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Estimation of Genetic Component Responsible for High Salt Tolerance in Indigenous Desert Bacterial Strain

by

Arooj Wajid

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

in the

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I would like to dedicate this thesis to my father Wajid Hussain.

CERTIFICATE OF APPROVAL

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Abstract

Climate change resulted in significant changes in weather conditions, precipitation, soil erosion, moisture, fluctuation, salinity, desertification and may other biotic and abiotic stresses. Salinity among the major problem of present day world, in saline soil concentration of salts is increased due different natural and anthropogenic factors. Irrigation is one of the major causes of salinity and this results in reducing the fertile land area available for crop production. As population of the world is increasing day by day but production of food is going low as there is less fertile land area available for crop production. Different microorganisms present in the soil are able to survive even in high salinity and such microbes also help to remediate extra solutes, salts ,ions or other contaminants present in the soil by a process known as bioremediation. A high salt resistant strain named as AR-6 present in the soil sample of desert Cholistan was isolated and identified by treating different strain from the sample with 0- 20mg/L Nacl. This strain was Gram positive, non-pigmented and non-spore forming. Genome analysis of the strain was performed in order to estimate genes, proteins or enzymes responsible for salt tolerance in them. Phylogenetic analysis based on 16S rRNA sequencing showed that the strain AR-6 belongs to family *Micrococcaceae* and it is related to genus *Arthrobacter* and it showed highest sequence similarity with *Arthrobacter ginkgonis*. G+C content of the strain AR-6 was 70%. Total sequence of gapped and un-gapped length of nucleotides present in it is 3,291,617. 50 number of scaffolds and contigs and 2,963 proteins are present in it. Proteins involved in membrane transport, potassium metabolism, nitrogen metabolism, ATP binding proteins, ABC transporters, polyphosphate kinases, transcriptional regulators are considered as responsible for high salt tolerance in AR-6. This study suggested that in future this high salt tolerant strain can be used as bio stimulant in the saline soil in order to reduce the salinity of the soil thus to increase crop production.

Keywords: Salinity, Bioremediation, Salt tolerance, Phylogenetic Analysis, salt tolerating genes, *Micrococcaceae*.

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Abbreviations

ABC	ATP Binding Cassete
BTEX	Benezene Toulene Ethylbenzene and Xylene
CSA	Chromogenic Salmonella Agar
ERF	Ethylene Response Factor
HAK/KUP/KT	High Affinity K+,K+ Uptake Transport
ISP-2	International Streptomyces Project 2 Media
ISP-3	International Streptomyces Project 3 Media
MNA	Monitored Natural Attenuation
NAD	Nicotinamide Di-nucleotide
PCR	Polemerase Chain Reaction
PRB	Permeable Reactive Barrier
R2A	Reasonor's 2 Agar
TMD	Transmembrane Domain
TSA	Tryptic Soy Agar

Chapter 1

Introduction

1.1 Background

The need for preservation of ecosystem is increasingly revealed within rising population of world and daily life demands supplies by industries and modern industrialized systems. Wars, earthquakes and tsunamis are calamities that are the reasons for further attention of cleaning of the polluted ecosystem. Bioremediation is one of the most economical and stable approach to cope this task; it is branch of environmental biotechnology in which various microorganisms are used to remediate the contaminated ecosystem. It has been recognized that role of microorganisms in the detoxification of polluted and contaminated soil is very unique and distinct.

Bioremediation is a modern term and it explains a process that has been existed since start of life. Bioremediation is recycling of organic matter of the, soil by using different strains of microorganisms. Organic matter of soil is converted into carbon dioxide by the actions of microorganisms. Role of bioremediation or bio reclamation is to eradicate toxification of man-made pollutants present in the soil. Bioremediation is a very efficient and environmentally acceptable process which is used for controlling salinization of agriculture soil [1]. Climate change resulted in significant changes in weather conditions, precipitation distribution, soil erosion,

moisture and temperature fluctuation, salinity and desertification, many biotic and abiotic stresses have been caused by these extreme environmental conditions. Human activities are also continuously adding salts in the soil and water, presence of salts in soil is considered as major stress that severely limits crop production. Salts are added in maximum amount in the soil that is under irrigated agriculture. Water resources are decreasing day by day and use of saline water for irrigation purposes is increasing saline and reducing the yield of crop production due to large amounts of salts are accumulated in root zone of plants. [2]. Essential mineral nutrients are needed for plants to grow and develop, a large number of soluble salts present in the soil are harmful for many plants. Salts can restrict plant growth more than any toxic substance can do on a world scale; salinity can affect more than 40% of irrigated land to various degrees [3]. World has used different irrigation technologies for irrigation of land. These technologies include direct irrigation with saline water and irrigation with fresh water and saline water [4].

Saline water can cause shortage of water resources, utilization of saline water with large amount of salt concentration and use of insufficient amount of saline water for irrigation may cause both the water and salinity stress and that leads to salinization of cultivated soil, and may cause serious problems to agricultural environment and ecosystem [5].

In saline soil many crops cannot survive or may survive only with decreased yields, to mitigate the harmful effects of salinity, measures such as reclamation of salinized soil and improvement of irrigation with saline water and cultivation of salt tolerant varieties of crops have been applied [6].

Soil salinization can occur due to primary and secondary process, salts are accumulated in the soil through natural mechanisms like maximum evapotranspiration and is termed as primary salinization, salts can be accumulated in soil by human activities, increased withdrawals from aquifers and is termed as secondary salinization. Chances of saline soil to occur mainly depends on geographical location of land, soils from sea-shore areas have more chances to be affected with events such as tsunamis and floods [7]. Salinization has several effects on agriculture

including productivity changes, it increases risk for plants having minimum tolerance , loss of biodiversity. Soil organisms play role in ecological processes like organic matter decomposition, nutrients cycling and maintenance of soil structure and salinity may effect the role of these microbes [8]. There is less studies on the affect of salinization but negative and harmful effects of salinty on reproduction and sustainability of soil invertebrate species have been reported [9]. Studies have been reported that soil salinization have decreased or delayed seed germination, plant growth and photosynthetic pigments. These effects can lead to yield of different commercial plants, for example 72-100% for rice, 5-79% for wheat, 26.2% for tomato. Salinization tolerance have been found in some soil inhabitants and they can survive salinity levels upto 70% (70g 1-1 assuming NaCl salt) [10]. Biological agents mainly algae, fungi, yeast or bacteria are used to clean up contaminated soil mainly highly saline soil. These microorganisms degrade environmental pollutants into a form that less toxic than the usual. This process promotes the growth of specific micro flora that is indigenous to contaminated soil that is able to perform their degradation role [11].

Such microbial consortium can be established by promoting growth of microbes through addition of nutrients, terminal electron acceptor or by maintaining temperature and pressure [12]. Microorganisms use contaminants present in polluted soil as nutrients for themselves in the process of bioremediation [13]. Microorganisms are stimulated to rapidly degrade harmful organic pollutants to a safe level for environment from soil, sediments, materials and ground water. Microorganisms required enzymatic apparatus during enrichment course and they attack on organic contaminants present in the soil with enzymatic actions, contaminants from soil either increase or decrease the rate of enzyme activity of microbes [14]. Bio augmentation is a process in which microorganisms are imported to contaminated site in order to enhance biodegradation of contaminated soil. This can decrease lag phase of microbe due acclimatization of specific contaminants. Bioremediation and bio augmentations are mainly a safe and natural process to reduce harmful contaminants [15]. Microorganisms that carry out biodegradation in different environments are: *Actinobacteria alcaligenes*, *Arthrobacter*,*Bacillins*,*Berijerinka*,

Flavobacterium, *Methyosinus*, *Mycrobactrium*, *Pseudomonas*, *Serration*, *Methyosinus*,*Pencillium*,*Nocardia*, *Phanwrochaete*,*Xanthofactor*. These hazardous organic compounds cannot be mineralized and degraded by only one microorganism, so complete mineralization is done by sequential consortium of microorganisms and involves synergism and co metabolism action [16]. Actinobacteria are known as extremophilic as they can survive in extreme environments such as saline, high-/low pH, low/high temperatures, low level of moisture and nutrients [17]. Actinobacteria are highly extremophilic and extremotolerant due to their physiology and metabolic flexibility. Old pattern of numerousness of actinobacteria in soil and fresh water habitats was broken when it was reported that actinobacteria are present in extreme environments. Few studies have been done to understand behavior, physiological and ecological role of actinobacteria to survive in highly extreme environment. Polyextremophile and polyextremotolerant are two species of actinobacteria that are able to survive under different extreme environments. Polyextremophile can exist in environments with different stresses, which includes thermoacidophile, thermophilic, radio tolerant, haloalkaliphilic, and thermoalkalolerant actinobacteria [18].

The extremophilic actinobacteria have different adaptive strategies to survive, which includes antibiosis, changing their metabolic modes from one form to another such as autotrophy, heterotrophy and saprobes. They synthesize specific enzymes to function properly under unfavorable condition such as high temperature, salinity and alkalinity ranges. Thermophilic bacteria may have hydrophobic interaction and disulfide bonds in their proteins, they have distinct proteins known as chaperons which help in folding partially denaturized protein. Different kinds of proteins are manufactured that binds to DNA and prevent their denaturation at higher temperature. Thermophilic chemolithoautotrophy utilizes sulfur as energy source under extreme conditions. Acidophiles and alkaliphiles have proton pumps in their membranes to regulate H^+ inside and outside of cell for maintaining pH. Alkaliphiles have negatively charged polymer in their cell membrane that help to stable it by reducing charge density at cell surface. Haloalkaliphiles are tolerant to salt environment by synthesizing and accumulating high number of solutes that

prevents desiccation through osmoregulation. Na^+/H^+ antiporter is present in their membrane which help to exclude high salt content from inside of the cell [19].

1.2 Problem Statement

Increased salinity is responsible for decline in soil fertility as well as loss of yield of edible crops, to explore the genetic factors in bacterial strain that are responsible for salt tolerance could be possible remedy to minimize the effects of salinity on soil.

1.3 Aim and Objective of Research Study

The aim of this study to know how bio-remediation is helping to increase yield of crops growing in highly saline environment. Bacterial 16s RNA strain will be isolated from desert sample and complete genome sequencing will be performed in laboratory to identify the strain.

1.4 Objectives of Research Study

- To isolate xerophytic bacteria from desert sample.
- To screen the strain with maximum salt tolerance range.
- To identify which strain will have maximum salt tolerance range.
- To sequence whole genome of strain for finding generic and protein profile of strain.

Chapter 2

Literature Review

2.1 Bioremediation

Environmental effluence has been amplified since past few eras due to increased human actions. Activities such as energy reservoirs, unsafe farming practices and speedy industrialization by human are major cause of environmental pollution. Major pollutants such as heavy metals, nuclear wastes, pesticides, greenhouse gases, and hydrocarbons are causing environmental problems due to their toxicities. The process of bioremediation (use of microbial organisms for remediation of polluted soil) has been proven reliable and effective due to its eco-friendly features. From past two decades there have been advances in bioremediation techniques which can reestablish polluted environments in an environment sociable level at very little cost.

Different techniques have been established by researchers for removal of pollutants from soil but due to nature or type of pollutant it is not possible to use a single technique. Indigenous microorganisms present in the soil are capable to solve most of the challenges allied with bioremediation and biodegradation [20]. Nature of pollutant is very necessary for the selection of process to remove pollutant. Pollutants such as: chlorinated substances, harmful, green-house gases, metals, hydrocarbons, nuclear water, plastics and sewage waste are required to remove

from environment. Bioremediation techniques can be divided into ex-situ and in-situ. These techniques can be chosen on the base of nature of the pollutant, degree & depth of pollutants, kind of environment, location of pollutant, charge rate and environmental policies [21, 22].

Different performance criteria (O_2 & nutrients concentrations), temperature (T), pH that determines rate of success of remediation processes have been given major considerations before starting a bioremediation project.

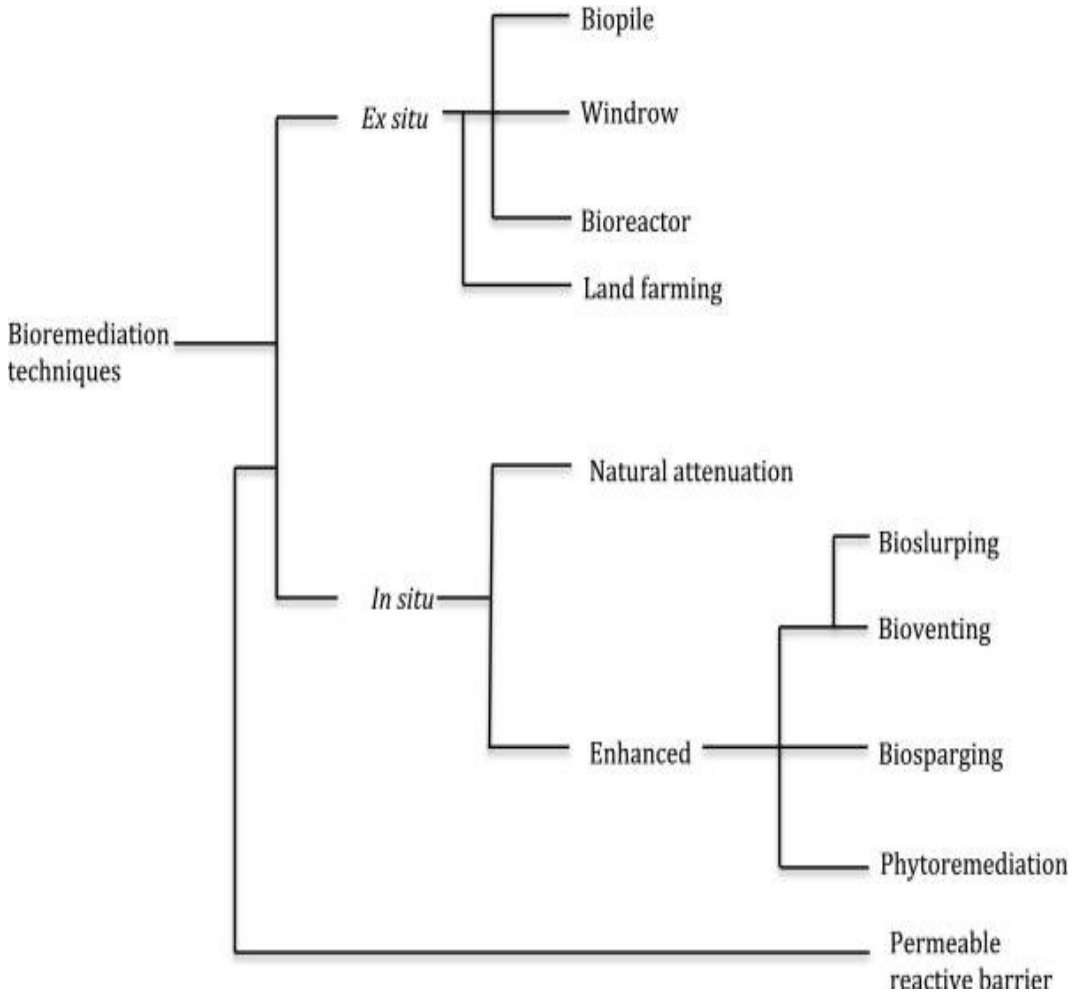

FIGURE 2.1: Hypothetical divergence of bioremediation techniques [21].

2.1.1 Ex-situ Techniques of Bioremediation

These techniques are done by extracting impurities from polluted sites and then transferring them to another place for dealing. Ex situ techniques generally based

on cost of treatment, depth of pollution, type of pollutant, degree of pollution, geographical location and geology of the polluted site.

2.1.1.1 Bio-Pile

Bioremediation that involves pilling of excavated polluted soil above ground level and it is followed by nutrients amendment, aeration and increases bioremediation activities by microorganisms termed as bio-pile mediated bioremediation. Components such as: aeration, irrigation, nutrients and leachate collection system and treatment bed are necessary for this technique.

Particular ex-situ technique is progressively used due to its features including cost effectiveness that enables effective bioremediation. Volatilization of low molecular weight pollutants is major limitation of application of bio-pile remediation technique & it can also be used to treat contaminated extreme environments such as very cold, hot, acidic or alkaline regions [23].

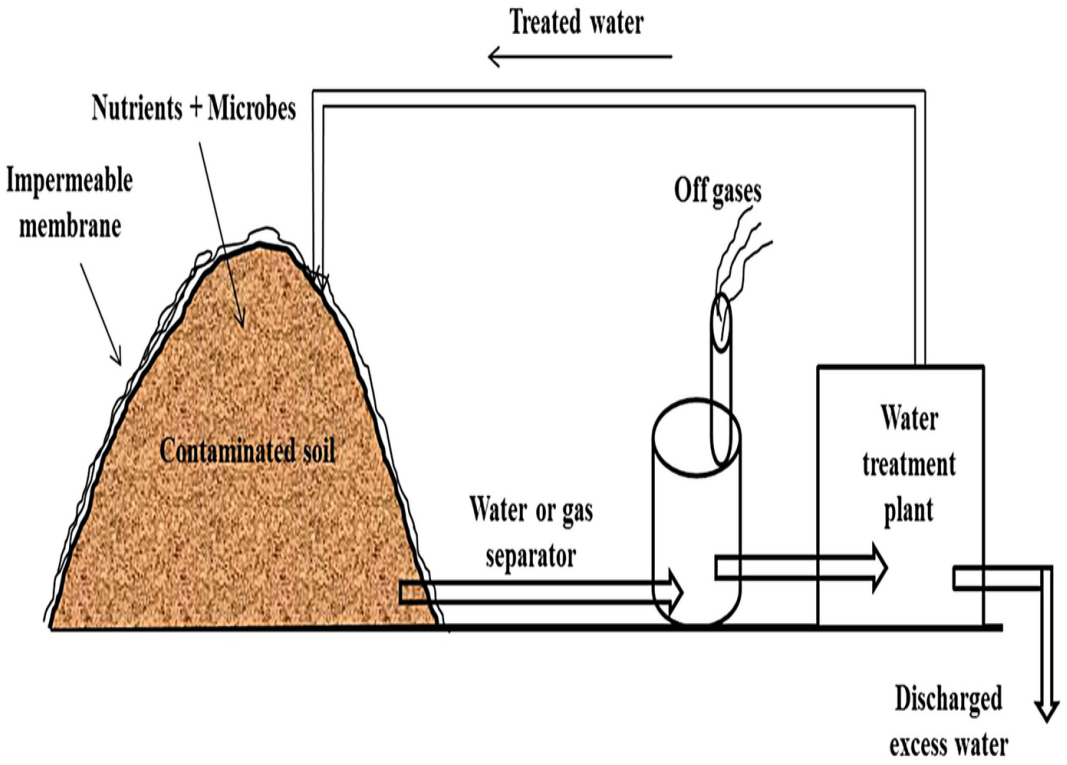

FIGURE 2.2: Bio Pile [23].

2.1.1.2 Windrow

Windrow is a bioremediation procedure in which pilled polluted soil is turned over on regular interval of time to help in increasing biodegradation activities of indigenous microorganisms.

This periodic rotation of polluted soil with water and increases aeration and speeding up the rate of microbial degenerative activities [24].

Assimilation, biotransformation and mineralization help to elevate rate of bioremediation. Windrow treatment have higher efficiency of hydrocarbon removal from the polluted soil than bio-pile treatment [25].

2.1.1.3 Bioreactor

Bioreactor is a vessel in which raw materials are converted into specific end products by following different biological reactions. Different operating modes for bioreactors are, which include: batch, fed-batch, sequencing batch, continuous and multistage bioreactors.

Operating mode of bioreactor is selected on the base of market economy and capital expenditure. Bioreactors mimics natural environment to support natural process of microorganisms grown in it. Contaminated samples can be added into bioreactor in dry form or in slurry form.

Bioreactors have many advantages as compared to other ex-situ bioremediation techniques as they have brilliant control on bioprocess parameters i-e temperature (T), pH, agitation and aeration rates. Ability to control and manage these parameters within bioreactors help reduce bioremediation time.

Precise bioaugmentation, nutrient addition, increased pollutant bioavailability, mass transfer is among the limiting factors of bioremediation process and can be effectively established in bioreactor and makes bioreactor-based bioremediation more effective [26].

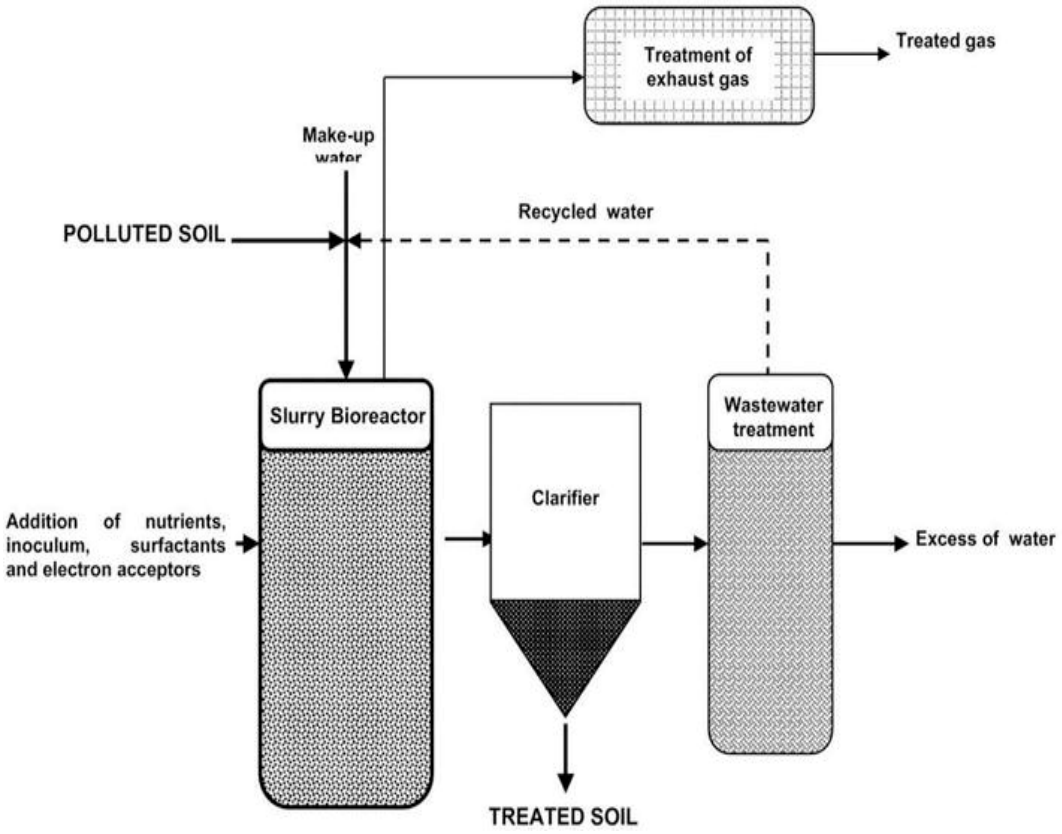

FIGURE 2.3: Bioreactor for treatment of polluted soil [27].

TABLE 2.1: Pollutants removed by using bio-reactor based bioremediation

Bioreactor	Samples Nature	Pollutants Nature	%age of removal of pollutants	Reference
Expanded granular sludge bed reactor	Laundry	Alkylbenzene sulfonate	92.9	[27]
Stir tank bioreactor	Crude oil polluted sediment	Petroleum Polyaromatic hydrocarbons	82- 97	[28]

Expanded granular sludge bed reactor	Laundry	Alkylbenzene sulfonate	92.9	[28]
Anaerobic sludge blanket	Bezene Toulene Ethylbenzene	Benzene, toluene, ethylbenzene, and xylene	51-86	[29]
continuous flow	Xylene (BTEX) containing H2O.			
Roller slurry bioreactor	Polluted or contaminated soil	2,4-dichloro phenoxyacetic acid	97-100	[30]
Packed-bed reactor	Amines	Mixture of sulfonated amines		[31]

2.1.1.4 Land Farming

Land farming is easiest bioremediation practices because of its small cost and less material requirements used for its processing. Land farming is regarded as ex-situ technique in most conditions while in other conditions it is known as in-situ technique.

This question arises due to place at which treatment of pollutants may occur. Land farming could be processed as ex-situ or in-situ techniques and it depends on the pollutants depth.

When excavated soil and pollutants is treated on site, it will be counted as in-situ technique. Its been reported that when pollutants are present <1m below the ground surface in this case bioremediation may proceed without excavation while

pollutants present >1.7m required to be transfer to the ground surface for effective bioremediation [32].

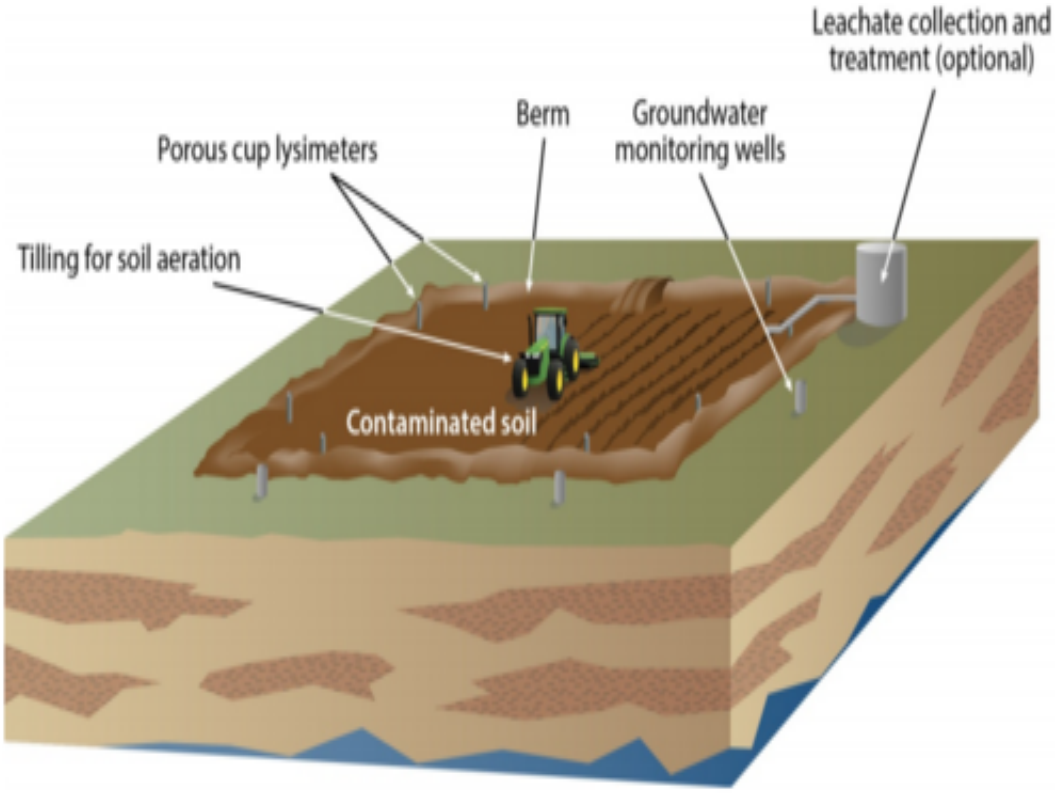

FIGURE 2.4: Land Farming [32].

2.1.2 In-situ Techniques of Bioremediation

In-situ techniques are involved in treatment of polluted materials at the place of pollution and it does not require any diggings therefore it may be processed by bit or no excursion to soil structure.

These in-situ methods ought to be less expensive as compared to ex-situ bioremediation techniques as there is no extra expenses for excavation process. Some in-situ bioremediation techniques, such as: bioventing, bio-sparging and phytoremediation might be enhanced but other techniques might process without any kind of enhancement in them.

In-situ techniques have been used since many years to treat different types of chlorinated solvents, dyes, heavy metals and hydrocarbons successfully [33,34].

2.1.2.1 Bio-venting

Bioventing involves measured stimulation of air-flow by sending oxygen to unsaturated zone in order to upsurge bioremediation by increasing activities of microbes living there. In this procedure of bioremediation several changes are carried by adding required amount of nutrients and moisture to increase the rate of bioremediation with goal to attain microbial conversion of pollutants to a harmless form. This technique has gained popularity amongst other in-situ bioremediation techniques [35].

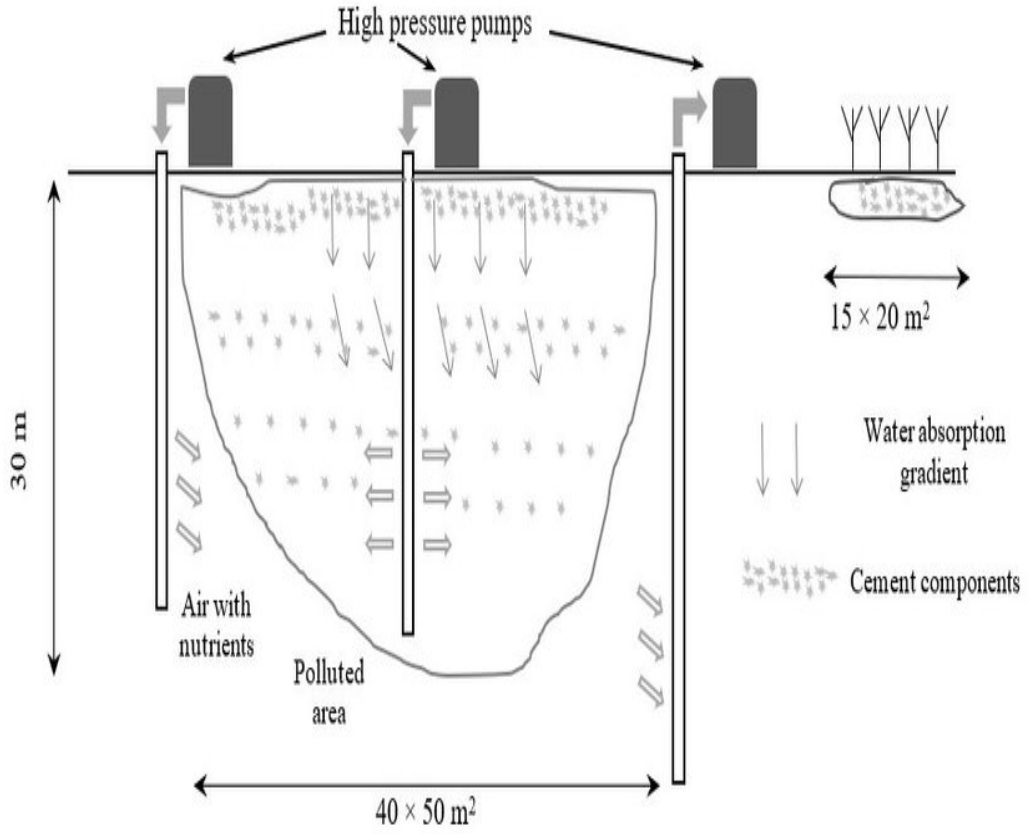

FIGURE 2.5: Bio-venting [35].

2.1.2.2 Bio-slurping

Bio-slurping incorporates other processes such as mechanically enhanced pumping, retraction of soil vapor and regeneration of soil-acquired compounds by providing indirect oxygen and promoting polluting environmental degradation. Flexible pollutants and flammable substances present in the soil can be removed using this bio slurping method [36]. This method uses slurp that enters the product layer and draws liquid from this layer in the same way that grass is used to draw liquid from any vessel. By pumping up the increase of free products occurs and even reaches the point where it is separated from water and air. This system can easily be used to act as a general biological technique to complete bioremediation [37].

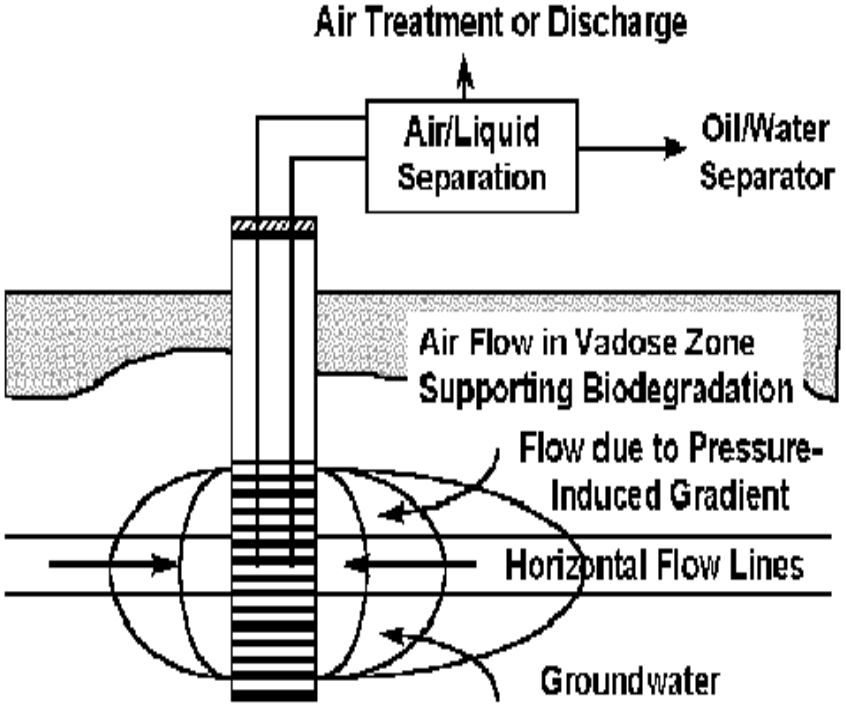

FIGURE 2.6: Process of Bio-slurping [37].

2.1.2.3 Bio-sparging

Bio-sparging is process closely related to bioventing technique in which air is presented into soil to increase rate of microbial actions to remove pollutants from contaminated spaces. In this technique: air is supplied at saturated zone that causes upward movement of easily evaporated organic compounds to enhance biodegradation. Bio-sparging effectiveness depends on two main factors firstly soil permeability that determines pollutants bioavailability to microorganisms and pollutant biodegradability [38]. This technique has been used to treat aquifers polluted with petroleum products such as kerosine and diesel [39].

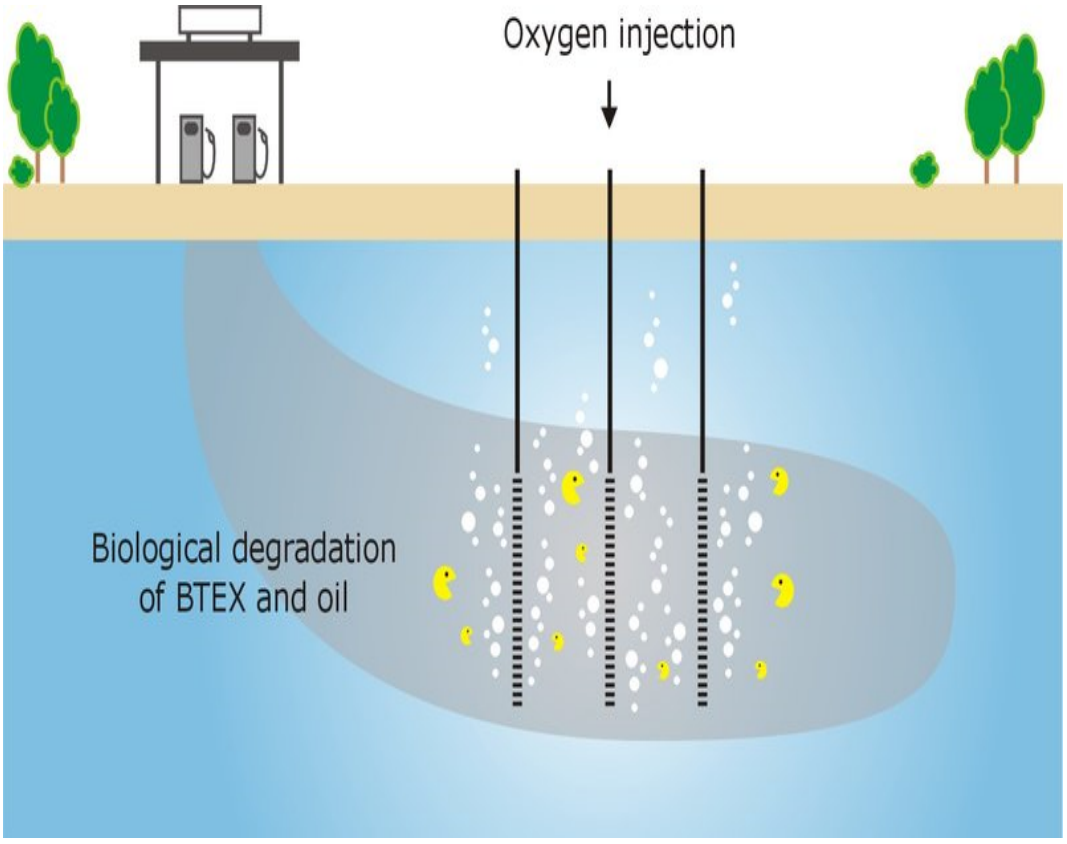

FIGURE 2.7: Process of Bio-sparging [39].

2.1.2.4 Phytoremediation

Phytoremediation is dependent on plants physical, biological, biochemical, chemical & microbial interactions in polluted spots to overcome the harmful possessions

of pollutants it is a process in which higher plants are used for environment friendly treatment or rehabilitation of soil which is polluted by organic and in organic compounds.

Extraction, degradation, filtration, stabilization and volatilization are different mechanisms that are involved in phytoremediation and they depend on the pollutant type. Pollutant may be of two types: heavy metal and organic. by using some plants alfalfa and willow organic pollutants can be removed by degradation, stabilization, rhizoremediation, and volatilization [40]. A plant can be chosen for phytoremediation on the basis of some important factors such as: root system that can be fibrous or tap depending on the depth of pollutant, toxicity of pollutant type to plant, plant survival rate and its adaptability to prevail environmental conditions, plant growth rate, site monitoring and above all time required to achieve the best level of cleanliness. Plant used for phytoremediation must be resistant to diseases and pests [41].

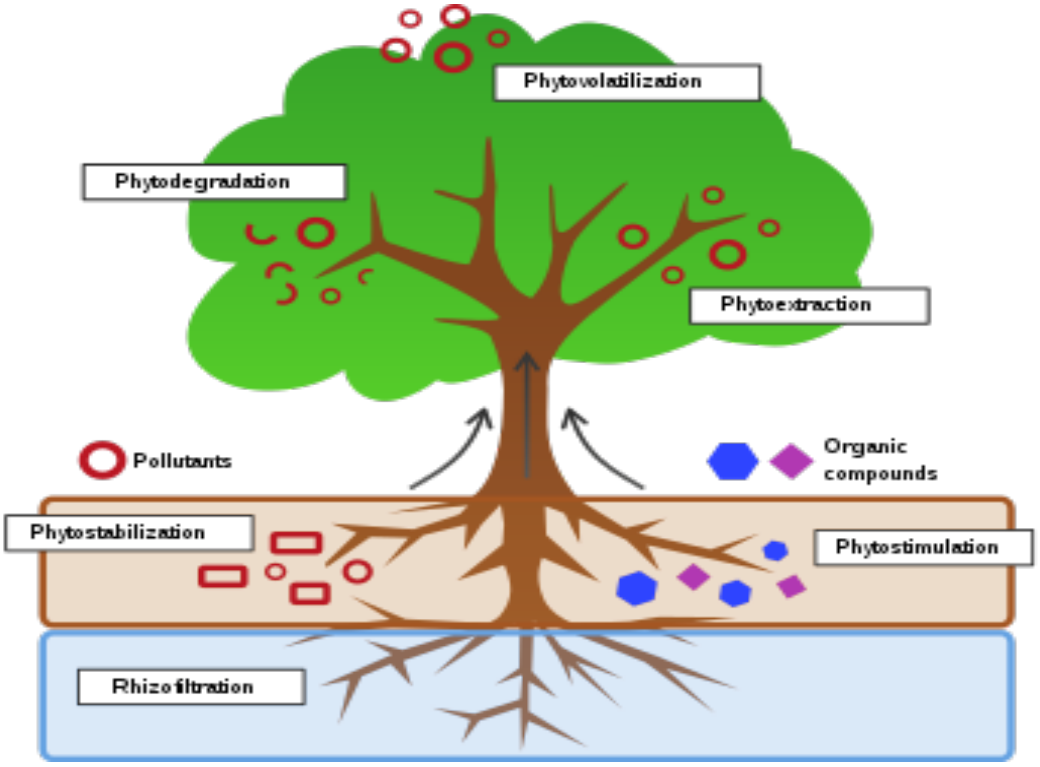

FIGURE 2.8: Process of Phytoremediation [41].

2.1.2.5 Permeable Reactive Barrier (PRB)

Permeable reactive barrier is technique considered as a physical process for treating contaminated ground water polluted with several kinds of pollutants having heavy metals & chlorinated compounds. It is reported that several biological reactions including degradation, precipitation, and sorption are mechanisms being used in PRB [42, 43]. Permanent or semi -permanent reactive medium made up of a zero-valent iron is used in this technique, which is submerged in the polluted ground water. Under natural gradient polluted water flow through the medium, pollutants get trapped and experience series of reactions which results in pollutant free water [44,45]. These barriers are so reactive that they are able to trap pollutants and allows the flow of water but not pollutants. This technique uses less energy input and it is readily available and approachable [46]. Pollutant type, biogeochemical & hydrogeological conditions, mechanical stability and cost are factors that explain effectiveness of technique in a better way [47].

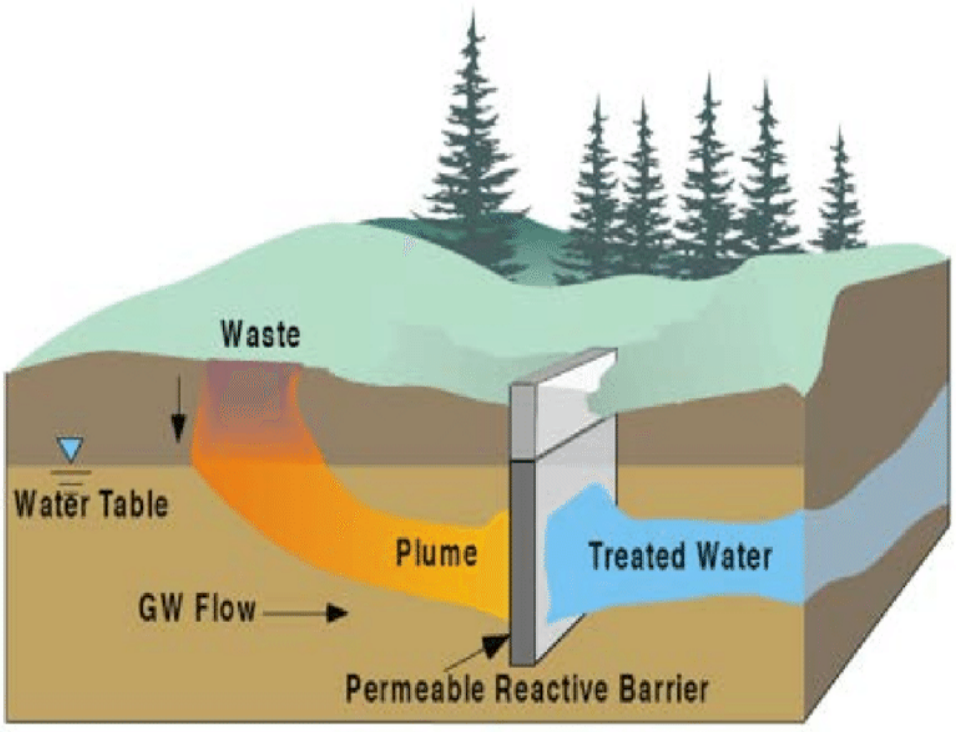

FIGURE 2.9: Process of permeable reactive barrier [42].

2.1.3 In-trinsic Bioremediation (IB)

Natural attenuation also termed as Intrinsic bioremediation (IB) is considered as in-situ bioremediation technique because it is involved in passive remediation of polluted sites without being used any peripheral force or human interfere.

This technique uses both the microbial aerobic & anaerobic processes to degrade polluting substance in the soil. This technique lesser costly as compared to other in-situ techniques due to absence of any external force. This technique is also termed as, monitored natural attenuation (MNA), as this process mut be monitored to check bioremediation is ongoing and sustainable [48].

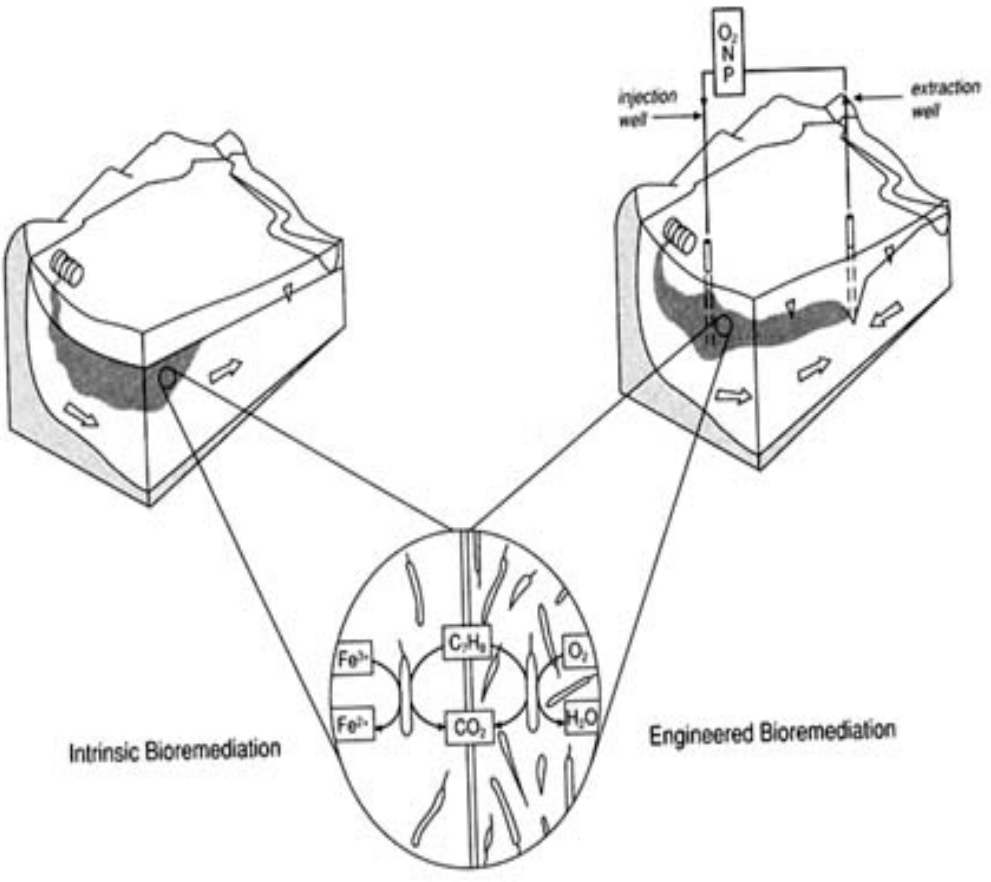

FIGURE 2.10: Intrinsic bioremediation [48].

2.1.3.1 Limitations of Intrinsic Bioremediation

No external force is used to accelerate the remediation process, intrinsic bioremediation takes extensive duration to achieve the target level of pollutant concentration. Risk calculation needs to be carried out before applying intrinsic bioremediation to make it sure that remediation time should be lesser as compared to time used for pollutants to reach exposure point. It has been reported tin studies that intrinsic bioremediation does not help to remove enough quantity of polyaromatic hydrocarbons [49].

2.2 Salinity of Soil

Some energy intensive activities such as mining, agriculture and deforestation impact on economies and causes directly or indirectly soil, air and land pollution. Large quantities of structureless geologic supplies are produced by mining and they contain significant number of toxic metals, lead, arsenic, cadmium in the form of primary or secondary minerals and that help to increase land pollution. In modern agricultural practices a vast quantity of commercial fertilizers and pesticides are used and they can pollute land, air and water.

Quality of land is indirectly affected by deforestation that increases the soil erosion and sediment transport. Invasive plants species are also affecting soil by creating soil conditions that may be toxic to other plants. These activities can affect soil salinity and acidity by releasing unwanted metals, salts, acid or acid- forming minerals [50].

Soil salinity is of two main types: primary & secondary. As a result of natural environmental conditions such as topography, rain, mineral composition of the earth natural salt gathering in the soil surface occurs and this may result primary salinity in the soil. Secondary salinity has anthropogenic causes: poor management in the face of undersupplied environmental conditions for agriculture, excessive use of fertilizers in the soil, poor management of irrigation water, use of saline water

& proper drainage system. It is estimated that about 67% of agricultural area is associated with salinity [51].

Water soluble salts are accumulated in soil regolith to level that proves to be dangerous for agricultural production, environmental health, and welfare. Land is degraded by salinity and this results in malfunctioning in plant crop growth and development and that result in reducing agricultural productivity about 9.5 107 hectares of area which is more than 7% of Earths surface [52]. As with increase in human population and development of industries, soil salinization is expected to increase, it is mandatory to locate and identify salt affected areas and determine their composition of salts in order to remediate that effected piece of land [52].

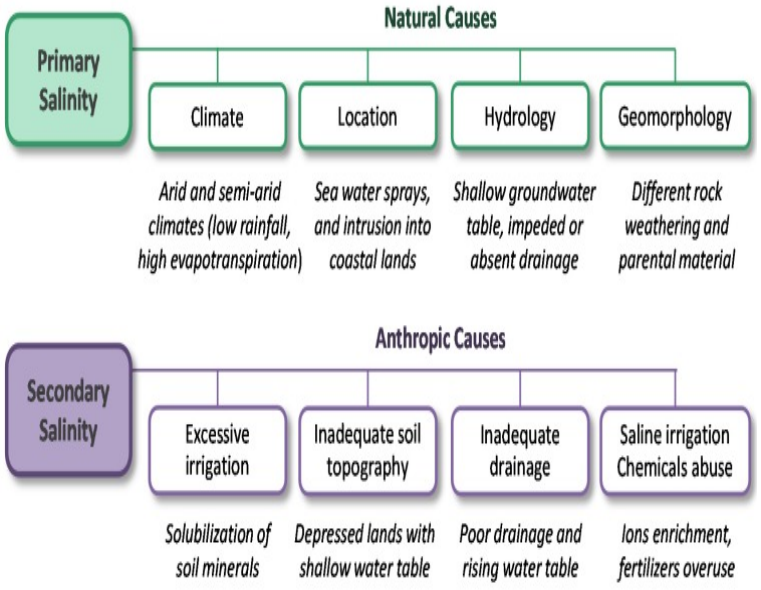

FIGURE 2.11: Primary salinity caused by natural ways; Secondary salinity caused by anthropogenic factors [51].

2.2.1 Irrigation and Salinity

Agricultural production is increased in areas where rainfall is less than normal rate, by using irrigation process but due to scarcity of fresh water farmers are compelled to irrigate plants using contaminated or poor-quality water [53]. Saline water is irrigated and it increases soil concentration in the agricultural lands, about 33% of agricultural lands are affected by salinization [54].

FIGURE 2.12: High Saline of soil [53].

Salinity has major effect on plants physiological processes: photosynthesis and transpiration are badly affected by salinity [55]. Soil water potential is reduced by salt stress and it result in ion toxicity and eventually plant death Decreased osmotic stress in plants reduces water uptake by plants which occurs usually by closing leaf stomata and decreasing transpiration, this result in decreasing rate of photosynthesis and this negatively affects plant growth. Physiological and biochemical metabolic disorders may result in plants due to excessive accumulation of salts in the soil, mainly due to osmotic effects, dehydration and nutritional imbalance and Na+ toxicity [56].

2.2.2 Winter Irrigation and Salinity

Arid regions receive less amounts of rainfall whereas a significant role is played by irrigation in the agricultural production of arid area. A large amount of water is consumed by irrigation and there are expected change in these trends in coming future. Sustained irrigation can bring severe land salinization in arid regions [57].

Winter irrigation stimulates salt leakage from the root zone: the need for water vapor permeability is low and loss of moisture is minimized, reduced salty winter irrigation has been suggested [57]. It has been reported that winter irrigation is

one of the most common Winter irrigation salt increases with increasing water irrigation volume [58]. Winter irrigation should be done when the soil starts to cool at night but melts during the day as the beneficial effects of winter irrigation come mainly from salt purification, which reduces salt. After the 150-mm winter irrigation application, the accumulated salt in the 0 60 cm layer can penetrate deep into the soil layers and that the salt content of the soil as measured by its electrical conduction decreases by about 0.2%. Therefore, the amount of water sprayed in advance can effectively prevent secondary salinity of the soil and improve land use efficiency should be considered when designing winter irrigation systems Although some of the best winter irrigation results have been confirmed by previous experimental studies and modeling simulations, most researchers have focused on how winter watering affects soil moisture and salinity [59].

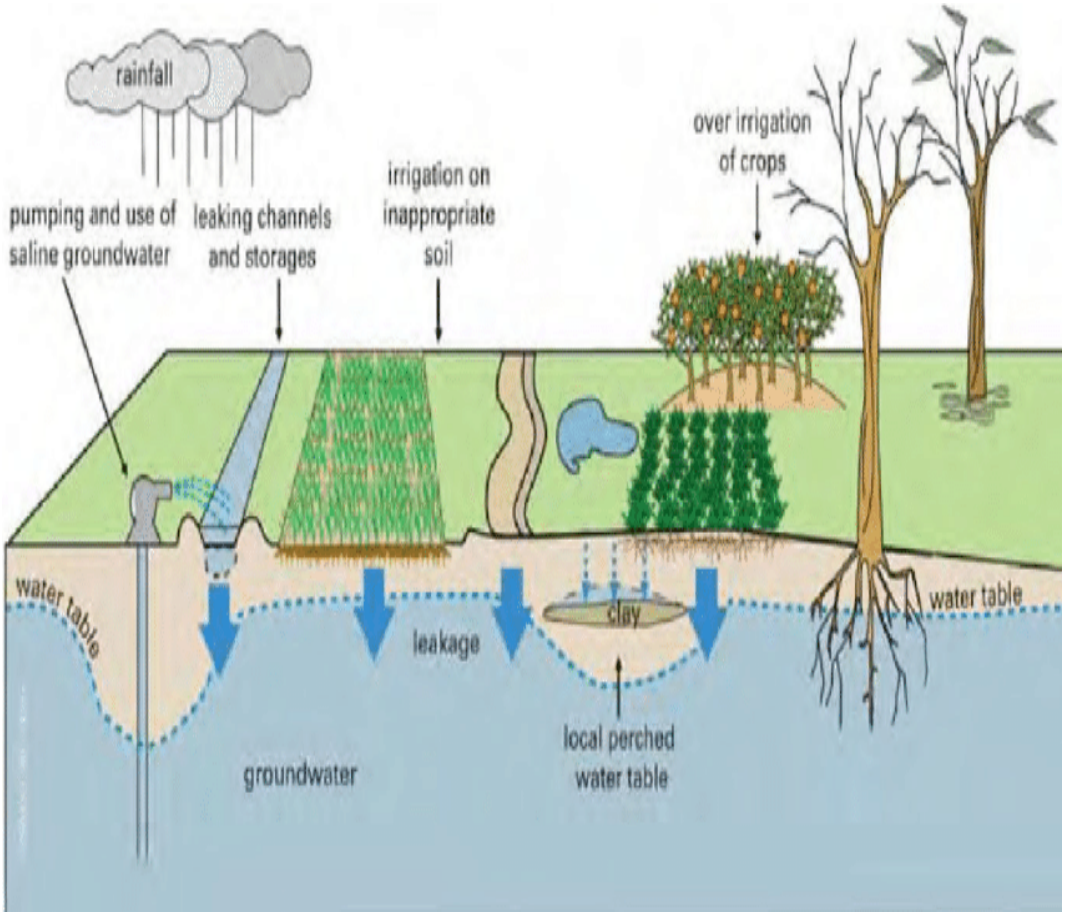

FIGURE 2.13: Causes of irrigation salinity [57].

2.3 Salt Tolerance

Plants salt tolerance is often defined as the degree of salts to which plants are exposed but plants survive in the presence of salts without reaching this salt in the leaves or roots. Salt tolerance is important in irrigated lands in semi-arid areas where it can exacerbate the problem of soil salinity.

The conditions of salinity by plants are variable, crop species have usually intolerance against the concentration of salts found in sea water. Traditional efforts to improve salt tolerance in plants met very little success due genetically and physiologically complexity of the trait [60].

There is ample evidence to support that salt tolerance is multi-genic trait, physiology of salt tolerance shows that the overall trait is determined by sub trait and which return is determined by any genes. These sub-traits usually include the ability to minimize pure accumulation of sodium or chloride ions and it choose potassium over sodium from a high sodium back ground [60].

2.3.1 Need of Salt Tolerance

Earth is considered as planet of salts, as most of its water contains Na 30g/L. Crops that are already present on the lands or may be grown in future are affected by salt solution badly. A huge area of land is affected by salinity and it pose a great threat to agriculture, as many crop plants except halophytes are not able to grow in high concentration of salts. So due to increased salinity in land, crops will fail to grow and consequently there will be less supply of food. Presently there is enough supply of food in the world, more than 900 million people are undernourished. But it is alarming that food availability will be minimum in coming future as the population of world is increasing day be day, it is expected the population of world will become 9.3 billion till 2050 [60]. To ensure food security crop production must be increased.

Dry lands and semi-arid areas cover half of the world and the production rate of these areas is very low, these areas can only be made more productive by irrigation. Irrigation increased over the past few years but there is a strong link between irrigation and salt. The question arises from the sustainability of irrigation to promote crop production. To overcome this problem, there is a need to increase salt tolerance in plants or to produce varieties that are more resistant to salt. Salt pressure can also be reduced if the soil is treated with pesticides that help the soil to absorb more salt in the soil and make the soil more fertile. In order to maintain global food production, it is necessary to increase the salt tolerance of plants.

2.3.2 Salt Tolerance in Plants

Important abiotic stresses that badly affect plant growth and productivity is salinity [61], use of poor-quality water for irrigation intensifies the salinity problem. Plants have ability to tolerate higher concentrations of salt in the form of overhaul of strength, growth, and yield. Salinity can affect plants through osmotic stress and/or ionic stress [62]. To overcome the effect of salinity stress plants developed physiological, biochemical responses either for tolerating or avoiding the stress. Common strategies of salt tolerance in plants are as: ion uptake by the roots, ion exclusion from the roots, ion accumulation in vacuoles of root or shoot cells, regulation of ion transport from root to shoot, increased tolerance to high concentrations of toxic ions, and accumulation of compatible solutes[63]. It is important to link the biochemical and physiological responses of plants underlying genetic mechanisms in order to solve this severe problem of salinity.

Plants exposed to salinity have a large number of proteins that are involved in ion exclusion, ion compartmentalization, detoxification effects of accumulated ions and regulation of gene expression. Few plants have genes with increased tolerance against salinity, changes in ethylene response factor gene *(MsERF11)* may led to enhanced tolerance to salt [64]. Transcription factor ALFin1 is over expressed in some plants such as alfalfa and this help to boost salt tolerance in them [65].

Genes have been explored in plants that may involve in the production of polyols, sugars, proline & betaines that help to control homeostasis and play important role in maintaining osmoregulation during stressful external environment [66]. Glutathione and proline (amin acid) are antioxidant and it is reported that they allow plants to tolerate the joint attack of metalloids and salinity [67].

2.4 Role of Microorganisms in Bioremediation

Unicellular prokaryotic microorganisms such as bacteria and archea live in various parts of the soil. They can show up in warm, humid areas full of vegetation in desert and arid areas. They play a vital role in cycling with biological decay because of their efficient body processes. They degrade compounds, modify minerals, treat waste and participate in a number of symbiotic reactions with plants, animals and other soil organisms [68]. Factors such as soil types, pH, moisture content, aeration, related composition of soil nutrients, climatic conditions help to retain many types of bacteria in the soil Different kinds of microorganisms are used to remove, reduce or transform pollutants of soil to less deleterious form by the process of bioremediation. Pollutants are isolated by living organisms through enzymatic pathways [69]. The use of microorganisms has many beneficial purposes as they are ubiquitous on the biosphere, their metabolic ability & nutritional versatility is impressive. They can divide in a very wide range of environmental conditions, by using relatively low-cost and low-technology techniques, generally has good public acceptance and can often be carried out on-site [70]. By last15 years bioremediation carried by using microorganisms became of great importance, in case of hydrocarbons, organochlorine compounds which are being in agriculture, heavy metals [71,72] [73,74] and in the recovery of environments with naturally high concentrations of salt or toxic amalgams.

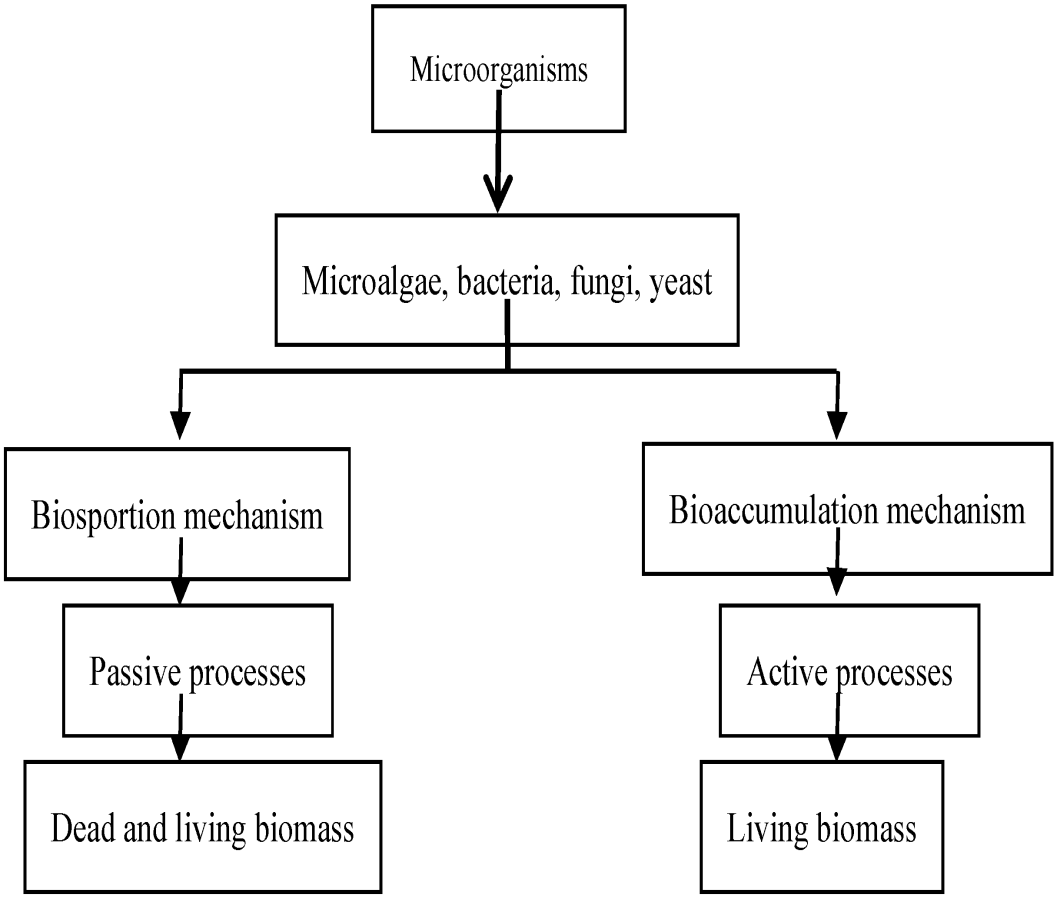

FIGURE 2.14: Role of micro-organism in bioremediation [68].

2.4.1 Microbial Mechanisms Used for Bioremediation

Microbes possess different mechanisms to survive in toxic environmental conditions [75], to break and convert harmful compounds into less toxic compounds, they release toxic components through intracellular & extracellular transport systems and produces appropriating compounds that help to binds and eradicate toxic agents from their interior and excrete extracellular chelating substances to destroy toxic substances [76,77].

They prevent the entry of toxic substances into the cell by gluing them to the cell membrane and inclusion bodies help to maintain cytoplasmic protection by retaining a large number of toxic substances [78]. Some microorganisms help in immobilizing the toxic elements by forming biominerals with them inside or outside their cells [79,80].

2.5 Proteins and Genes Involved in Salt Tolerance in Microorganisms

Mechanisms of salt tolerance is present in all living organisms with minor changes from a microbe to higher plants. Different abiotic stresses affects the cellular gene expression system of living organisms, so this results in up or downregulation of different genes of living systems.

Differential screening, differential display or microarray analysis have been used to isolate and and study the genes involved in different abiotic stress signaling pathways [81]. Studies suggested that some cellular response to salinity stress are same both in prokaryotes and eukaryotes including plants.

Different experiments also proved that functionally analogous stress tolerant genes are present in both unicellular organisms and plants [82]. Salinity tolerant genes *(RPL30E, Chla/bBP and FDH)* are isolated from plants and it has been shown that these three genes have same response against salts in bacteria too. RPL30E shows high salinity tolerance in bacteria and its expression level is increased in high saline environment. There is difference between ribosomal structure of prokaryotic and eukaryotic cell, expression of this protein is not working by translational processes but it confers tolerance by different pathways yet to be studied. Some salinity induced genes are also responsible for cold and drought stress tolerance [83]. Different researchers have isolated a cDNA clone from a seaweed that would be able to express salinity tolerance in bacteria. Role of *FDH* genes is in the synthesis of long chains of lipids that are found in the cuticle and epidermis of living organisms and this shows similarity to large groups of proteins that are related to beta-ketoacyl-CoA synthases and chalcone synthases. *FDH* is involved in epidermal cell interactions and thus blocks organ fusion at early stages of development. About 20 genes showing similarity with the function of FDH have been identified. *FDH* genes have also shown responsibility of salt resistance in bacterial cell too [81].

Chapter 3

Methodology

3.1 Proposed Diagram

Fig**3.1** shows the detail outlines of our research methodology.

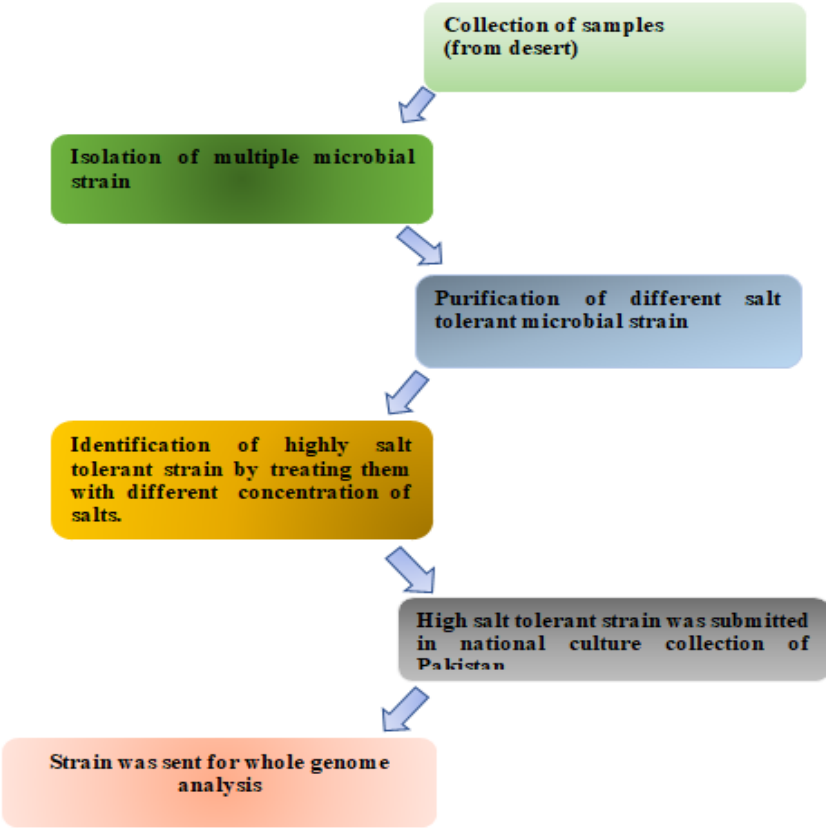

FIGURE 3.1: Prposed diagram

3.2 Sample Collection

Microbes living in the harsh environment have the ability to survive in extreme temperature and drought conditions, desert soil are rich source of novel strains of these strange species. Cholistan desert in Pakistan is unique and unfamiliar area, situated in south of district Bahawalpur, Punjab. It covers the Thar desert in Sindh between longitudes $69^o52^{'}$ to $73^o24^{'}$ E and latitudes $28^o42^{'}$ to $29^o25^{'}$ N present an altitude of 89m above sea level. Climate of this desert is categorized as harsh, precipitation is very scarce, relatively low humidity, long dearth season and high rate of evaporation. Soil of this desert is categorized as very saline although it has rich diversity of different microbial strains [84].

Soil samples (25) were collected in polythene bags from Cholistan desert, samples were collected from a depth of 0.2 m and 1-2 km distance from each other. Physical and chemical treatments were applied, heat treatment was given at 37^oC-40^oC for 3 days and chemical treatment was given by mixing the soil sample with $CaCO_3$ at room temperature for one week.

FIGURE 3.2: Chloistan Desert View [84].

3.3 Isolation and Purification of Microbial Strain

There is need to isolate bacteria from their environment in order to determine their species and function in their environment. It is obligatory to dilute soil sample prior to isolating bacteria from sample because a tiny amount of sample can comprise up to millions of bacteria.

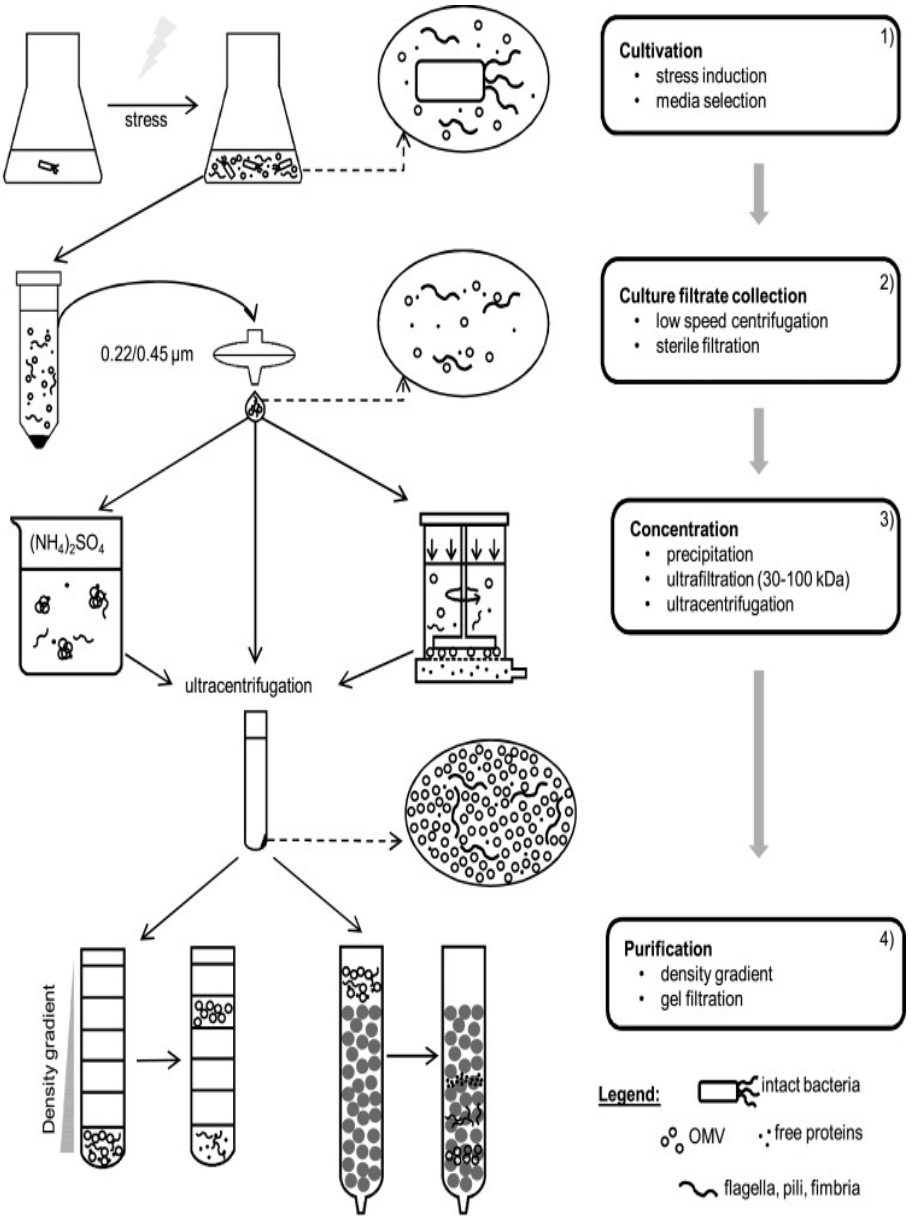

FIGURE 3.3: Procedure of isolation and purification of microbial strain

For the isolation of bacteria, 100 ml. of distilled water in the graduated cylinder was measured and then it was added to sterile bottle. 1g of soil sample was weighed

and added into the bottle of distilled water. Bottle was tightly packed and shaken to thoroughly mix the solution.

3.4 Media Preparation

A specific mixture of nutrients and other substances known as culture media supports the growth of microorganisms.

Different kinds of culture media are being used for the growth of microorganisms in the laboratory. Following culture media was used for the growth of strains isolated from sample.

3.4.1 International *Streptomyces* Project 2 Media

ISP-2 medium is used for culturing different strains of bacteria, this medium contains extract of yeast and malt.

For the culturing of strains ISP-2 medium was prepared by adding 4g of yeast extract powder, 10g of Malt extract powder and 20g of agar in liter of distilled water. This medium was autoclaved for some minutes to sterilize it.

3.4.2 Functional Domain Identification of Targeted Proteins

ISP-3 3 media also known as oatmeal media; this media was prepared by boiling 20g of white oats in 1L distilled water for 20 minutes. These oats were then filtered by using cheesecloth. After adjusting pH to 7.2, solid agar and 1mL of traces salts (0.1 g F_eSO_4 x $7H_2O$,0.1g M_nCl_2 x $4H_2O$,0.1g Z_nSO_4 x $7H_2O$) solution was added in medium, medium was autoclaved for some minutes to sterilize it.

3.4.3 International *Streptomyces* Project 7 Media

ISP-7 media was prepared by dissolving 15g glycerol, 0.5g L-asparagine, 0.5g L-tyrosine, 0.5 Nacl, 0.5 g $MgSO_4$ x $7H_2O$ and 20g agar in 1L distilled water, pH of the medium was adjusted to 7.2 to 7.4. To sterilize, this medium was autoclaved.

3.4.4 Chromogenic *Salmonella* Agar (CSA) Media

Chromogenic salmonella agar medium is used in labs for identification of Salmonella strain from samples. CSA medium was prepared by dissolving 5g of chromogenic mixture, 5g of bacteriological agar and 5g of meat in 1L distilled water. Mixture was boiled at 80^{o}C with continuous agitation. Then medium was cooled at 40-50^{o}C to solidify it.

3.4.5 Tryptic Soy Agar (TSA) Media

TSA medium is commonly used growth medium for isolation, cultivation, storage, and transportation of pure culture of microorganisms. TSA medium was prepared by dissolving 3g of Tryptic Soy Broth (TSB) powder in 100ml of distilled water. This powder was gently mixed in the water. This medium was autoclaved at 121^{o}C for 10-15 minutes. After removing from autoclaved, this medium was cooled at room temperature.

3.4.6 Reasoners 2 Agar Media (R2A)

R2A medium was prepared by dissolving 0.5g of casein hydrolyze, 0.5 dextrose, 0.5g yeast extract, 0.25g casein peptones, 0.25g meat peptone and 0.15g agar in 1L distilled water.

Medium was heated on 80^{o}C with constant agitation to dissolve all the components completely. Prepared medium was autoclaved at 121^{o}C for 10-15 minutes for sterilization.

3.5 Strain Culturing

Test tubes (6) were labeled and 9 ml of distilled water was added into each of test tube by using pipette: tubes were tightly capped and swirled until solution was mixed.

Several dilutions in the test tubes were made in order to isolate the strains. By using a new pipette samples were taken from tubes and spread into all above mentioned media.

Plates were incubated at 37^{o}C, and 45^{o}C. Growth was checked after every 24 hours. Among isolated strains 20 distinguishing colonies were purified into their respective media. All the strains were streaked repeatedly in their respective media to ensure purity. The purified strains were stored in 35%(W/V) glycerol at -20^{o}C for further studies.

3.6 Identification of Highly Salt Tolerant Strain

About 20 salt tolerant strains were isolated and purified to identify which strain has high salt tolerance.

All these strains were treated with different concentration of NaCl from 1% to 20% with 1% increment each and strain that survived at maximum salt concentration was isolated, it was a 16S rRNA strain and it was named as AR-6. Strain was sent to National culture of Pakistan (NCCP).

3.7 Phenotypic Characterization

Colony morphology of the strains was observed on respective media at 37C. Growth at various temperatures 4^{o}C, 10^{o}C, 15^{o}C, 20^{o}C, 28^{o}C, 30^{o}C, 33^{o}C, 37^{o}C, 40^{o}C, 45^{o}C, 50^{o}C was observed for one week. Strains were also checked between pH 4-11 using buffer system [81] for four days.

TABLE 3.1: Recipes of buffer against selected pH range

S.N	pH Range	Buffer
1	4.0-5.0	0.1M Citric acid/0.1M Sodium citrate
2	6.0-8.0	0.1M KH2PO4/0.1M NaOH
3	9.0-10.0	0.1 M NaHCO3/0.1M Na2CO3
4	11.00	0.05 M Na2HPO4/0.1 M NaOH

3.8 16S rRNA Gene Sequencing and Phylogenetic Analysis

The earliest identification of strain AR-6 was done on the base of 16S rRNA gene sequences. Genomic DNA of strain AR-6 was extracted and PCR amplification was performed. By using EzTaxon-e server , obtained sequence of AR-6 strain was equated with available 16S rRNA gene sequence of authentically named species in the server. By using MEGA 7 software package phylogenetic analysis was performed. Multiple alignment of the sequences was performed by using CLUSTAL X programme. Kimuras two parameter model was used as distance matrices to construct neighbour- joining tree. With 1000 replications bootstrap analysis was performed to determine support of each clade. With maximum-likelihood (ML) and maximum parsimony (MP) validity of the neighbour-joining tree was evaluated.

Chapter 4

Results and Discussions

4.1 Isolation and Purification of Bacterial Strain

Soil samples were collected at 1-2 km distance from Cholistan desert. Sample were collected by using clean, dry and sterile polythene bags by using sterile spatula. Different physical and chemical treatments were given to sample for about one week. 100ml of distilled water was measured in a graduated cylinder, pH was adjusted 4.0-11 by using 0.1ml of concentrated H_2SO_4, this water was then transferred into sterile beaker. About 1g of soil was taken from collected sample and was transferred into beaker having distilled water. 6 test tubes were taken,9ml of distilled water was added into each of test tube,1 ml of water was taken from beaker having soil sample by using sterile pipette. Pipette was emptied into test tube, similarly several dilutions in the test tube were made in order to purify strains of bacteria and then these strains were spread on culture media for their growth.

4.1.1 Temperature Tolerance

Growth of 20 bacterial strain was checked at 4^oC, 10^oC, 15^oC, 20^oC, 28^oC, 30^oC, 33^oC, 37^oC, 40^oC, 45^oC, 50^oC for one week respectively. Most of the strains showed

significant growth at optimum temperature range 37C while some strains showed maximum growth when temperature was increased up to 45^{o}C. Purpose of this experiment was to isolate the bacterial strain showing maximum growth at high temperature. Result of this experiment is summarized in **Table 4.1**.

TABLE 4.1: Temperature tolerance of bacterial strains from 4^{o}C to 50^{o}C

Strain No.	4	10	15	20	28	30	33	37	40	45	50
AR-2	-	-	-	+	+	+	+++	+++	++	-	-
AR-3	-	-	-	+	+	+	+++	+++	++	-	-
AR-4	-	-	-	+	+	+	+++	+++	++	-	-
AR-5	-	-	-	+	+	++	+++	+++	++	+	-
AR-6	-	-	-	+	+	++	+++	+++	++	+	-
AR-7	-	-	-	+	+	+	+++	+++	++	+	-
AR-8	-	-	-	+	+	++	+++	+++	++	++	-
AR-9	-	-	-	+	+	++	+++	+++	++	+	-
AR-10	-	-	-	+	+	++	+++	+++	++	+	-
AR-11	-	-	-	+	+	+	+++	+++	++	+	-
AR-12	-	-	-	+	+	++	+++	+++	++	+	-
AR-13	-	-	-	+	+	++	+++	+++	++	+	-
AR-14	-	-	-	+	+	++	+++	+++	++	++	-
AR-15	-	-	-	+	+	+	+++	+++	++	+	-
AR-16	-	-	-	+	+	++	+++	+++	++	+	-
AR-17	-	-	-	+	+	+	+++	+++	++	-	-
AR-18	-	-	-	+	+	++	+++	+++	++	+	-
AR-19	-	-	-	+	+	+	+++	+++	++	+	-
AR-20	-	-	-	+	+	++	+++	+++	++	+	-

+++ (Significant Growth)

++ (Slow Growth)

+ (Very Slow Growth)

4.1.2 pH Tolerance

Bacteria experience sensitiveness towards different pH range, mostly bacteria show effective growth at neutral or 6-7.5 pH. Low or acidic pH may not allow bacteria to continue their growth whereas high or alkaline pH also have adverse effect on the growth of bacteria. Growth rate of different bacterial strain was checked at 4-11 pH range by using buffer systems mentioned in **Table 4.2**. Some strains showed little or no growth at low pH whereas some strain including AR-6 showed maximum growth even at alkaline 9-11 pH.

Table 4.2: Estimation of pH range of Isolates from 4 to 11

Isolate no.	pH Range			
	4.0-5.0	6.0-8.0	9.0-10.0	11.00
AR-1	-	++	-	-
AR-2	-	++	+	+
AR-3	-	++	-	-
AR-4	+	++	+	-
AR-5	++	++	++	-
AR-6	-	+	+++	+++
AR-7	++	+	-	-
AR-8	-	+	++	+++
AR-9	-	++	+	-
AR-10	-	++	+	-
AR-11	+	++	+	-

AR-12	+	++	+	-
AR-13	+	++	+	-
AR-14	+	++	+	-
AR-15	+	++	+	-
AR-16	+	++	+	-
AR-17	+	++	+	-
AR-18	+	++	+	-
AR-19	+	++	+	-
AR-20	+	++	+	-

+++ (Significant Growth)

++ (Slow Growth

+ (Very Slow Growth)

- (No Growth)

4.1.3 Salt Tolerance

Salt tolerance test was performed using 20 different microbial isolates in order to check which of them have high resistance against salts. These purified strains were treated with 1-20% of NaCl concentration with 1% increment. It is known salts act as selective mediator for the growth of bacteria and high concentration of salts may slow down or inhibit the growth of bacteria, only bacteria that may have mechanism for salt resistance are able to survive in high salt concentration. From 20 strains only few including AR-6 were able to survive at high concentration of salts, AR-6 survived in 20mg/L concentration of NaCl and those that showed significant growth at high concentration of salt isolated for sequencing of genome.

Table 4.3: Salt tolerance (NaCl %)of Isolates from 1% to 20 % with 1 percent increment

	Salt Tolerance NaCl %																			
Isolate No	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
AR-1	++	++	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AR-2	++	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
AR-3	++	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AR-4	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AR-5	++	++	++	++	++	++	++	++	+	+	+	+	+	+	-	-	-	-	-	-
AR-6	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
AR-7	++	++	++	++	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
AR-8	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	-	-	-
AR-9	++	++	++	++	++	++	++	++	++	+	+	+	+	+	+	+	-	-	-	-
AR-10	++	++	++	++	++	++	+	+	+	+	+	+	+	+	+	-	-	-	-	-
AR-11	++	++	++	++	++	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
AR-12	++	++	++	++	++	++	++	++	++	+	+	+	+	+	+	-	-	-	-	-
AR-14	++	++	++	++	++	++	++	++	++	++	+	+	+	+	+	-	-	-	-	-

TABLE 4.4: Continue from previous page: Salt tolerance (NaCl %) of Isolates from 1% to 20 % with 1 percent increment

	tolerance (NaCl %) of Isolates from																			
Isolate No	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
AR-15	++	++	++	++	++	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
AR-16	++	++	++	++	++	++	++	+	+	+	+	+	+	-	-	-	-	-	-	-
AR-17	++	++	++	++	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
AR-18	++	++	++	++	++	++	++	+	+	+	+	+	-	-	-	-	-	-	-	-
AR-19	++	++	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
AR-20	++	++	++	++	++	++	+	+	+	+	+	-	-	-	-	-	-	-	-	-

++ (Significant Growth)

++ (Slow Growth)

- (No Growth)

4.2 Phylogenetic Analysis of Strain

The evolutionary history of strain AR-6 was directed using the Maximum Likelihood method based on the Tamura-Nei model [84]. The tree with the highest probability of log (-13181.85) is shown in **Fig 4.1 and 4.2**. The first heuristic search trees were automatically detected using Neighbor-Join and BioNJ algorithms in a two-dimensional matrix considered using Maximum Composite Likelihood (MCL), and then selecting topology with the highest log entry value. The tree is weighed on the scales, the length of the branch being measured by the number of inserts in each place. The analysis involved 23 nucleotide sequences. Codon's included positions were 1 + 2nd + 3rd + Noncoding. All positions containing spaces and missing data have been deleted. There were a total of 1197 posts in the final database. Evolutionary analysis was performed at MEGA7 [85].

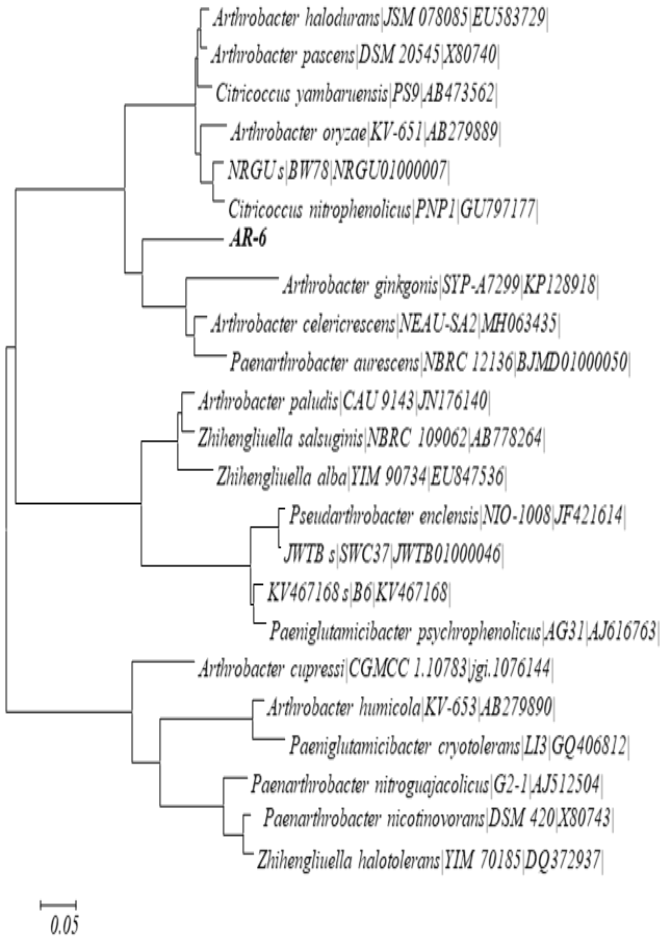

FIGURE 4.1: Evolutionary relationships of taxa

FIGURE 4.2: Molecular Phylogenetic analysis by Maximum

4.3 Analysis of Draft Genome

Bio sample SAMD00178857 was sent under the Bio project PRJDB8509 with accession number BKDJ01000001 for whole genome sequencing, genome of strain AR-6 was sequence using illumine Hiseq-2500 by PE125 strategy and obtained reads were assembled into 50 contigs by using SOAPdenove software (http: //-soap.genomics.org.cn/soapdenovo.html). GeneMarks server (http://opal.biology-gatech.edu) was used to perform gene prediction. After phylogenetic analysis based on 16s rRNA whole genome sequencing it was resulted that this strain was belong to family *Micrococcaceae* and it was related to genus *Arthrobacter*. It has highest sequence similarity with *Arthrobacter ginkgonis*. This strain AR-6 has 70.0 mol% DNA G+C content. Total length of the bacterial sequence is 3,291,617 bp

& 2,963 protein with 50 number of contigs, 50 scaffolds are present in bacterial genome. Ungapped length in the 3,292,577,4 spanned and none of unspanned gaps are present in the genome. By putting many little overlapping sequences of DNA into a larger sequence a contig is formed, contig is basically a physical map that is formed by joining little sequences of DNA that may overlap, and by joining them together to make a larger sequence. 50 Contigs of AR-1664 were represented in **Table 4.5**.

Table 4.5: Showing 50 Contigs of AR-1664

S.N	Accession No	Name	Proteins	Length
1	BKDJ01000001.1	Sequence01	527	573,704
2	BKDJ01000002.1	Sequence02	237	267,966
3	BKDJ01000003.1	Sequence03	242	254,703
4	BKDJ01000004.1	Sequence04	193	230,183
5	BKDJ01000005.1	Sequence05	177	203,588
6	BKDJ01000006.1	Sequence06	179	192,879
7	BKDJ01000007.1	Sequence07	170	185,344
8	BKDJ01000008.1	Sequence08	173	184,104
9	BKDJ01000009.1	Sequence09	152	178,140
10	BKDJ01000010.1	Sequence10	97	109,905
11	BKDJ01000011.1	Sequence11	92	96,385
12	BKDJ01000012.1	Sequence12	88	94,124
13	BKDJ01000013.1	Sequence13	86	92,934
14	BKDJ01000014.1	Sequence14	84	89,258
15	BKDJ01000015.1	Sequence15	70	80,712
16	BKDJ01000016.1	Sequence16	67	68,647
17	BKDJ01000017.1	Sequence17	54	58,297
18	BKDJ01000018.1	Sequence18	50	52,174
19	BKDJ01000019.1	Sequence19	37	41,102
20	BKDJ01000020.1	Sequence20	33	39,683

21	BKDJ01000021.1	Sequence21	33	38,892
22	BKDJ01000022.1	Sequence22	28	37,878
23	BKDJ01000023.1	Sequence23	24	27,594
24	BKDJ01000024.1	Sequence24	19	19,738
25	BKDJ01000025.1	Sequence25	15	15,898
26	BKDJ01000026.1	Sequence26	8	9,556
27	BKDJ01000027.1	Sequence27	9	8,083
28	BKDJ01000028.1	Sequence28	5	7,299
29	BKDJ01000029.1	Sequence29	5	6,521
30	BKDJ01000030.1	Sequence30	1	3,242
31	BKDJ01000031.1	Sequence31	0	2,188
32	BKDJ01000032.1	Sequence32	1	2,073
33	BKDJ01000033.1	Sequence33	1	1,856
34	BKDJ01000034.1	Sequence34	0	1,448
35	BKDJ01000035.1	Sequence35	0	1,383
36	BKDJ01000036.1	Sequence36	0	1,231
37	BKDJ01000037.1	Sequence37	0	1,182
38	BKDJ01000038.1	Sequence38	0	1,126
39	BKDJ01000039.1	Sequence39	1	1,108
40	BKDJ01000040.1	Sequence40	0	1,091
41	BKDJ01000041.1	Sequence41	0	1,071
42	BKDJ01000042.1	Sequence42	1	1,051
43	BKDJ01000043.1	Sequence43	1	958
44	BKDJ01000044.1	Sequence44	0	902
45	BKDJ010000451	Sequence45	0	858
46	BKDJ01000046.1	Sequence46	0	850
47	BKDJ01000047.1	Sequence47	1	783
48	BKDJ01000048.1	Sequence48	1	752
39	BKDJ01000049.1	Sequence49	0	599
50	BKDJ01000050.1	Sequence50	1	574

4.4 Mechanisms Responsible for Salt Tolerance in Strain

Bacteria involved in salt tolerance may exhibit different mechanisms including specific membrane, draining out of salts or ions by salt efflux, changes of intracellular environment by gathering non-toxic hydrogen and carbon osmolytes and by producing those enzymes or proteins that are responsible for high salt tolerance. Salt tolerant bacteria may survive in saline environment by three mechanisms i-e pumping out the cell, intracellular adaptation process and cell wall construction [86].

High amount of energy is cast-off to produce osmolytes that are able to protect the cells from high concentration of salts. To bear osmotic stress caused by high concentration of NaCl bacteria may modify their gene expression and produce specific stress proteins. Internal amount of organic osmolytes can reach up to 1M under salt stress in some halophilic bacteria and it has significant function in lowering melting temperature of DNA and stabilizing the double helix of DNA [87].

4.5 Osmolytes (Amino Acid and their Derivative) Responsible for Salt Tolerance

It has been reported that amino acids, quaternary amines, sugars, compatible solutes prevent bacteria from degenerative processes and allow bacterial cell to grow under stressful osmotic conditions [88]. Genome analysis of strain AR-6 showed that it contains about 288 Glutamine, Proline, phenylalanine glutamate, aspartate, asparagine, histidine, arginine, lysine, threonine, methionine, and cysteine containing proteins (7 proteins out of 288 are shown in **Table 4.6**). These amino acids and their derivatives act as organic osmolytes and as mention earlier accumulation of these osmolytes help in tolerating the stressful extracellular environment.

Proteins and compatible solutes exert help in changing the solvent structure and it may bring a minor change in the dynamic characteristics of proteins except altering the protein structure itself. These osmolytes help in balancing the different macromolecular structures that may be alter or degenerate due to physiological stress caused by salinity. Such organic solutes or osmolytes are generated in cytoplasm of and they help to preserve enzyme or cell organelles against dehydration caused by salinity [89].

TABLE 4.6: Amino acid and their derivatives responsible for salt resistance inAR-6

Amino acid and their derivatives responsible for salt resistance inAR-6			
S.N	Accession No.	Length	Protein name
1	GER21672.1	638	Glutamine-fructose-6-phosphate aminotransferase
2	GER21755.1	528	GMP synthase glutamine-hydrolyzing¿glutamine-hydrolyzing
3	GER22502.1	501	Glutamine ABC transporter permease
4	GER22503.1	252	Glutamine ABC transporter ATP-binding protein
5	GER23873.1	218	Glutamine ABC transporter permease
6	GER24190.1	298	Release factor glutamine methyltransferase
7	GER24395.1	474	Glutamine synthetase

4.6 Role of potassium(K^+) Transporters in Salt Tolerance

Living cells exhibit potassium homeostasis as a central attribute. It reacts to bring changes in osmotic strength, pH and cell volume balancing. It also effects membrane potential and membrane related functions. Potassium is known as important factor in tolerating salinity, drought and other extreme conditions in living organisms. Several numbers of genes encoding K^+ transporters and channels are identified [86]. High affinity potassium transporters 5 (HAK5) has been identified as high affinity K^+ that belongs to KUP/HAK/KT families. It helps in K^+ accumulation by genetic mapping of natural variations potassium concentration in cell. These transporters help in homeostasis of salts and ions such as K^+/Na^+ in the internal environment of the cell and also help living cells to tolerate high concentration of ions and salts [89].

Genomic analysis of strain showed that it has 5 K^+ regulating proteins and they may be involved in the regulation of salts and help in salt resistance.

Table 4.7: K+ Regulating protein of AR-6

S.N	Accession No.	Length	Protein name
1	GER21504.1	606	2-succinyl-5-enolpyruvyl-6-hydroxy-3 -cyclohexene-1- carboxylate synthase
2	GER21505.1	256	ATP-binding protein
3	GER21506.1	311	ABC transporter permease
4	GER21507.1	309	ABC transporter substrate-binding protein
5	GER21508.1	460	Isochorismate synthase

4.7 Role of Sodium (Na^+) Transporter in Salt Tolerance

Sodium transporter or Na^+ circuit has an important role between exergonic and endergonic membrane reactions of some bacteria. These sodium channels or transporter help in uptake of sodium in and out of the cell and thus maintain the internal environment of living organisms. Some bacteria use Na^+ ion gradient to pump sodium outside by oxaloacetate decarboxylase. These Na^+ transporter helps in reduction of NAD^+. These Na^+ transporter help to drive solute uptake, locomotion and ATP synthesis. In some strains of bacteria ATP production is totally dependent on the Na^+ transporter or Na^+ gradient [90]. From previous studies it is reported that Na^+ transporter or channels help in maintaining the homeostasis in bacteria by moving out all the extra salts and ions out of the cell and it also helps to synthesize adequate amount of ATP so that bacteria living in extreme can survive there. Genome analysis of bacteria strain AR-6 showed that there is 7 Na^+ dependent transporters and Na^+ independent transporter is present, these Na^+ transporter help the strain to survive in high salinity.

Table 4.8: Sodium transporter responsible for salt tolerance in strain AR-6

S.N	Accession No.	Length	Protein name
1	GER21515.1	525	Sodium-dependent transporter
2	GER22254.1	505	Sodium-independent anion transporter
3	GER22650.1	454	Sodium proton antiporter
4	GER23146.1	499	Sodium proline symporter
5	GER23717.1	468	Citrate sodium symporter
6	GER23722.1	511	Sodium solute symporter

7	GER23913.1	487	Putative sodium-dependent alanine carrier protein

4.8 Role of ABC Transporter in Salt Tolerance

ATP-binding cassette (ABC) is a protein that is present ubiquitously from prokaryotes to eukaryote, this protein family is very large and its members are present in all living organisms. ABC protein perform different cellular function in living organisms. These ABC transporters help to transport various substrates in and out of the cell membrane including conjugated molecules, inorganic acids, salts, sugars, peptides, secondary metabolites, amino acids and drugs. ABC Protein structure is composed of characteristics modular structure, it consists of two structural regions: hydrophobic trans membrane domain (TMD) which is composed of 6 membrane spanning helics & cystolic domain, and other region is involved in ATP binding and it is called as nucleotide binding domain (Nucleotide-binding domain).

ABC carriers allow the transport of materials unrelated to structure and function, raising the question of whether a single protein can bind and transport different fragments. The proposed construction of ABC carriers and the presence of two homologous units of TMD NBD suggest that it is possible for each to contribute to the binding and transport of various chemical classes [91,92,93].

ABC-Transporter showed resistant against drugs and salts, mechanisms against drugs are discovered in some species but mechanisms against salt tolerance yet have to be discover.

Genome analysis of strain AR-6 showed that about 125 ABC-Transporter proteins are present in this strain and they may play a role in tolerance of salt. Out of 125 ABC-transporter proteins 10 are given in the **Table 4.9**.

Table 4.9: List of ABC-Transporter

S.N	Accession No.	Length	Protein name
1	GER21506.1	311	ABC transporter permease
2	GER21507.1	309	ABC transporter substrate-binding protein
3	GER21520.1	280	L-cystine ABC transporter ATP-binding protein YecC
4	GER21521.1	212	ABC transporter permease
5	GER21522.1	225	L-cystine ABC transporter permease
6	GER21522.1	225	L-cystine ABC transporter permease
7	G ER21546.1	796	Exconuclease ABC subunit A
8	R21771.1	294	Putative amino acid ABC transporter, substrate binding protein
9	GER21781.1	598	Peptide ABC transporter substrate-binding protein
10	GER21783.1	361	ABC transporter permease

4.9 Transcriptional Regulator in Salt Tolerance

Transcriptional regulators are proteins present in eukaryotic and prokaryotic cell and their role is to activate or inhibit DNA by binding to specific DNA sequences.

These transcriptional regulators help in regulation of ion transporters that play an important role in ion homeostasis and control ion flow from external environment to internal environment of living cell.

Proteins of ion transporter are transcriptionally regulated by Cis-elements. The presence of many cis-elements helps to cross talk between different signal transduction pathways, a specific stress signaling pathway can be activated by depending on the cis-elements. This stress signaling pathway evoke a physiological change towards maintain ion homeostasis to mitigate the stress response [94,95].

Previous studies have been reported that transcriptional regulator paly a necessary role in alleviating the environmental stresses. They can help living cell to tolerate harsh environmental conditions such as salinity, alkalinity, high temperature etc [96,97].

131 transcriptional regulatory protein have been discovered from genome analysis of strain AR-6 and these transcriptional regulators may help to survive this strain in high concentration of salt. 10 out of 131 transcriptional regulators have been presented in **Table 4.10**.

Table 4.10: List of Transcriptional regulators

S.N	Accession No.	Length	Protein name
1	Ger21514.1	468	Pucr family transcriptional regulator
2	Ger21518.1	262	Iclr family transcriptional regulator
3	Ger21527.1	145	Transcriptional repressor
4	Er21557.1	236	Transcriptional regulatory protein glnr

5	Ger21769.1	165	Putative transcriptional regulator, asnc family protein
6	Ger21822.1	206	Tetr family transcriptional regulator
7	Ger21867.1	222	Transcriptional regulatory protein
8	Ger21869.1	148	Transcriptional regulator
9	Ger21901.1	239	Iclr family transcriptional regulator
10	Ger21901.1	239	Iclr family transcriptional regulator

4.10 Hypothetical Proteins

Hypothetical proteins are those proteins that may be predicted in genome analysis of an organisms, there are large number of hypothetical proteins due to lack of experiential work and evidence for them. About 20%-40% hypothetical proteins are present in any newly sequenced genome. Instead of different techniques to express the product of genes but still it is not an easy process to assign these protein functions. In whole genome analysis of AR-6 it is revealed that there are 1059 hypothetical proteins present in the sequence that makes 35% of the whole proteins present in the strain.

As this strain have high salt tolerating ability so it is assumed that there are some hypothetical proteins responsible for resistance against salt and making strain to survive in harsh conditions. 10 out of 1059 proteins are listed in **Table 4.11**.

Table 4.11: List of hypothetical proteins

S.N	Accession No.	Length	Protein name
1	GER21805.1	211	Hypothetical protein
2	GER21806.1	296	Hypothetical protein
3	ER21811.1	315	Hypothetical protein
4	GER21820.1	120	Hypothetical protein
5	ER21830.1	339	Hypothetical protein
6	GER21849.1	368	Hypothetical protein
7	GER21851.1	306	Hypothetical protein

8	ER21868.1	90	Hypothetical protein
9	GER21878.1	150	Hypothetical protein
10	GER21889.1	269	Hypothetical protein

4.11 Important Genes Responsible for High Salt Tolerance in Strain

Analysis of proteins present in AR-6 strain showed that it has maximum of 1349 protein present in it from which maximum count was of amino acid & their derivates related proteins. Proteins involved in Co-factors, vitamins, prosthetic group, pigments, potassium metabolism, RNA metabolism, membrane transport, virulence disease and defense, nitrogen metabolism, potassium metabolism, DNA metabolism, fatty acid metabolism, sulfur metabolism, dormancy, sporulation, stress response, regulation and cell signaling, nucleoside and nucleotides, cell division, cell signaling are present in strain. Number count of all the mentioned proteins or subsystem feature is presented in **Figure 4.3**. From previous studies it has been reported that microbes can survive in high saline environment by possessing different mechanisms they may convert or break salts present in their surrounding by converting them into less toxic form, different extracellular and intracellular transport are also involved in degrading or blocking all the harmful agents such as excessive salt so they may not enter in the internal environment of cell [76]. Protein involved in membrane transport, potassium metabolism, sulfur

metabolism, and nitrogen metabolism are studies in depth and they are responsible for salt tolerance in AR-6.

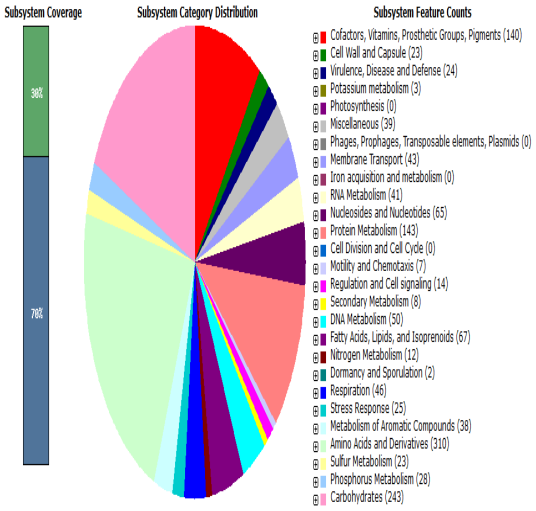

FIGURE 4.3: Subsystem feature count of strain AR-6

4.11.1 Membrane Transport

Membrane has major role in transport of minerals, ions salt across the cel. Protein count of strain AR-6 shown that 36 proteins categorized into ABC transporters,protein secretion system type-II, proetins involed in transloaction of material across the cytoplasmic membrane,cation transporters, uni-Sym and antiporters are presnt in membrane of strain AR-6, these proteins or transporters are involeved in transloaction of salts ions or minerla from intracellular to extracellular and vice verca.

ABC transporters are involed in transport of different substrate across membrane of cell by utilizing energy provinded by ATP. Cation transporters are involved in the translocation of Mg^{+} Cu^{+} across the membrane.

Uni-Sym and anti-porter are also present in the strain and their role is mediate the flow of Na^{+} and H^{+} in and out of the cell. These antiporter are considered responsible for mainting homeaostatsis in starin AR-6 and thus it helps the strain to survive in maximum salinty.

TABLE 4.12: Proteins involved in membrane transport

S.N	Subcategory of proteins	Subsytem or genes	Function
1	Protein secretion system, Type II	Widespread colonization island	Predicted ATPase with chaperon activity
2	ABC Transporters	Oligopeptide transport system permease protein	Oligopeptide transport system protein OppB
3	ABC Transporters	ABC transporter dipeptide	Dipeptide transport ATP binding protein
4	ABC transporters	ABC transporter dipeptide	Dipeptide-binding ABC transporter, periplasmic substrate binding component
5	Cation transporter	Magnesium and cobalt transport proetin	Magnesium transport
6	Cation transporter	Copper resistance protein	Copper transport
7	Uni- Sym- and Antiporters	Multi-subunit cation antiporter subunit A	Na+/ H+ transport
8	Uni- Sym- and Antiporters	Multi-subunit cation antiporter subunit B	Na+/ H+ transport

4.11.2 Nitrogen Metabolism

Bacteria play a major role in fixing nitrogen present in the soil and surrounding by process of nitrogen fixation and assimilation. Then these bacteria convert this nitrogen into nitrates or nitrites and supply it to plants.

Bacteria use ammonium as major source of nitrogen. Bacterial cell will start getting nitrogen from ammonium if it is present in ample amount and it will suppress other source of nitrogen such as amino acid, inorganic compounds and urea etc. Ammonia can fixed be fixed by the help of glutamate dehydrogenase.

Nitrogen containing salts can also be fixed by bacteria, genome analysis of strain AR-6 shown that there are different proteins involve in the nitrogen metabolism and these proteins or genes are responsible for the survival of bacteria in the high saline environment.

TABLE 4.13: Proteins involved in nitrogen metabolism

S.N	Subcategory of proteins	Subsytem or genes	Function
1	Nitrogen metabolism no subcategory	Nitrate/nitrite transporter	Nitrate and nitrite ammonification
2	Nitrogen Metabolisms no subcategory	Nitrite reductase (NADPH small subunit)	Nitrate and nitrite ammonification
3	Nitrogen Metabolisms no subcategory	Glutamine synthetase type I.	Ammonia Assmiliation
4	Nitrogen Metabolisms no subcategory	Glutamate synthetase type I	Ammonia assimilation
5	Nitrogen Metabolisms no subcategory	Glutamate ammonia-ligase adenyltransferase	Ammonia assimilation
6	Nitrogen Metabolisms no subcategory	Ammonium transporter	Ammonia assimilation
7	Nitrogen Metabolisms no subcategory	Glutamate synthase [NADPH] large chain	Ammonia Assimilation

4.11.3 Phosphorus Metabolism

Phosphorus is one of the most important component present in living organisms as it is found in nucleic acid, lipopolysaccharides, phospholipids and in various solutes present in cytoplasm. Instead of this phosphorous is present in different rocks and stones, phosphorus content in soil is increasing day by day by using different fertilizer for crop production.

And high content of phosphorus can affect plants badly. Many soil microorganisms possess mechanisms to accumulate phosphate in the form of polyphosphate and it help to remove extra phosphate present in the surrounding environment [93].

Genome analysis of strain AR-6 shown that it has different proteins / genes that are involve in the phosphorus metabolism and these may help the strain to metabolize different phosphorus salt and help bacteria to survive in high salinity.

Table 4.14: Protein involved in phosphorus metabolism

S.N	Subcategory of proteins	Subsytem or genes	Function
1	Phosphorus Metabolism no subcategory	Phosphate regulon transcriptional regulatory protein	High affinity phosphate transporter and control of PHO regulon
2	Phosphorus Metabolism no subcategory	Phosphate transport system permease protein	High affinity phosphate transporter and control of PHO regulon
3	Phosphorus Metabolism no subcategory	Phosphate transport ATP-binding protein	High affinity phosphate transporter and control of PHO regulon

4	Phosphorus Metabolism no subcategory	Phosphte transport system permease	High affinity phosphate transporter and control of PHO regulon
5	Phosphorus Metabolism no subcategory	Phosphate ABC transporter, periplasmic phosphate-binding protein	High affinity phosphate transporter and control of PHO regulon
6	Phosphorus Metabolism no subcategory	Polyphosphate kinase	High affinity phosphate transporter and control of PHO regulon
7	Phosphorus Metabolism no subcategory	Inorganic pyrophosphatase	High affinity phosphate transporter and control of PHO regulon
8	Phosphorus Metabolism no subcategory	Phosphate starvation-inducible protein PhoH, predicted ATPase	High affinity phosphate transporter and control of PHO regulon

4.11.4 Potassium Metabolism

Potassium homeostasis is among central feature of living cell it effects different functions of membrane and membrane potential. It also helps to change in osmotic strength, pH and volume of cell. It also to balance cell volume and bring new cell equilibrium. Potassium efflux was changed by adopting downstream effector by different signaling system.

Different potassium gated channels have been found in different bacteria and they regulate the flow of potassium ion in and out of the cell membrane. These gates also help in the regulation of potassium solutes and help in maintain the homeostasis of cell [94]. Protein involved in the potassium homeostasis have been discovered in the genome of bacterial strain Ar-6 and these proteins may involve in the resistance of strain against salinity.

TABLE 4.15: Proteins involved potassium metabolism

S.N	Subcategory of proteins	Subsytem or genes	Function
1	Potassium metabolism-no subcategory	Large conducatnce mechanosensitive channel	Potassium homeostasis
2	Potassium metabolism-no subcategory	FKBP-type peptido-prolyl cis-trans isomerase	Potassium homeostasis
3	Potassium metabolism-no subcategory	Potassium voltage-gated channel subfamily	Potassium homeostasis

Chapter 5

Conclusion and Future Prospects

The aim of this study is to isolate and identify high salt tolerant strain from desert sample and to estimate the genes or proteins responsible for salt resistance in it. Different strain from the sample were purified from culture media and high salt tolerant strain AR-6 was isolated by treating them with high concentration of salts and this strain showed significant growth (+++) at 20mg/L NaCl.

AR-6 showed significant growth(+++) at 9-11 pH and temperature 40^oC. Genome analysis of AR-6 showed that total sequence length of AR-strain is 3,291,617, total un-gapped length 3,291,577, total number of contigs 50 and 2963 genes are present in it.

Proteins such as ABC transporter, polyphosphate kinase, cation transporter, glutamate synthetase type I, potassium transporter, transcription regulators have shown responsibility for salt tolerance in AR-6.

Information obtained in this study suggest that this strain can be used in future as a bio stimulant for bioremediation of highly saline soil in order to increase crop production by increasing soil fertility.

It also suggest that enzymes or genes responsible for salt tolerance can be extracted and by introduction them into crop producing plant can make these plants to survive and give good yield in high saline environment.

Following are the future prospectus:

- Enzymes responsible for salt tolerance can be extrcated from this strain and can be introduced into plants to increase their resistance against salts.
- This strain can be used as biostimulant to degrade or remove excessive salts present in the saline soil and to lower the concentration of salts present in them.
- From phylogenetics analysis, closest relative of this strain can also be studied to compare salt tolerance range in them.

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