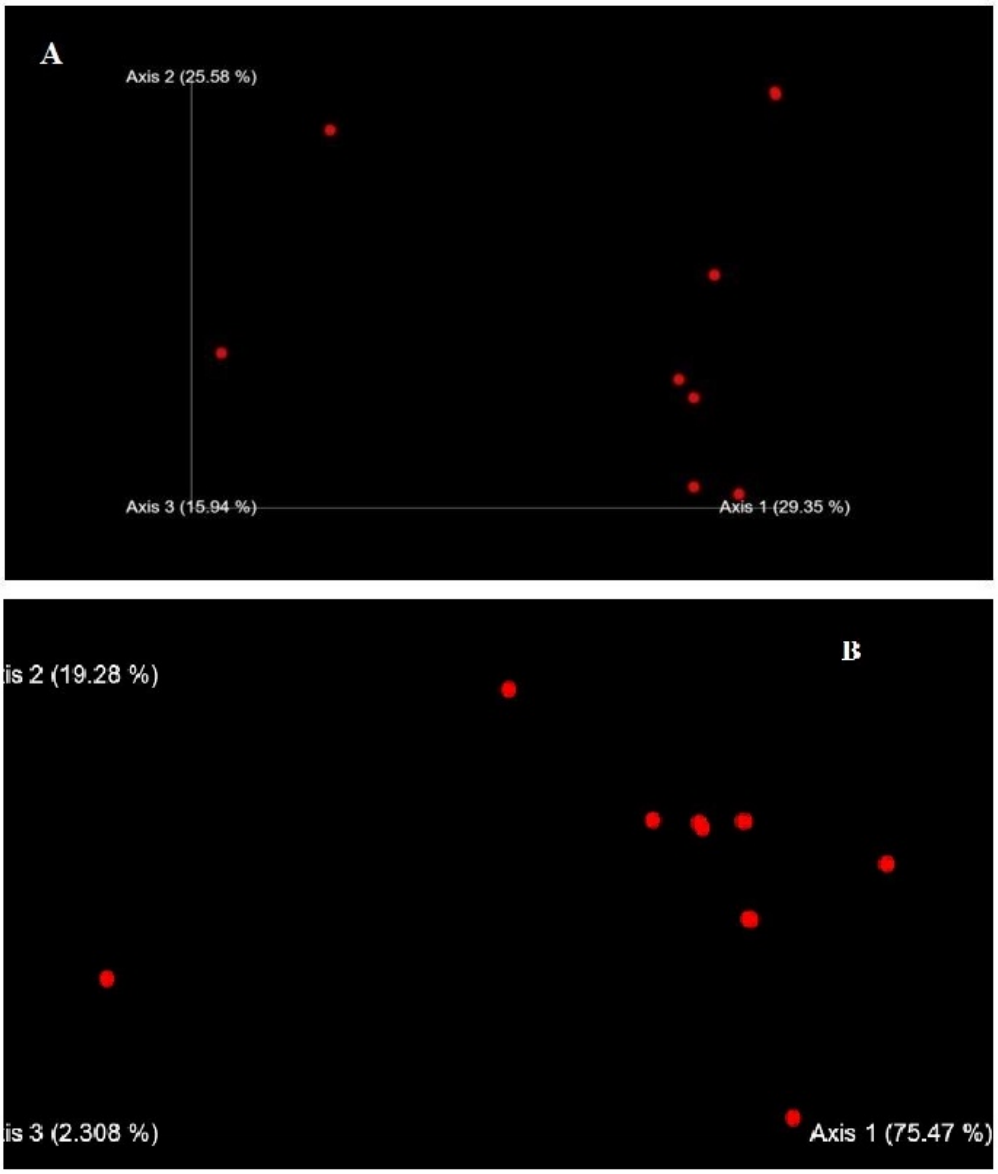

FIGURE 4.17: (A) is showing weighted unifrac emperor plot with Axis 1, Axis 2 and Axis 3 at 29.35%, 25.58% and 15.94% respectively and (B) is showing unweighted unifrac emperor plot with Axis 1,2 and 3 at 75.47%, 19.28% and 2.3% respectively

Figure 4.17 is representing Emperor plot which has the ability to visualize gradients, visualize different principal coordinates axes. Our data is presented in the form of parallel coordinates and is showing taxa as red dots. Environmental samples dynamics are adjusted in the varying sizes. Weighted unifrac emperor plot shows distance between sampling point which is smaller between samples from

the same point, suggesting the community composition of samples from the same point. Unweighted unifrac emperor plot shows tight clustering that indicate phylogenetic association of microbial communities. In figure all spheres are of almost same size that showed the effects of metabolites equally on distribution of OTUs in three-dimensional space.

4.5.3 Demultiplexed Sequence Counts Summary

Table 4.9: Demultiplexed sequence counts summary

Minimum:	84502
Median:	87306.5
Mean:	86934.5
Maximum:	88981
Total:	1043214

Table 4.10: Shows per-sample sequence counts of 12 samples.

Sample name	Sequence count
CN2	88981
C1	88680
C2	88039
RJ1	88039
BH2	88039
NP1	87438
RJ2	87175
CN1	87175
NP2	85700
BH1	84723
PN2	84723
PN1	84502

Table 4.9 show demultiplexed sequence counts summary obtained after data analysis table shows minimum sequence count is 84502, median value of sequence count is 873036.5, mean value of sequence count is 86934.5, maximum value of sequence count is 88981 and total sequence counts are 1043214. Table 4.10 shows per sample sequence counts of 12 samples higher number of sequence count is observed in CN2 sample.

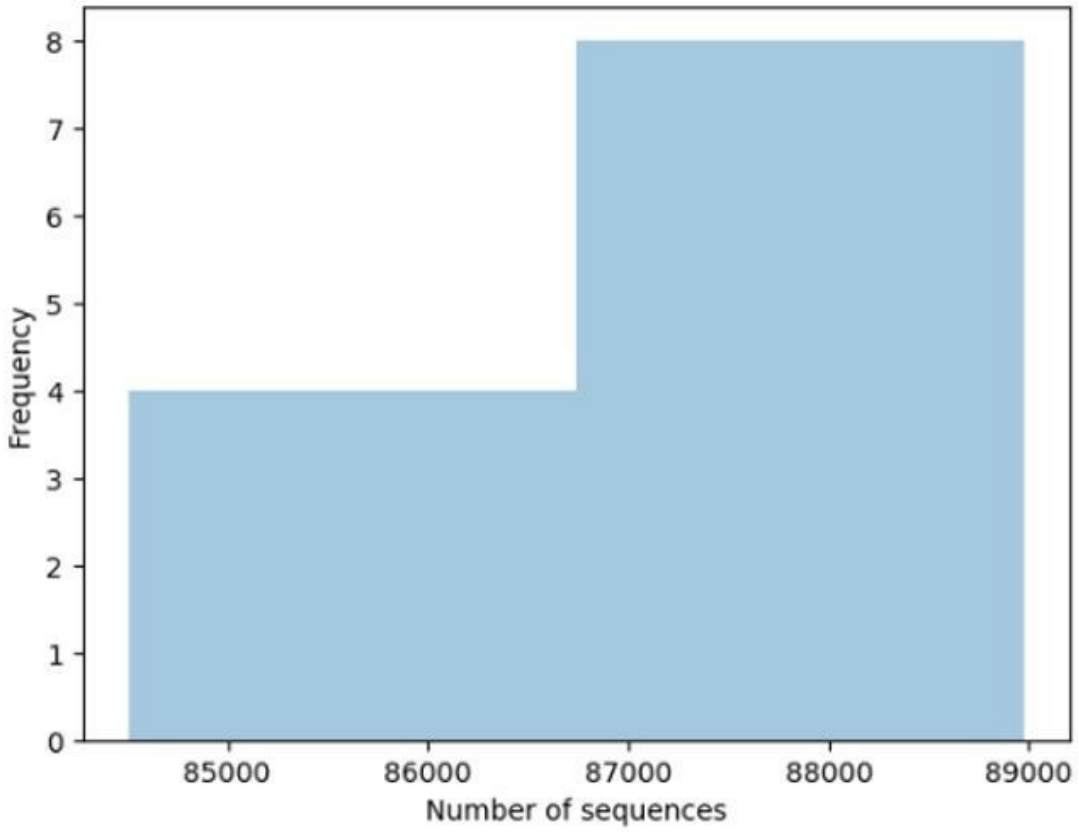

FIGURE 4.18: Shows per-sample sequence counts of 12 samples.

In figure 4.18 graph depicted the per sample sequence counts higher number of sequence count is observed in CN2 samples shows that this sample contains maximum numbers of different microbial and archaeal species as compared to other samples.

4.6 Discussion

Majority of Archaeal, bacterial and SRB phyla were identified from the oil field samples. Two phyla of archaea namely *Euryarchaeota* and *Thaumarchaeota* were identified. Among phylum *Euryarchaeota* genus *Halorubrum, Methanobacterium, Methanosarcina, Methanomassiliicoccus* were identified and among phylum Thaumarchaeota class of Soil Crenarchaeotic Group (SCG) archaea are present in different samples. Bacterial phylum *Acidobacteria, Actinobacteria, Armatimonadetes, Bacteriodetes, Chlamydiae, Chlorobi, Chloroflexi, Cyanobacteria, Deferribacteres, Deferribacteres, Deinococcus, Elusimicrobia, Firmicutes, Fusobacteria, Gemmatimonadetes, Ignavibacteriae, Latescibacteria, Nitospirae, Percubacteria, Planctomycetes, Proteobacteria, Saccharibacteria,* and *Verrucomicrobia,* are present [103].

Table 4.4 shows taxonomy of archaea in five different fields and in control sample, above results depicted that there are number of archaea's present in the oil fields. Phylum *Euryarchaeota* (Family *Halobacteriaceae*) shows its abundance in BH1 field, 5 OTUs' were present. Family *Mthanobacteriaceae* shows its abundance in NP1 (31 OTU's), PN1 (20 OTU's), BH1 (16 OTU's) and RJ1(31 OTU's) [104]. Family *Methanosarcinaceae* is only found in PN1 (4 OTU's). Family *Thermoplasmalates Incertae* Sedis in BH1 (6 OTU's). Most of the archea isolated from oil fields belong to Euryarchaeota phylum [103-105].

The most diverse group of microorganisms in archaea is related to phylum *Euyarchaeota* that has adapted most extreme environments. This phylum includes *thermophiles, mesophiles,* and *psychrophiles.* Some microbes including both aerobes and anaerobes live at extreme temperatures from 41 to 122 degree Celsius. Acidophiles members can live in highly acidic and halophytes in high salty environments. These microbes are different from others on the bases of ribosomal RNA and unique DNA polymerases [106-107]. Members of this phylum has diverse appearance and metabolic properties. They are in rods or cocci shapes, either Gram-positive or Gram-negative. They can be methanogens, halophytes and sulphate reducers. They are found in oceans. Although marine members of this phyla are difficult to culture but genomic sequence studies suggest that they are motile

heterotrophs [106],[111]. Euryarchaeota are also habitants of several moderate environments like water springs, marshlands, soil and rhizospheres. They are also known as highly adaptable e.g *halobacterials* order can live and grow in high salt concentrations to slow salt concentrations such as in sea water. Members of this phyla lack defence mechanisums against oxidative stress (ROS) [109-111].

Phylum *Thaumarchaeota* is indentified in PN1(10 OTU's), RJ1 (10 OTU's) and C1 (482 OTU's) of Soil Crenarchetic Group, 25 OTU's of genus *Candidatus Nitrososphaera* were also identified in C1. *Thaumarchaeota* members are ammoniaoxidizing organisms that live in soil, marine and hot springs habitats [112-122]. They are autotrophs and fix cabondioxide and few of them are dependent on other bacteria or small amounts of organic matter. Members of this phyla are also capable of oxidizing methane. Marine Thaumarchaea members produce nitrous oxide that is greenhouse gas and has role in climate change.

Among all phylum's the bacterial phylum's dominating one is *Acidobacteria, Actinobacteria, Bacteriodetes, Firmicutes, Planctomycetes, Protobacteria*, and *Chloroflexi*. Detail of these phylums . *Acidiobacteria* is abundant phylum in natural ecosystem. NP1 shows 1280, PN1 13343, BH1 2178, CN1 157, RJ1 2404, C1 763 OTU's of *Acidobacteria*. It is observed that exopolysaccharide producing bacteria has longer viability in soil. Due to high synthesis of exopolysaccharide they are dominant in acidic and chemically polluted environments with heavy metals, petroleum compounds. These bacteria are considered to be important contributors for ecosystem [99],[11],[121]. It is clear that *Actinobacteria* are nitrogen fixing bacteria. Actinobacters that have characteristics of fungi as well has role in recycling biomaterials are widely distributed in terrestrial and aquatic environments. In NP1 3552, PN1 211, BH1 2866, CN1 3732, RJ1 3808, C1 3314 OTU's of *Actinobacteria* were identified. More than 10,000 bioactive metabolites are found to be produced by these bacteria that are useful in natural products with potential applications [98]. For Example, streptomyces species are industrially important microorganism due to several useful bioactive natural products.

The species of *Bacteroidetes* play important role in protein metabolism by proteolytic activity. Some species can be utilized as source of urea as nitrogen cycle. In NP1 159, PN1 260, BH1 211, CN1 6938, RJ1 480, C1 448 OTU's of Bacteroidetes were identified. Significant abundance of phylum chlorofexi were also observed, in NP1 4375, PN1 4212, BH1 373, CN1 17, RJ1 4050, C1 568 OTU's of Chloroflexiare present [120].

The *Firmicutes* have mostly Gram-positive members that produce endospores that are resistant to desiccation due to which they can survive in extreme conditions. They are found in various environments. In NP1 162, PN1 188, BH1 272, CN1 156, RJ1 146, C1 118 OTU's of Firmicutes were identified. *Proteobacteria* are ubiquitous in oil reservoirs over all temperature ranges. Species of *Proteobacteria* are al Gram-negative. In NP1 17337, PN1 15552, BH1 20090, CN1 31296, RJ1 31097, C1 15573 OTU's of *Protobacteria* were identified. *Planctomycetes* are also present in NP1 193, PN1 216, BH1 200, CN1 210, RJ1 211, C1 316 OTU's represent significant abundance of these bacteria [121].

Above table 4.6 shows the abundance of Sulphur reducing bacteria among different phylums. Phylum *Acidobacteria* (specie uncultured Desulfovigra sp) shows 10 OTUs' in NP1 sample. Phylum *Firmicutes* (Genus *Desulfoporosinus*) 7 OTU's were found in RJ1. *Desulfosporosinus* is a genus of strictly anaerobic, SRBs found in microbial communities are associated with mining environments and are involved in bioremediation process of metal contaminated water and sediments. These environments are enriched with SO , sulphate reduction leads to precipitation of metal sulphides and immobilization of toxic harmful metals. Recently *Desulfosporosinus* bacteria are identified as key players in microbial sulphate reduction in peatlands. 19 OTU's in NP1,8 in RJ1 of Genus *Desulfotomaculum* were present. *Desulfotomaculumare* Gram positive, obligate and anaerobic soil bacteria. SRBs can be identified the release of hydrogen sulphide gas with its rotten egg smell. They are endospore forming bacteria. The bacteria use H2 that is generated in environment by the energy released from radioisotopes as well as by other chemical reactions to form Hydrogen sulphide that replaces the hydrogen

bonds produced by photosynthesis. This discovery is promising as it proves that organisms cab obtain energy from sources other than sun [123].

Desulfotomaculum are curved, straight rods, highly resistant and free-living bacteria that also fix atmospheric nitrogen. They hay peritrichous flagella and are motile, present in inhabitants of soil, water, geothermal run off, insects and in rumens. They also cause sulphide stinker spoilage of canned foods [96].

Phylum *Proteobacteria* (class *Deltaproteobacteria*, Genus *Defluviicoccus*) represent 22 OTU's in PN1, 28 in BH1, 31 in RJ1. *Deltaproteobacteria* is anaerobic, Gram negative class of bacteria. Many genera of this class act as essential contributors to sulphur cycle. Genus H16 (Family *Desulfurellaceae*) shows 8 OTU's in PN1, 4 in CN1, 6 in C1, 14 in NP1, 20 in BH1, 15 in RJ1. The family Desulfurellaceae is part of the class *Deltaproteobacteria* in the phylum *Proteobacteria*, Members of the family are mostly obligately sulfur respiring, though *Desulfurella propionica* can use thiosulfate as an electron acceptor besides elemental sulfur. During growth, organic substrates are completely oxidized with CO_2 and H_2S as products [122].

Genus *Geoalkalibacter*, family *Desulfuromonadaceae*, class *Deltaproteobacteria*, phylum *proteobacteria* members of the family are anaerobic with some tolerance to oxygen. They are found in anoxic enveironment such as freshwater and marine sediments. They play a vital role in degradation of organic matter and are involved in syntrophic associations especially with methanogens and phototrophic green sulphur bacteria. 33 OTU's of this family were found in NP1, 65 in PN1, 31 in BH1, 16 in RJ1 [98].

Genus *Geobacter* of *Proteobacteria* include species that are anaerobic that have capabilities of bioremediation. *Geobacter* is first organism that have ability to oxidize organic compounds and metals such as iron, radioactive metals and petroleum compounds. *Geobacter* species can respire on graphite electrode. They are found in soil and aquatic sediments [71],[79]. 10 OTU's were found in NP1, 12 in C1, 2 in RJ1. 9 OTU's of Genus *Geothermobacter* in C1 were found. Genus *desulfovigra* are strict anaerobes, many members of *Syntrobacteraceae* are SRBs. Some species

are also motile due to presence of one polar flagellum. They show significant abundance 98 OTU's in NP1, 316 OTU's in PN1, 104 OTU's in BH1, 137 OTU's in RJ1 and 4 OTU's in C1.

Chapter 5

Conclusions and Recommendations

The basic task of MEOR technology is to resolve the problem of microbe's regulation based on the findings of some trials, namely the diversity and distribution of microbes related to the MEOR mechanism and their function on the MEOR effect by the use of nutrients injected into the reservoir. In general, biotechnology and microbiology, which have an impact on the development of the oil / gas field, will be able to provide originality dimensions in order to overcome the many issues involved in the EOR.

Current study was carried out by using six water samples, two from each site Nandpur, Panjpir and Bahu oil and gas field and four soil samples, two from each site chak Nourag and Rajian. AAS Aanalyst 7000 model was used in determining the content of Pb, Cr, Cd, Ca, Fe, Na, Cu, Mn, and K from the soil samples. AAS Analyst 7000 model used in determining the content of calcium, magnesium, potassium, sulfate, phosphate, nitrate, nitrite, copper, iron, manganese, lead, chromium, cadmium, and zinc from the water samples. Metagenomics analysis was done at sequencing depth of 85000 to 89000. Results showed highest pH (8.5) in RJ1 and lowest pH (6.3) in BH1. Electrical conductivity revealed in BH2 and lowest in C2. Highest sulphur concentration 520ppm in NP1 and lowest concentration of Sulphur

6.3 ppm were observed in C1. Alpha and beta-diversity analysis revealed highest concentration of nitrates and sulphur in PN1 and NP2. Archaeal rich diversity of phylum *Euryarchaeota* and *Thaumarchaeota* and bacterial phylum Acidobacteria, Actinobacteria, Bacteriodete, *Firmicutes, Planctomycetes, Protobacteria* and *Chloroflexi* were found dominating in reported samples.

Most of these innovations hinder the further production of oilfields due to their economic limits. The Microbial Enhanced Recovery of Petroleum (MEOR) technique has been suggested for many years as a cheap and effective solution to enhancing oil recovery, as its different processes do not necessarily rely on oil prices. Microbes are rich in diversity within the specified reservoirs. Activated microbes may generate multi-functions during oil displacement. Microbes could freely move inside the porous medium. Development of MEOR method is impossible without the knowledge of physiochemical and microbiological condition of the oil field. This in silico work will be verified for effective concentration of active functional genes in lab. We don't see many characterized strains in our data profile but sulfur activity is high that most probably is because of presence of active genes in natural environment. Difference in activity of sulfur reduction on site and off site will be determined, it will help us in estimating exact amount of bio stimulation which needed to be added in reservoirs for enhanced sulfur reducing activity and metabolism of oils.

Over nearly a century, MEOR's potential developments have been surpassed by others on the grounds of their economic efficiency and environmental protection. Gradually, tertiary oil recovery technology is becoming an effective technique, particularly for the exploitation of high-water and heavy oil reservoirs. In order to overcome its shortcomings by promoting more industrial applications, the implementation of MEOR could, in the future, concentrate on the following aspects:

1. Development of a practical microbial library, the nature and distribution of indigenous microorganisms under target oil reservoirs should be investigated and evaluated using molecular cloning technology. Microbial candidates could then be isolated and classified on the basis of their different

oil-displacement functions.

2. Specific analysis of the MEOR system: a mechanism research will rely on one or more specific microorganisms. Such cells could be chosen from a generic microbial collection that could have different oil-displacement roles. Consequently, their MEOR functions are examined, including structural gene expression, functional enzyme development and essential biochemical pathways. Functional genes involved in metabolism of specific complex of petroleum are in need to be explored.

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