CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Screening of Bacteria for Enhanced Surfactant Activity and Their Role in Oil Recovery from Oil Reservoirs

by

Sajida Tayyaba

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

in the

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Dedicated to Almighty ALLAH and the Holy Prophet Muhammad (P.B.U.H) and My Loving Family

CERTIFICATE OF APPROVAL

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Abstract

Demand of energy and production of oil is becoming less and experiencing decline in many parts of World. Some factors are included by which oil production is declining. From these factors, one is the increasing population growth. Therefore, there is a need to find out an alternative ways to recover oil from existing oil fields. Bacterial activities playing important role in oil reservoirs since long time but our knowledge regarding nature and diversity of bacteria is still poor, and their metabolic phenomena is largely ignored. Microbial Enhanced Oil Recovery (MEOR) is a useful process to increase oil recovery from a reservoir after primary and secondary recovery operations using organisms and their metabolites. Stimulation of bacteria for the production of biosurfactant and degradation of heavy oil fractions by indigenous microorganisms can raise the fluidity and decrease the capillary forces which retain the oil into the reservoir. Low energy requirement and independence of the price of crude oil are advantages of MEOR over conventional EOR. Biosurfactants degrade complex organic substances and make them more mobile with use in oil recovery for pumping of crude oil. This work has conducted to isolate, purify and characterize bacterial strains from soil samples of OGDCL oil refinery to check their biotransformtion capability and metal tolerance for enhanced surface properties by identifying fatty acid profile. Fatty acids are identified and characterized by GC-MS. Strains with maximum metal tolerance and highest biotransformation were checked for presence of specific fatty acid. These candidates which have ability to tolerate metal and biotransform, can be used in MEOR methods after screening and checking their growth kinetics.

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Abbreviations

CEOR	Chemical Enhanced Oil Recovery
EOR	Enhanced Oil Recovery
GC-MS	Gas Chromatography Mass Spectrometry
HPAI	High Pressure Air Injection
MEOR	Microbial Enhanced Oil Recovery
MIC	Microbiology Influenced Corrosion
MTC	Metal Tolerance Concentration
NAPLS	Non Aqueous Phase Liquids
PAH	Polycyclic Aromatic Hydrocarbon
TEOR	Thermally Enhanced Oil Recovery

Chapter 1

Introduction

1.1 Background

Economic development of the world drives by oil which is a vital source of energy oil recovery is composed of primary and secondary phases. In primary recovery method oil and gas is produced through the usual force energy of the reservoir as well as oil wells are stimulated to inject the fluids in secondary phase to recover the production of petroleum from reservoirs [1]. 5-10% of oil is recovered in primary oil recovery method while secondary phase of oil recovery which includes other recovery efficiencies recoveres other 10-40% of oil [2]. In this way raw petroleum (crude oil) which is remained in the reservoirs after ordinary oil recoveries methods formulate 2/3 of the total oil reserves [3-5]. Some factors which forms high capillary forces that capture oil in small holes within the reservoirs are little permeability high thickness of oil and the high interfacial tensions between hydrocarbon and aqueous phases are responsible for the poor oil recovery from reservoirs. It results rise in energy demand which eventually leads to decline in energy. Manipulation of energy reserves in established reservoirs is required to fulfill upcoming energy demands. demands [2-5]. Pakistan's oil and gas resources are speckled all over the country together with lots of potential oil reservoirs. Approximately 1000 oil and gas exploratory wells have been drilled in Pakistan having depth ranging from

230 meters to more than 6400 meters [6]. Pakistan is an oil importing country because its oil production is less than its requirements [7]. In Pakistan around 300 million barrels of oil reserves has been recorded as on June 2006. The largest oil producing fields are located in the Southern Indus Basin whereas major part of oil production comes from Southern half of country. Since late 1980s, there were no new oil fields discovered in Pakistan as a result oil production in Pakistan has remained fairly flat with around 60,000 bb/day. Government has encouraged personal firms to build up domestic manufacturing capacity [8]. Pakistan‘s known approximate total oil reserves are 27 bb and approximate recoverable reserves are 936 Million barrels. Presently Pakistan is producing around 69,286 bbls /day with primary or secondary recovery [9].

1.2 Microbially Enhanced Oil Recovery MEOR

MEOR is an imperative tertiary oil retrieval skill which denotes a profitable and environmental friendly substitute to chemical enhanced oil recovery. Bacterial strains are recycled in microbial enhanced oil recovery to produce substances which are similar to the compounds used in chemical enhanced oil recovery. Method to enhance the retrieval of oil from short and marginal reservoirs and life of these compounds are extended microbes be able to manufacture beneficial products using little price substances or raw materials so MEOR is fewer expensive technology than CEOR. Moreover microbial products are decomposable and have little toxicant [10].

1.3 Biosurfactant Production by Bacteria and Oil Recovery

Bio-surfactant characterizes one of the most encouraging technique to recover a considerable extent of the remaining oil from develop oil fields [11]. Bio-surfactants

are diverse collection of surface active molecules having both hydrophilic and hydrophobic domains and produced by microorganisms. These water loving and water hating domains of molecules allow them to screen at the interface between fluid phases with altered amount of polarity such as oil water or air water interafaces. As biosurfactants have all these features that is why they can decrease surface and interfacial tension and form stable emulsions where compounds of hydrogen and carbons can be solubilized in water or water in hydrocarbons [12,13]. Bio-surfactants produced by microbes have the ability to create the low interfacial tension between the compounds which are chief components of petroleum and natural gas and the aqueous phases required to assemble entrapped oil [13].

Biosurfactants play vital role in application of MEOR methods and can be good substitutes of man-made surfactants because of their specific activity low lethality and high biodegradability and effectiveness at extreme conditions of temperature, pressure, pH, and salinity [14-17].

1.4 Primary Approaches for Administration of Biosurfactants On-Site

There are two primary approaches that can be accepted for the utilization of biosurfactants in improved oil regaining. Biosurfactants can be delivered ex situ and infused into the reservoirs; or they can be created in situ by indigenous or infused microorganisms stimulated by the expansion of chosen supplements into the well [1]. Ex-situ production of biosurfactant is costly because capital bioreactors are required for this task item purification and an introduction into oil containing rocks. The second alternative is progressively ideal from commercial perspective however requires the utilization of microorganisms fit for creating sufficient measures of biosurfactant inside the supply [10].

1.5 Functioning of MEOR and Bacteria

Use of microorganism is another mechanism of MEOR in which these microorganisms reduce dense lubricant (oil) fraction. Accordingly the oil thickness diminishes and it turns out to be more fluid lighter and increasingly significant. Multiple mechanisms with groups of microbes which have various characteristics such as capacity to reduce thick oil fractions and microbial surfactant manufacturing can be an efficient approach for EOR [18]. There are various microorganisms which have the ability to destroy the compounds of hydrogen and carbon utilizing them as carbon sources. Throughout the past period remarkable outcomes of bacterialn-alkane deprivation have been stated [19,24].

Specifically strains of *Gordonia amicalis* can degrade huge n-alkanes in oxygen presence and oxygen absence conditions. *Pseudomonas fluorescens* degrade compounds of hydrogen and carbon with chain lengths somewhere in range of C_{12} and C_{32}; denser n-alkanes (C_{36}-C_{40}) can be corrupted by *Pseudomonas aeruginosa* strains and a *thermophilic Bacillus* strain that degraded long chain of carbons ($C_{15} - C_{36}$) but not short chains (C_8-C_{14}) n-alkanes has been stated [25-28]. Utilization of biosurfactant for production of native *Bacillus* strains to reduce the higher fractions of raw petroleum and upgraded its flow qualities has also been concentrated for an oil or petroleum reservoirs [29].

1.6 Major Problem Areas

Major problem associated with MEOR is the struggle of separating microbes that can develop and produce preferred metabolic items under the ecological conditions which normally exists in oil reservoirs; as limited environmental parameters are manipulated because many anaerobic reservoirs have high salinities and temperatures due to which alot of reservoirs are limited where microbes are utilized for in

situ handling. *Bacillus subtilis* and *Bacillus licheniformis* strains have been insulated frequently from oil reservoirs, thus apparently can stand with the reservoir conditions [30,31].

In this project Bacterial strains were isolated from deep oil well reservoir. Strains were screened for their heavy metal tolerance and salt tolerance. Fatty acid profiles of selected strains were observed for identification of major bio-surfactants responsible for enhanced recovery of oil from cavities. It gave insight into the factors which reduce thick lubricant fractions and helps in attaining appropriate applicants for use in microbial enhanced oil recovery.

1.7 Problem Statment

Increasing demand of energy and inefficient recovery methods for crude oil creates the need to find out an efficient ways to recover oil from oil fields. It compelled us to explore indigenous oil reservoir's bacteria and their capability for the production of biosurfactant and degradation of heavy oil fractions. Factors behind enhanced surfactant activity were identified. Fatty acid profile of strains with highest biotransformation rates were measured by GC-MS. Specific fatty acids which could play important role in oil recovery by raising the fluidity and decreasing capillary forces (which retain the oil into the reservoir) were found.

1.8 Aims and Objectives

This examine includes the subsequent goals:

1. Isolation of natural micro flora from cavities of deep oil reservoirs of OGDCL oil refineries.

2. To check chromium salt tolerance of the strains.

3. To check the biotransformation capability of the strains with significantly high salt tolerance.

4. To Identify the fatty acid compounds responsible for enhanced surfactant activity which results in break down of link between hydrogen-carbon and the aqueous phases necessary to organize trapped oil.

Chapter 2

Literature Review

2.1 Background

Manufacturing of oil has been declining whole over the world and main oil fields in the North Sea are also suffering from this decline [32]. Due to global population growth,energy demands are increasing and there is difficulty to discover fresh oil fields which can be used as substitute source to the oppressed oil pitches. Accordingly, it is necessary to invent another skill to rise oil regaining from remaining oil pitches everywhere the biosphere. About 80-90% of worldwide energy is produced from fossil fuels and about 60% is with oil and gas [33]. About 30-40% of oil can be recovered by artificial lift and natural drive, whereas 15-25% of oil recovery can be done by water flooding and pressure maintenance in which water injection leaves approximately 35-55% of oil as remaining oil in the reservoirs [34]. Residual oil around 2-4 trillion barrels or around 67% of total oil reserves keeps the main focus of many enhanced oil recovery technologies [35,36]. Enhanced oil recovery (EOR) or tertiary recovery approaches of oil are used to raise the manufacturing of crude oil and also use to recover the residual oil from oil reservoirs. Chemical flooding,miscible CO_2 injection and thermally enhanced oil recovery which uses heat for further oil recovery from reservoirs are the most common tertiary oil recovery methods [37]. Current main and subordinate oil recovery methods leaves about

2/3 of the original oil in the reservoir so residual oil in huge amount is recovered by these EOR methods. Microorganisms are used in microbial enhanced oil recovery method to draw out residual oil from the pools. By this low cost technology,upto 50% of residual oil can be recovered [1-2]. Lesser porousness of reservoirs, huge stickiness of basic oil and great oil-water stiffnesses are core interferences of an efficient oil which produces high capillary forces by which oil is retained inside the reservoir rocks which can be repressed by MEOR [38].

2.2 Enhanced Oil Recovery and Its Status

Oil companies and authorities has a major concern to increase oil recovery from aging resources because maximum oil of the whole world is produced from well-estsblished oil fields. Furthermore,due to new discoveries,produced reserves replacement rate has been falling progressively in the most recent decades. Consequently, increasing retrieval aspects from established fields under the main and secondary formation will be dangerous to fulfill the increasing vitality demands in upcoming centuries. It is certified that enhanced oil recovery plans are greatly inclined by funds and crude oil charges [39].

The commencement of EOR developments depends on the preparation and readiness of stockholders to accomplish enhanced oil recovery threat and financial revelation,and also the accessibility of extra appealing asset choices. In reality since 2002 enhanced oil recovery gas infusion developments exceed thermal tasks in the past three periods [40]. Though, thermal plans have established an increase to some extent, meanwhile 2004 because of the development of High Pressure Air Injection (HPAI) extends in light oil supplies. Compound enhanced oil recovery strategies still have not trapped the attention of oil firms with only two tasks stated in 2008 [41-43]. In the whole world,unrefined oil is upto 67% of the total petroleum reserves in reservoirs, which shows incompetence of primary and secondary oil production techniques.

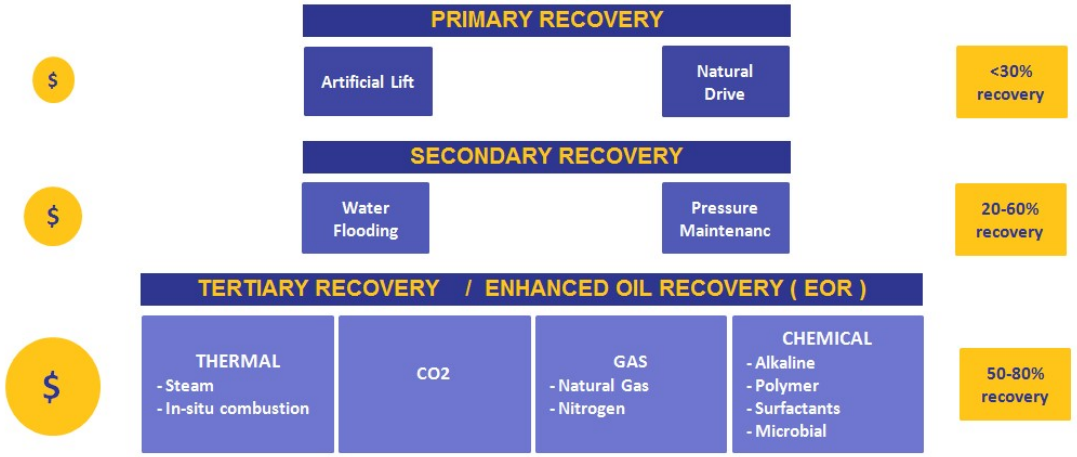


FIGURE 2.1: Different Methods of Enhanced Oil Recovery

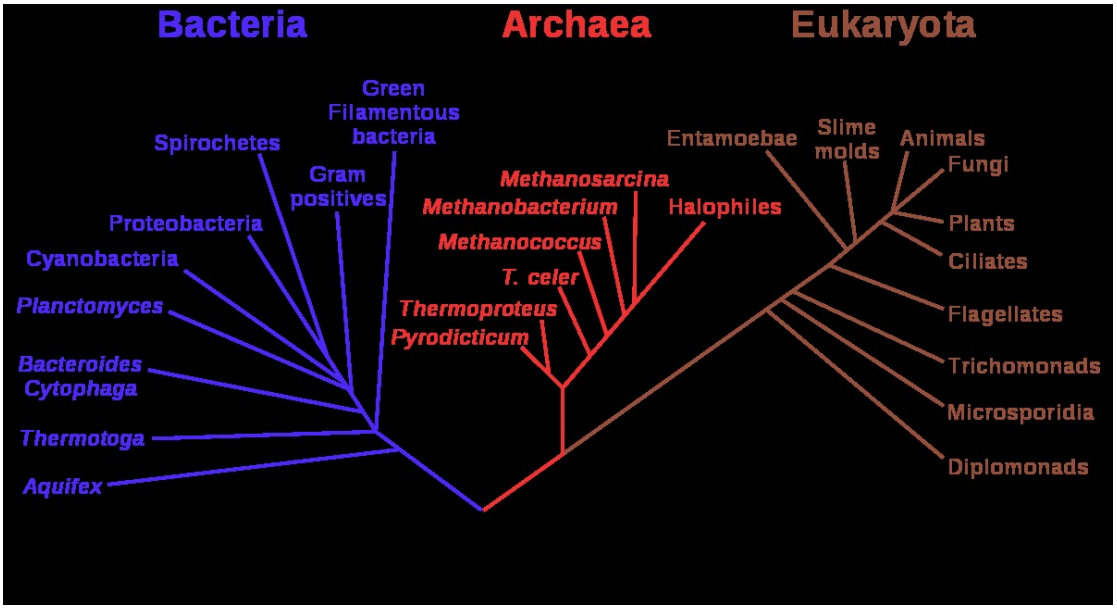


FIGURE 2.2: The Prokaryotic Microbes e.g, Bacteria and Archaea Live in All Environment But They Also Live in Extreme Environment. They Can be Found In Bottomless Aquatic Apertures To Rocks In Holes 6 km Below The Soil's Surface As Well As In Oil Reservoirs [45].

Chemicals injection (polymres or surfactants), gases (carbondioxide, hydrogen, carbon or nitrogen), or steam into the reservoir can help to achieve the extraction of the trapped oil. The synthetic substances (chemicals) used for enhanced oil recovery should be adaptable with the corporeal and biochemical surroundings of oil reservoirs. The changing penetrability of oil repositories is additionally an apprehension in enhanced oil recovery forms. When water is introduced to

dislocate the oil, it specially moves through parts of maximum porousness and avoids abundant oil. Thus,the traditional enhanced oil recovery approaches to improve the trapped unrefined oil appear not to be very efficient [44].

2.3 Microbial Enhanced Oil Recovery (MEOR)

There are three stages by which oil is recovered named as primary, secondary and tertiary oil recovery. While tertiary oil recovery technique used microorganisms or microbes to recover oil from oil wells. So microbial enhanced oil recovery is a tertiary oil retrieval practice. Primary recovery of oil recovers only 12-15% of oil in the wells without introducing other substances into the wells. While an additional 15-20% of oil from oil wells drives out by other substances and water flooding into the wells which is known as secondary oil recovery. An additional trapped oil from oil wells is extracted by the last phase of oil recovery which is tertiary oil recovery technique and it includes microbial enhanced oil recovery. As MEOR process has some advantages such as it forms an emulsion among two liquid states which decreases an interfacial stiffness and blocking the high porous zones. As bacterias are smaller in size and they have negative charge on their cell surface so in *In situ* microbial enhanced oil recovery initially bacterias which are injected with water into the wells move into the high porous regions and then they grow and block those high porous zones. Consequently an additional effective retrieval of oil can be attained by this development as it increases the sweep efficiency [46-47].

Chemical enhanced oil recovery is an expensive technology therefore MEOR can substitute CEOR. Microbes are able to produce beneficial products by fermentation of inexpensive substrates or raw materials. Chemicals used in chemical enhanced oil recovery processes are very expensive so in microbial enhanced oil recovery, selected microbial strains produces the substances which are similar to those chemicals used in CEOR procedures to rise the oil retrieval from washed-out and peripheral reservoirs. In addition to it, microbial products are decomposable and are little toxicant [6]. Microbial innovations are getting to be endorsed all

around as valuable and eco-accommodating ways to deal with improve oil generation [48-49].

2.3.1 A Short History and Early Development

MEOR study was little known after Beckman's discovery but until Zobell and his researchers made a great contributions on laboratory study in 1947 [50]. After that other contributions are also done by researchers to gain the study of MEOR [51,52].

European countries performed substantial MEOR investigation in 1960s and 1970s. Due to the petroleum crisis in the 1970s the MEOR research was improved and later become substantiated. EOR processes maintained by research projects worldwide [1]. In 1970 a new stage of microbial technology development started in Russia mainly in two laboratories:

1. The Department of Geological Microbiology at the Institute of Microbiology Academy of Sciences of the USSR headed by Kuznetsov.
2. The Laboratory of Microbial Biochemistry and Physiology of Microorganism Academy of Sciences of the USSR organized by Ivanov.

These research groups were united under the basis of the Institute of Microbiology,Russian Academy of Sciences in 1986. 1990's era is well-known as an important year for MEOR expansion . MEOR had developed a scientific and interdisciplinary method for enhanced oil recovery by the end of the 1990s [51].

J.W.Beckman et al. studied and discovered that microbes can discharge trapped oil in permeable rock formations, bacterial formation of hydrogen sulfide in the waters of the Aspheron oil fields and also reported that Sulphate Reducing Bacteria (SRB) reside in samples from 67 wells located in California and Illinois. Others innovation established into novel information of improvement of oil creation consuming a bacterial network or MEOR. Meanwhile Bastin findings donated the

information of biodeterioration of constituents or disintegration because of the occurrence and metabolic activities of microbes which are usually stated as Microbiologically Influenced Corrosion(MIC) [53-58].

2.3.2 Mechanisms Involved in MEOR

Microbial enhanced oil recovery is advanced technology by which oil is recovered in trapped formations inside the reservoirs [59]. In this technology nutrients and bacterias are added into oil reservoirs to proceed it [60]. For MEOR technology growth of microbes,either indigenously or exogenously to the reservoir is very significant as it produces valuable constituents.

- Gases
- Organic acids
- Solvents
- Polymers
- Surfactants

The above mentioned constituents play substantial part for the retrieval of remaining oil as they have beneficial effect on the formation water characteristics oil and gas mixtures as well as to transform the features of the reservoir medium [61-64].

Table 2.1: Useful Products and Activities of Microbes For Enhanced Oil Recovery

Products	Utilization in MEOR
Gas	Improved pressure oil swelling, interfacial tension and sickness decrease.
	Permeability decrease due to dissolution of carbonate rock in interaction with carbon dioxide
Emulsifiers	Oil emulsification wax and paraffin control
Hydrocarbon	Paraffin control viscosity alteration, methane production
Polymers	Stickness alterartion of injected water
Biomass	Selective plugging , wettability modification
Acids	Recover actual porousness by liquifying carbonate swift from aperture throat , emulsification increase porosity.

*Adapted from Mclnerney et al. [52] Sen [2] Lazar [1].

The advantage of each substance is listed in (TABLE 2.1). Despite the fact that many substances have been identified and many mechanisms have been proposed,the effectiveness of each substance or mechanism for different reservoir parameters is still debatable [65,66]. It is most likely that several microbial processes act synergistically to enhance the flow of oil [52]. The widely known mechanisms by which microbes can affect the fluid-fluid and fluid-porous media interaction can be categorized into three major processes:

1). Changes in flow behaviour due to bioclogging or selective plugging [67-69].

2). Alteration of rock wettability [70,71].

3). Reduction of interfacial tension (IFT) [65,66].

Kaster et al. and Jackson et al. have also reported that formation of emulsions may also be important [72,73].

2.4 MEOR Consequences

MEOR depends upon two complete clarifications.

1. Oil development through permeable media is enhanced through changing the two phases characteristics of oil-water reserves. In this framework movement of microorganisms changes.
    - Smoothness
    - Thickness decrease miscible flooding
    - Dislodging effectiveness(decline of interfacial strain porousness increases)
    - Mobility ratio(flexibility control selective plugging)
    - Pressure of reservoir
2. Oil upgrading is the second known clarification on which MEOR depends. In this agenda due to microorgsnisms involved in MEOR lighter oil is produced

from degradation of thick oil. Rather it helps to remove sulfur from heavy weight oil just for example the exclusion of heavy metals.

Persistent exploration and effective utilizations,certify the way that MEOR can be seen as a powerful innovation regardless of the current contradiction by a few groups [74-76]. However effective microbial enhanced oil recovery field applications announced are understandable and available data to help financial compensations is inadequate. MEOR is a gifted upcoming exploration technology through excessive partiality as recognized by the Oil and Gas in the 21st Century Task Force [76]. MEOR is another innovation that can aid in improving the 377 billion barrels of oil which is unrecoverable via others predictable skills [2].

2.5 Microbes Importance In Bio Degeneration of Heavy Weight Basic Oil

Oil exploitation is a significant part of the microbial enhanced oil recovery by which the oil's consistency and the solidification point are decreased which thus expand the oil's stream *in situ*. Heavy oil which is ironic in gum and asphaltene having features for example solidifying fact little stream capacity tough oil regaining, and high restoration cost [77].

Microbes works in following two ways to improve the corporeal features of heavy oil:

- They degrade heavy weight oil and reducing molecular weight of crude oil.
- The by products which are produced by metaboilc processes of microbes such as biosurfactants,acid and gas,decrease the viscosity of heavy oil.

Heavy oil contains gum and asphaltene which have high molecular weight and polarity, in the mean time they also make the oil recovery difficult [78]. Generally microorganisms barely destroys them. Assortment of organisms from conditions

well-off in oil and completed a progression of investigations utilizing blended bacterial associations which can viably destroy crude oil such as gum and asphaltene [79]. These microorganisms decrease the consistency and the point of solidification of thick oil and in this way refine the physical and synthetic characters of crude oil [80].

As microbial consortia have different properties such as they are able to degrade crude oil and can produce biosurfactants thus applying these diverse methods gives useful effects for enhanced oil recovery [18]. Various microbes used hydrocarbons as sole carbon sources and they have the ability to degrade them [19]. Sabirova et al. reported that microbial n-alkane degradation has stimulating outcomes throughout the previous eras [22].

2.5.1 Investigation on Different Strains of Bacteria

Gordonia amicalis strain shave been reported an extensive researches which have shown that it is a powerful decomposer of larger n-alkanes in the presence and absence of oxygen conditions [25].

TABLE 2.2: Shows Different Bacterial Strains and Degradation of Hydrocarbon Chains

Bacterial strains	Chain length	References
Pseudomonas	Degrade lighter chains of carbon (C_{12}-C_{32}) and heavier chain of carbons (C_{36}-C_{40})	[26,27]
Thermophilic Bacillus	Break down long chains of carbon length from (C_{15}-C_{36}) but not short chains of hydrocarbons(C_8-C_{14}).	[28]

Bacillus strains has been reported to produce biosurfactant which has the ability

to destroy the advanced portions of thick oil then support for improvement of its drift features for a fuel reservoir in the Daqing Oil field [29]. Microbial enhanced oil recovery team in the Sultan Qaboos University Oman originate group of *Bacillus* strain which was taken from oil contaminated soil can break down compounds of hydrogen and carbon with the chain length of (C_{50}-C_{70}) to (C_{11}-C_{20}). These microorganisms have enzymes for degradation of petroleum hydrocarbons. Alkanes such normal, branched, cyclic paraffins, both paraffinic and aromatics are degraded by some microorganisms [81-83].

2.5.2 Alkanes and Petroleum Organization

Alkanes in the chain length of C_{10}-C_{26} are considered to be easily degraded while harmful and low molecular mass compounds such as benzene, toluene and xylene are degraded by acquatic microorganisms. Branched and condensed ring structures are not easily degraded by microbes therefore, for the complicated ring structures of hydrocarbons biodegradation rate would be low as compared to the rates of the simpler hydrocarbons originate in petroleum. In case of highly branched methyl quantity or complicated aromatic rings biodegradation rate would be slow.

Petroleum comprises several complexes of diverse organizational densities. Crude oil is not completely degraded and expresses as black tar which has high fraction of asphaltic compound. So the residual combination needs additional biodegradation. Residual mixture keeps no toxic effects on environment, beacuase the noxiousness and bioavailability of residual combination is very low [84]. Hydrocarbon-degrading microbial population is 10% of the entire bacterial population. Metabolic pathways of hydrocarbon biodecomposition have been cl- arified [85]. Microbes oxidize the oil by an enzyme oxigenases to degrade the hy- drocarbons as it is a major step of biodegradation.

2.5.3 Additional Biodegradation Mechanism

Saturated acids are modified to carboxylic acids which are further biodegraded by β-oxidation (it is a catabolic process in which fatty acids are decomposed into smaller molecules to form acetyl-CoA enzyme which then enters the CA cycle). Normally aromatic hydrocarbon rings introduce a hydroxyl group to form diols which are further broken down to intermediates of TCA cycle. Remarkably intermediates produced by activities of microbes has different arrangement of atoms generally cis-diols which are physically sedentary. As microbes are main degraders of hydrocarbons in acquatic environment so aromatic hydrocarbon products do not produce chemicals harmful for life. Completely biodegraded compounds of hydrogen and carbons produces biologically safe substances for example:

- Carbon dioxide
- Water
- Cell biomass

2.6 Surfactants

Surface active agents are the fundamental constituents in domestic cleaners. Domestic cleansers include washing cleaners (for example laundry powder, clothing cleanser, laundry cleanser, laundry glue, and clothing tablets) household washing materials (for example Cleansers, Floor Cleansers, Lavatory Fine and Fresh appliances washing) and individual toiletries (for example Cleansers, Bath Gel, Face Wash). Advancement of domestic cleansers additionally promote the development of surface active agents.

Moistening and emulsifying are the outstanding actions of surfactants due to which they use in household work. These agents containing waste water are released into the atmosphere in vast amount which is damaging marine life contaminating

water and threatening human health. Consequently monitoring and controlling the release of surfactants in environmental water is significant.

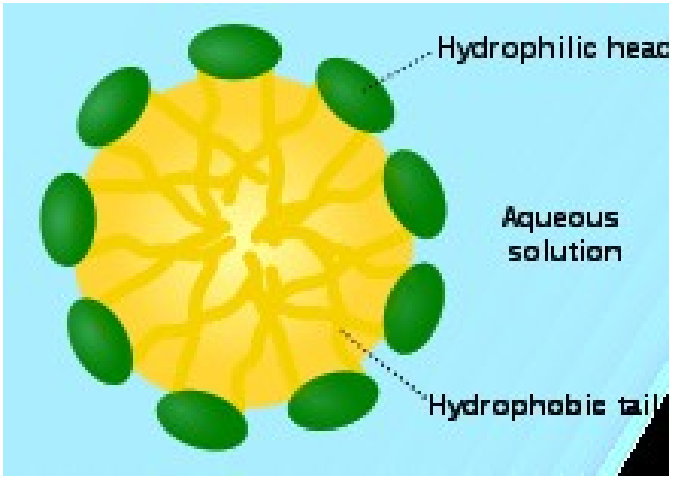


FIGURE 2.3: Structure of Surfactant

2.6.1 Classification of Surfactants

There are several types of surfactants,and they are categorized by

- Use
- Properties
- Biochemical structure

The surfactants grouping relies upon

- Water dissociation and the
- Structure of hydrophilic group

According to the water-soluble, surfactants can be characterized into:

- Ionic surfactants
- Nonionic surfactants

Ionic surfactants can be further classify in the following groups

- Negatively charged
- Positively charged
- Amphoteric surfactants
- Special features or new type surfactant is as unique surfactant [86].

2.7 Biosurfactants

These are the compounds having a polar water-soluble group attached to a water-insoluble hydrocarbon chain formed in living surfaces,generally on cell surface of microbes or expelled outside the cell water hating and water loving moieties which enables to aggregate between liquid state,consequently diminishing the surface and interfacial pressure correspondingly. They can diminish the surface and interfacial pressure utilizing indistinguishable components from synthetic compounds surfactants [87].

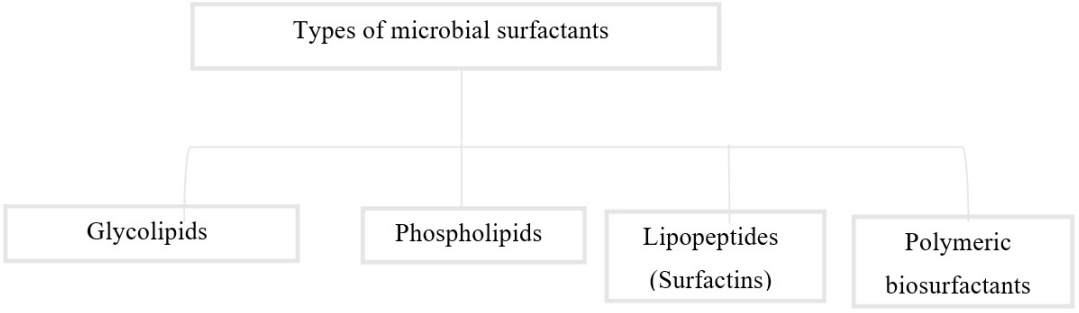


FIGURE 2.4: Types of Biosurfactants

Surfactants are the energetic components create in chemicals and cleansers with the ability to assemble at the air-water interface and are consistently used to segregate smooth materials from a particular media as a result of the manner in which that they can increase liquid dissolvability of Non Aqueous Phase Liquids (NAPLS) by diminishing their surface and interfacial tightness at air water and

water oil interfaces [88]. Microbial surfactants are basically described on the basis of their compound structure and their origin. The standard types of microbial surfactants are given in Figure 2.4.

The most prominent identified compounds in which carbohydrate is attached by glycosidic bond are:

- Rhamnolipid
- Sophorolipid
- Trehalolipid

Industrial, agricultural, food, cosmetics and pharmaceutical industries are the sites where these surfactants are widely used but maximum composites are manufactured synthetically and possibly make ecological and toxicological complications because of the fixed and persistent flora of these substances [89]. As biotechnology became an advanced technology so consideration has been compensated towards an eco friendly procedures to form numerous kinds of biosurfactants using microbes [90].

2.7.1 Use of Biosurfactants in Petroleum

Biosurfactants and bioemulsifiers are inventive molecules and helpful results that microbial innovation can offer in this field such as biofouling and bio-errosion of hydrogen and carbon compounds inside the reservoirs for upgradation of petrol [91]. Moreover these surface active molecules plays vital part in petroleum extraction, transference, cleansing and upgrading and petrochemical engineering.

TABLE 2.3: Series of Biosurfactant Utilization In Oil Manufacturing [92]. Steps Which Are Involved In The Production Chain of Petrol.

Abstraction	Modification of reservoir wettability
	Oil viscideness lessening
	Piercing sludge
	Control of paraffin/tar deposition
Transference	Oil stickness lessening
	Oil suspension maintenance
	Paraffin /asphalt depositon
Oil tank and cleaning of container	Oil thickness decrease
	Greasy mud emulsification

2.7.2 Biosurfactants Application in MEOR

Microorganisms are used in MEOR and their metaboliltes are utilized to increase oil production from reservoir. These surface active metabolites are generally utilized in oil retrieval in current eras. Solid phase acidification is an important mechanism for the release of oil. Some microorganisms for example:

- *Bacillus subtilis*
- *Pseudomonas aeruginosa*
- *Torulopsis bombicola*

are considered towards exploiting of hydrogen and carbon compounds & crude oil as sole carbon sources and aimed at clean oil spills [93].

This project relates to screening of bacteria for enhanced surfactant activity and their role in oil recovery from petroleum reservoirs. Bacterial flora were isolated

from deep reservoirs and their metal tolerance for chromium salt and biotransformation capability of the strains were checked, fatty acid compounds were identified which were responsible for enhanced surfactant activity which results in break down of link between hydrocarbons. All strains were identified with distinct fatty acid profile and enhanced biotransformation capability.

Chapter 3

Materials and Methods

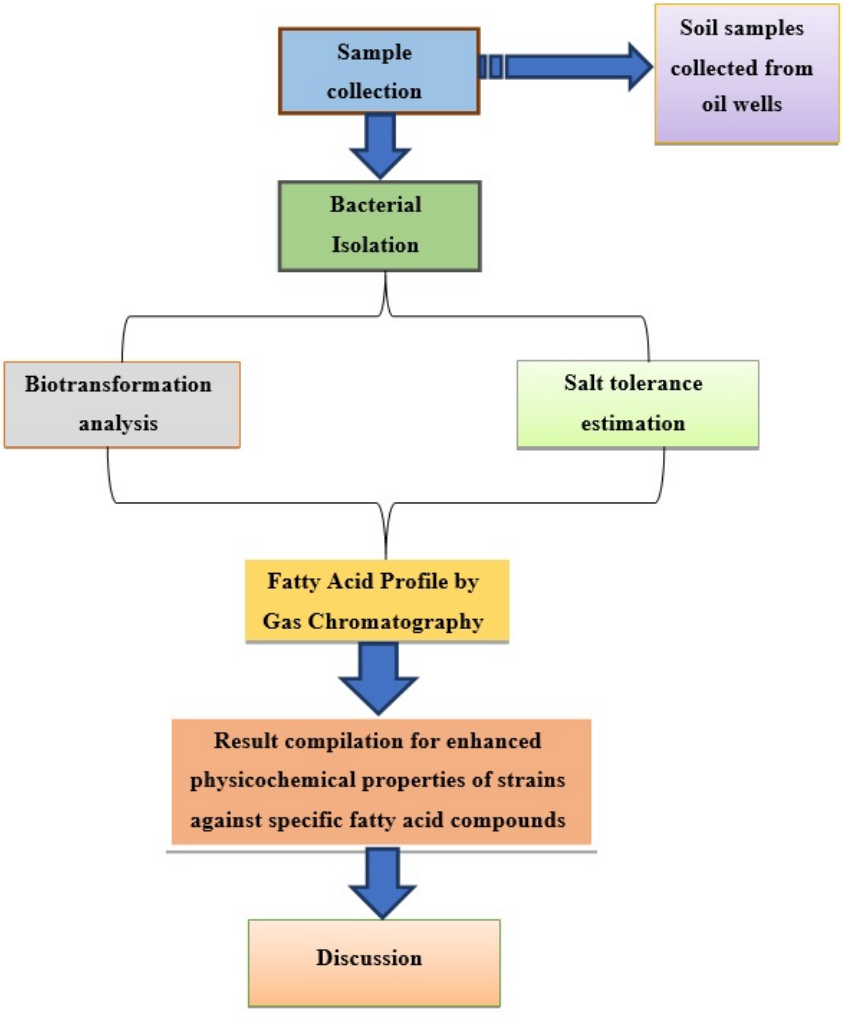


FIGURE 3.1: Overview of Methodology

3.1 Samples Collection

The soil samples were collected from deep oil well cavities of Rajian oil field and Chak Naurang oil field Chakwal District, Punjab. There were about four soil samples collected from each site, two from the Rjian well 8 at a depth of about 3550 m and 2000m. The other two from Chak Naurang well 5A at a depth of about 2515 m and 1000m. The samples were provided by Oil and Gas Development Company Ltd. Samples were collected in falcon tubes and were preserved in refrigerator and then utilized for bacterial isolation [31].

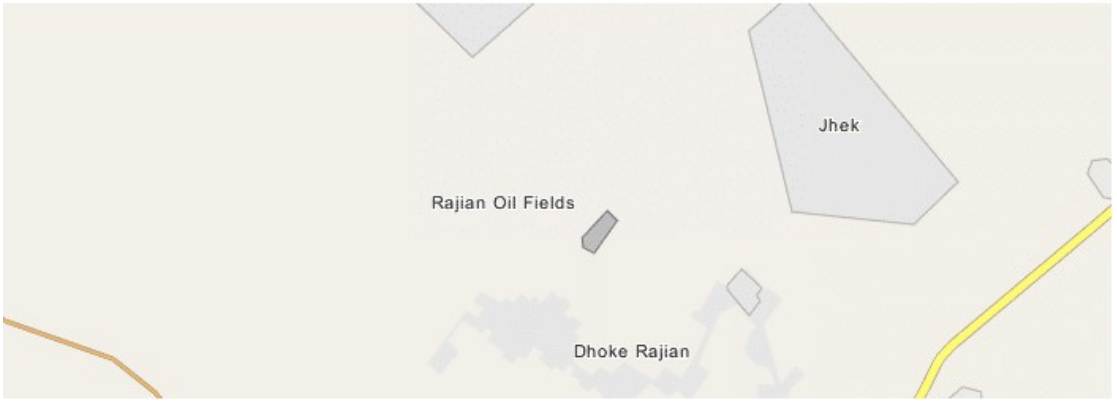


FIGURE 3.2: Sit Map of Rajian Oil Field

FIGURE 3.3: Site Map of Chak Naurang Oil Field

3.2 Isolation of Bacteria

3.2.1 Media Preparation

MSM medium, BHM, TSA, T5 and XYL media were used for isolation of bacteria which degrades hydrocarbon. Concentration of all medias were following (g/I). The pH was adjusted to 7 [94].

TABLE 3.1: Composition of MSM Media

Materials	g/l
KH_2PO_4	0. 17g
K_2HPO_4	0. 43g
$MgSO_4$	0. 025g
$Cacl_2$	0. 005g
NH_4N0_3	0. 25
$FeCl_3$	0. 0007g
Glucose	1g
Nacl	1g
Agar	5g

TABLE 3.2: Composition of BHM Media

Materials	g/l
$Mgso_4$	0. 05
$Cacl_2$	0. 005
KH_2PO_4	0. 25
K_2HPO_4	0. 25
NH_4NO_3	0. 25
$Fecl_3$	0. 01
Agar	5g

Table 3.3: Composition of T5 Media

Materials	g/l
Glucose	0. 25g
Starch	0. 25g
Yeast Extract	0. 5g
Tryptone	0. 125g
$MgSo_4$. 7H20	0. 125g
Trace salt solution	1ml
$CaCo_3$	0. 125g
Agar	5g

Table 3.4: Composition of TSA Media

Materials	g/l
Tryptone	0. 125g
Glucose	0. 25g
Yeast extract	0. 5g
$CaCo_3$	0. 25g
Trace salt solution	1ml
Agar	5g

Table 3.5: Composition of XLY

Materials	g/l
Cellulose	2. 5g
Tryptone	2g
Trace salt solution	1ml
$CaCo_3$	0. 125g
Agar	5g

3.2.2 Media Inoculation

The bacterial isolation was carried out by suspending soil samples (1g) from each site in 50 ml of sterile Phosphate Buffer Saline in a beaker and kept on the shaker for 15 minutes. 0.5 ml of dilutent from beakers of different samples was transferred using a micropipette to the petri plates and spreading was done using a glass spreader. The inoculum was spreaded on all media plates and kept in the incubator at 37 ℃ for about 24 to 48 hours. The bacterial colonies obtained were separated depending upon various parameters: appearance, shape and texture etc. and were sub-cultured on BHM, MSM, T5 and XYL agar media plates to obtain pure cultures for further experimentation. The plates containing purified colonies were further stored at 4 ℃.

3.2.3 Glycerol Stocks Preparation

The pure bacterial isolates were stored in glycerol stock solution. The glycerol stocks were prepared by mixing a loopful of bacteria with 1ml of 20% sterile glycerol in 2 ml eppendorf tubes and stored at -20 ℃ [95,96].

3.3 Morphological Characterization of Bacteria

The bacterial isolates were observed for colony color, pigmentation, colony size, colony margin and texture) and Gram's nature.

3.4 Biotransformation Analysis

Bacterial strains were screened for ability to use crude oil as sole C source. For this analysis crude oil was added from 5% to 8% onwards until unless cells stop showing any signs of growth. Growth curve was drawn for absorbance to observe

increase in growth and biotransformation of strains was determined by observing change in absorbance for 2 days [97].

3.4.1 Growth on Oil Agar Medium

The isolated bacteria were streaked on a plate of mineral salts medium (MSM), T5 and XYL oil agar medium containing 5% - 8% crude oil as a sole source of carbon. The bacteria were streaked on the media plates on which they had shown growth previously. The plates were incubated at 37 ℃. Visual observation after 2 days at 37 ℃ was used to determine the bacterial growth.

3.4.2 Growth In Oil Broth

The bacterial isolates showing the ability to grow in the maximum percentage of crude oil on oil agar plates were further studied for their crude oil utilizing ability. Further analysis was done by measuring the crude oil utilizing capability through spectrophotometer. A loopful of fresh oil medium grown culture was inoculated into 5mL of mineral salts medium (MSM), XYL and T5 media plus crude oil depending on the maximum crude oil percentage on which bacteria had showed growth. Two controls were setup negative control containing the same amount of MSM, XYL and T5 and crude oil but no bacterial culture and positive control containing the same amount of MSM, XYL and T5 and crude oil with *Bacillus megaterium*. Test tubes were incubated with shaking at 200 rpm for 2 days at 37 ℃. The residual crude oil was determined by reading the optical density with a UV-Visible spectrophotometer at 600nm.

3.5 Salt Tolerance Estimation

Salt tolerance of the bacterial strains were estimated against $CrSO_4.7H_20$ from 120mg/L to onwards 180mg/L. Measurement of optical density at wavelenghth

600 nm by a spectrophotometer was used for determining the growth of bacteria after 24 hours.

3.6 Fatty Acid Profile by Gas Chromatography

GC-MS of moderately purified biosurfactant was analyzed using gas chromatography [98]. GC-MS helped in estimation and identification of type of fatty acid series responsible or enhanced surfactant property of selective strains.

Chapter 4

Results and Discussion

4.1 Isolation of Bacteria

There were about 47 isolates obtained as a result of isolation from the soil samples. The isolates also showed morphological variations, which showed that they were different from each other. The table given below shows the characteristics of isolates. Information related to strain, color pigmentation, texture, margins, media opted, temperature and incubation time are given in the table 4.1.

4.2 Biotransformation Analysis

Biotransformation analysis started by adding 5% of crude oil onwards until unless cells stop showing any sign of growth. Then with every percentage of crude oil their absorbance was checked by making broth and plates were kept for 2 days (48 hrs) in incubator at 36℃. The bacteria isolated during this study were further checked for their ability to grow on Mineral Salts Media (MSM), T5 (TSA) and XYL media with different concentrations of crude oil as a sole carbon source ranging from 5% to 8%. Crude oil utilization was determined through growth on the solid media. The crude oil utilizing ability was determined by first growing the bacteria on oil agar media and then by determining this ability quantitatively.

Table 4.1: Physical Characteristics and Features of Strains at 37 ℃ After 48 Hours

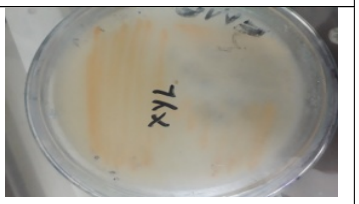
Strain code	Color	Pigmentation	Texture	Margin	Size(mm)	Media Opted	Grams Nature	Figure
S1A	Light white	Light white	Mucoid	Undulate	Small 2−Jan	BHM	−	
S1B	Light orange	Light orange	Smooth	Entire	Small 2 −Jan	MSM	+	
S1C	Yellow	Yellow	Smooth	Entire	Medium 3 −Feb	XYL	+	

Table 4.1 Continued From Previous Page

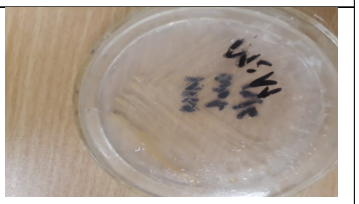
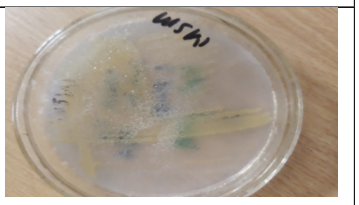
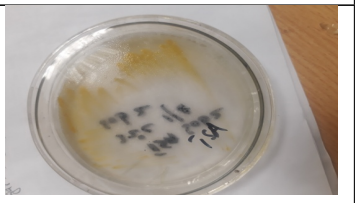
Strain code	Color	Pigmentation	Texture	Margin	Size(mm)	Media Opted	Grams Nature	Figure
S1D	Light pink	Light pink	Rough	Undulate	Small 2 −Jan	MSM	+	
S1E	Yellow	Yellow	Smooth	Entire	Medium 3 −Feb	MSM	−	
S1F	Yellow	Yellow	Viscid	Raised margin	Medium 3 −Feb	TSA	+	

Table 4.1 Continued From Previous Page

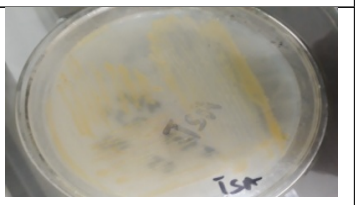
Strain code	Color	Pigmentation	Texture	Margin	Size(mm)	Media Opted	Grams Nature	Figure
S1G	Light pink	Light pink	Rough	Undulate	Small <1	MSM	+	
S1H	Yellow	Yellow	Smooth	Entire	Medium 3 −Feb	TSA	+	
S1I	Dirty White	Dirty white	Rough	Entire	Medium 3 −Feb	XYL	+	

Table 4.1 Continued From Previous Page

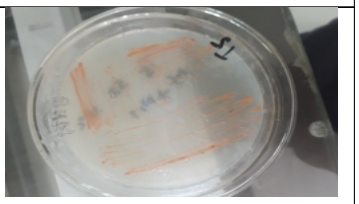
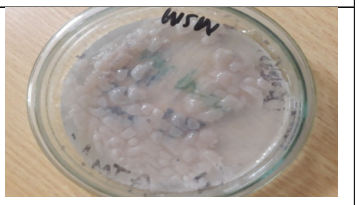
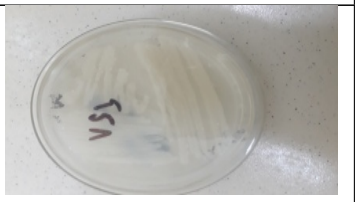
Strain code	Color	Pigmentation	Texture	Margin	Size(mm)	Media Opted	Grams Nature	Figure
S1J	Light orange	Light orange	Smooth	Entire	Medium 3 −Feb	T5	+	
S1K	Light white	Light white	Sticky	Raised margins	Medium 3 −Feb	MSM	−	
S1L	White	White	Rough	Entire	Medium 3 −Feb	TSA	+	

Table 4.1 Continued From Previous Page

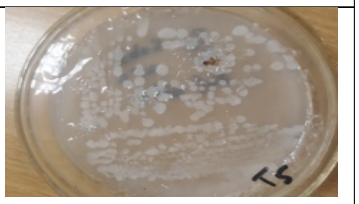
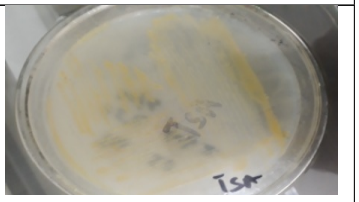
Strain code	Color	Pigmentation	Texture	Margin	Size(mm)	Media Opted	Grams Nature	Figure
S1M	White	White	Smooth	Raised margins	Large ¿3	T5	–	
S1N	White	White	Sticky	Undulate	Small 2 –Jan	BHM	–	
S1O	Yellow	Yellow	Smooth	Entire	Medium 3 –Feb	TSA	+	

Table 4.1 Continued From Previous Page

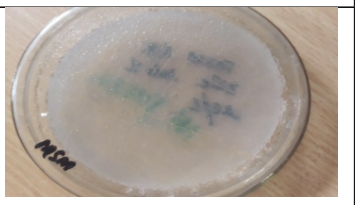
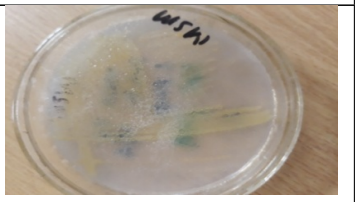
Strain code	Color	Pigmentation	Texture	Margin	Size(mm)	Media Opted	Grams Nature	Figure
S1P	White	White	Smooth	Undulate	Medium 3 −Feb	TSA	+	
S1Q	Light Yellow	Light Yellow	Rough	Undulate	Small 2 −Jan	MSM	−	
S1R	Yellow	Yellow	Viscid	Entire	Medium 3 −Feb	MSM	−	

Table 4.1 Continued From Previous Page

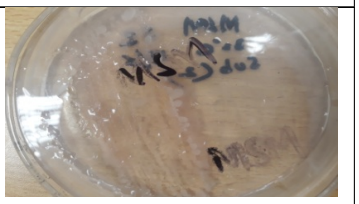
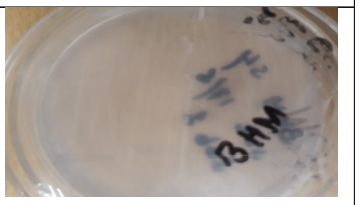
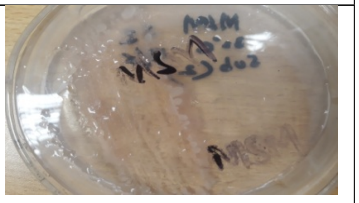
Strain code	Color	Pigmentation	Texture	Margin	Size(mm)	Media Opted	Grams Nature	Figure
S1S	Orange	Orange	Smooth	Entire	Medium 3 −Feb	T5	+	
S1T	Light pink	Light pink	Rough	Raised margins	Small 2 −Jan	MSM	+	
S1U	Transparent white	Transparent white	Sticky	Raised margin	Small 2 −Jan	BHM	−	

Table 4.1 Continued From Previous Page

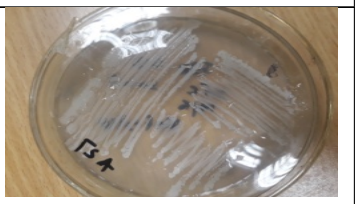
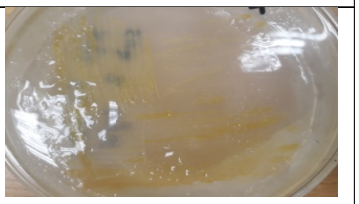
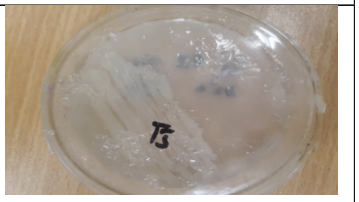
Strain code	Color	Pigmentation	Texture	Margin	Size(mm)	Media Opted	Grams Nature	Figure
S1V	Light Pink	Light pink	Rough	Raised margins	Small 2 −Jan	MSM	−	
S1W	White	White	Smooth	Entire	Medium 3 −Feb	TSA	−	
S1X	Light orange	Light orange	Smooth	Entire	Medium 3 −Feb	TSA	+	

Table 4.1 Continued From Previous Page

Strain code	Color	Pigmentation	Texture	Margin	Size(mm)	Media Opted	Grams Nature	Figure
S1Y	White	White	Smooth	Entire	Medium 3 −Feb	XYL	+	
S1Z	Light yellow	Light yellow	Smooth	Entire	Medium 3 −Feb	XYL	−	
S2A	White	White	Smooth	Undulate	Medium 3 −Feb	T5	−	

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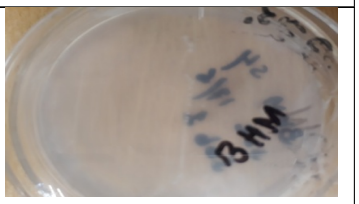
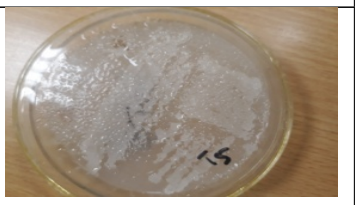
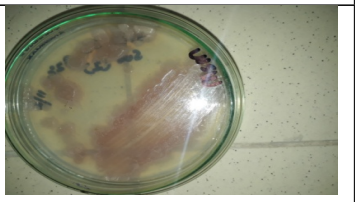
Strain code	Color	Pigmentation	Texture	Margin	Size(mm)	Media Opted	Grams Nature	Figure
S2B	White	White	Viscid	Undulate	Medium 3 −Feb	T5	+	
S2C	White	White	Sticky	Undulate	Small 2 −Jan	BHM	−	
S2D	White	White	Smooth	Entire	Medium 3 −Feb	T5	+	

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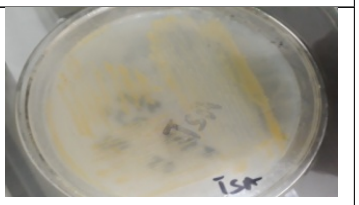
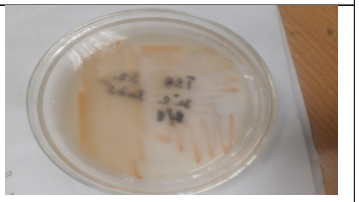
Strain code	Color	Pigmentation	Texture	Margin	Size(mm)	Media Opted	Grams Nature	Figure
S2E	Pink	Pink	Sticky	Undulate	Medium 3 −Feb	MSM	−	
S2F	White	White	viscid	Undulate	Small 2 −Jan	MSM	−	
S2G	Yellow	Yellow	Smooth	Entire	Small 2 −Jan	TSA	+	

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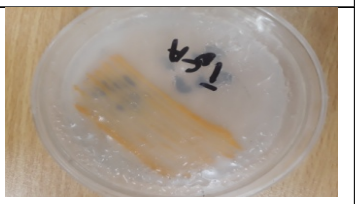
Strain code	Color	Pigmentation	Texture	Margin	Size(mm)	Media Opted	Grams Nature	Figure
S2H	Orange	Orange	Smooth	Entire	Medium 3 −Feb	TSA	−	
S2I	Orange	orange	Smooth	Raised	Medium 3 −Feb	TSA	+	
S2J	Light orange	Light orange	Smooth	Raised	Medium 3 −Feb	T5	+	

Table 4.1 Continued From Previous Page

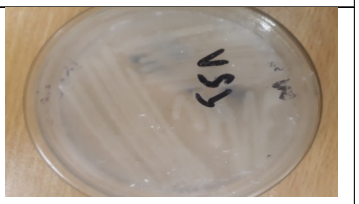
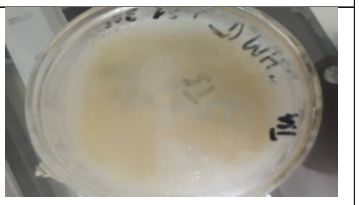
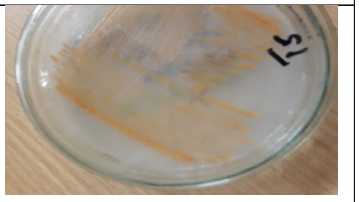
Strain code	Color	Pigmentation	Texture	Margin	Size(mm)	Media Opted	Grams Nature	Figure
S2K	Dirty white	Dirty white	Smooth	Entire	Medium 3 −Feb	TSA	+	
S2L	Half White	Half White	Viscid	Entire	Medium 3 −Feb	TSA	+	
S2M	Yellow	Yellow	Smooth	Entire	Medium 3 −Feb	TSA	+	

Table 4.1 Continued From Previous Page

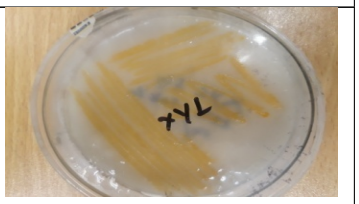
Strain code	Color	Pigmentation	Texture	Margin	Size(mm)	Media Opted	Grams Nature	Figure
S2N	Yellow	Yellow	Smooth	Entire	Small 2 −Jan	TSA	+	
S2O	Orange	Orange	Smooth	Entire	Medium 3 −Feb	T5	+	
S2P	Light yellow	Light yellow	Smooth	Raised margin	Small 2 −Jan	XYL	+	

Table 4.1 Continued From Previous Page

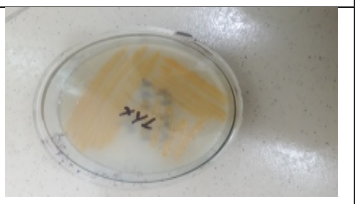
Strain code	Color	Pigmentation	Texture	Margin	Size(mm)	Media Opted	Grams Nature	Figure
S2Q	Yellow	Yellow	Smooth	Entire	Medium 3 −Feb	XYL	+	
S2R	Orange	Orange	Rough	Entire	Medium 3 −Feb	TSA	+	
S2S	White	White	Smooth	Entire	Medium 3 −Feb	T5	+	

Table 4.1 Continued From Previous Page

Strain code	Color	Pigmentation	Texture	Margin	Size(mm)	Media Opted	Grams Nature	Figure
S2T	Yellow	Yellow	Smooth	Entire	Medium 3 −Feb	XYL	+	
S2U	White	White	Smooth	Entire	Medium 3 −Feb	XYL	+	

4.2.1 Growth on Oil Agar Medium

The crude oil utilizing ability of bacteria was evaluated by growth of the isolates in oil agar media. Following 21 isolates out of 47 showed growth in crude oil media. The tables given below show the various concentrations of crude oil on which growth of isolates was observed.

4.2.2 Biotransformation with 5% Crude Oil

TABLE 4.2: Growth of Isolates In Oil Agar Media Containing 5% of Crude Oil

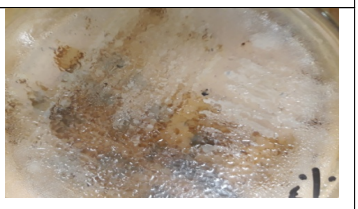
Strain code	Media opted	Growth	Figure
S1Y	XYL	High	
S1Z	XYL	Low	
S2T	XYL	High	
S2P	XYL	High	
S2E	MSM	High	

Table 4.2 Continued From Previous Page

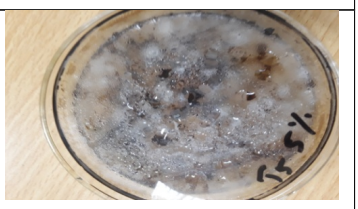
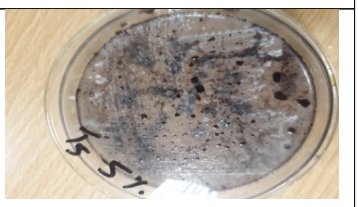
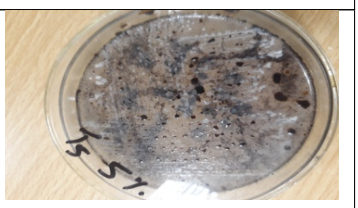
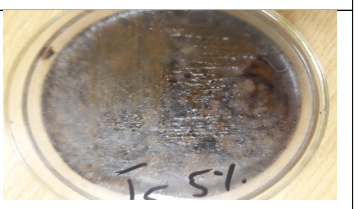
Strain code	Media opted	Growth	Figure
S1X	T5	High	
S1F	T5	High	
S2S	T5	Moderate	
S2B	TSA	Moderate	
S2O	TSA	Moderate	
S1J	T5	Moderate	
S1I	XYL	Moderate	
S1S	T5	High	

Table 4.2 Continued From Previous Page

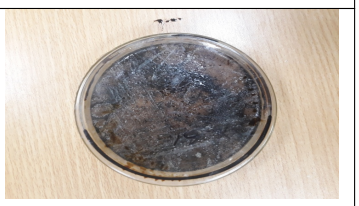
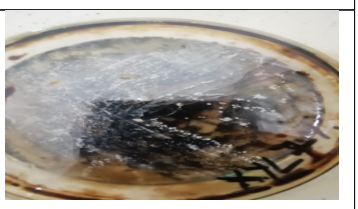
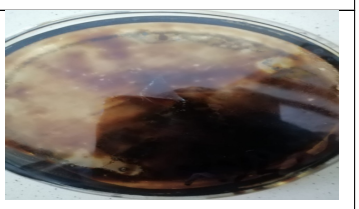
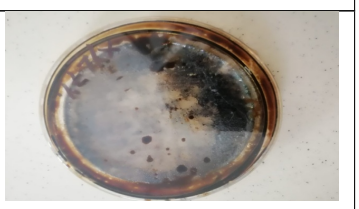
Strain code	Media opted	Growth	Figure
S1T	MSM	Low	
S1D	MSM	Low	
S1G	MSM	Low	
S1Q	MSM	Low	
S2A	TSA	Low	
S2D	T5	Low	
S2J	T5	Moderate	
S1C	XYL	Low	

4.2.3 Biotransformation with 6% Crude Oil

TABLE 4.3: Growth of isolates in oil agar media containing 6% of crude oil

Strain code	Media opted	Growth	Figure
S20	T5	Moderate	
S2B	TSA	High	
S1X	TSA	High	
S1J	TSA	Moderate	
S1S	T5	High	
S2T	XYL	High	
S1I	XYL	Moderate	

Table 4.3 Continued From Previous Page

Strain code	Media opted	Growth	Figure
S2S	TSA	High	
S1Q	MSM	Low	
S1G	MSM	Low	
MSM	S2E	Low	
S2P	XYL	High	
S1Y	XYL	High	
S1F	T5	High	

4.2.4 Biotransformation with 7% Crude Oil

TABLE 4.4: Growth of Isolates In Oil Agar Media Containing 7% of Crude Oil

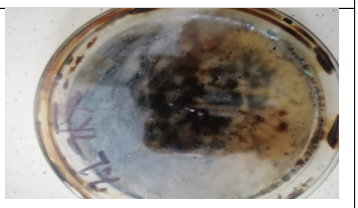
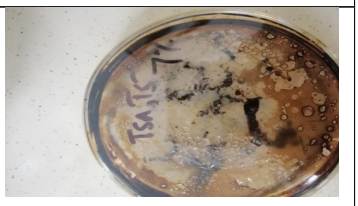
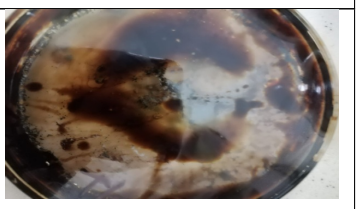
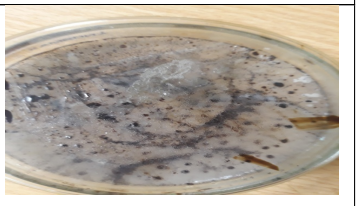
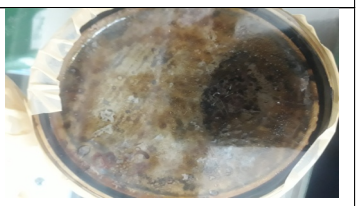
Strain code	Media opted	Growth	Figure
S2P	XYL	High	
S2O	T5	Moderate	
S1I	XYL	Moderate	
S1F	T5	High	
S1Y	XYL	High	
S2T	XYL	High	
S2B	TSA	High	
S1X	TSA	High	

Table 4.4 Continued From Previous Page

Strain code	Media opted	Growth	Figure
S1J	T5	Moderate	
S1S	T5	High	
S2S	T5	High	

4.2.5 Biotransformation with 8% Crude Oil

TABLE 4.5: Growth of Isolates In Oil Agar Media Containing 8% of Crude Oil

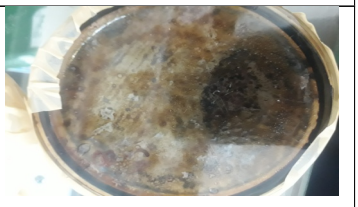
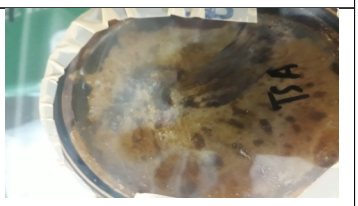
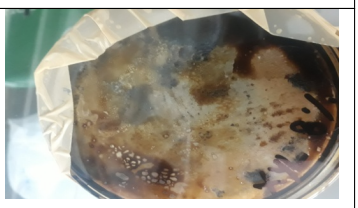
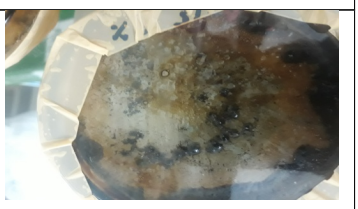
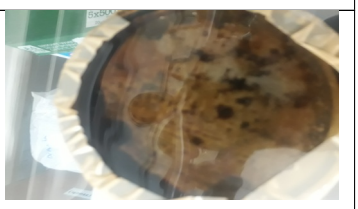
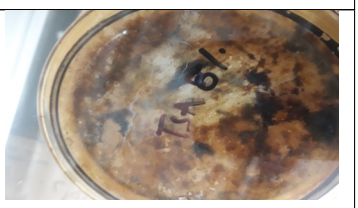
Strain code	Media opted	Growth	Figure
S2T	XYL	High	
S2O	T5	Moderate	
S1F	T5	High	

Table 4.5 Continued From Previous Page

Strain code	Media opted	Growth	Figure
S1S	TSA	High	
S1X	T5	High	
S2B	TSA	High	
S1I	XYL	Moderate	
S1Y	XYL	High	
S2S	T5	High	
S2P	XYL	High	
S1J	TSA	Moderate	

The isolates that showed growth on concentrations of crude oil ranging from 5% to 8% were selected and analyzed for their crude oil utilizing ability. Spectrophotometer was used to analyze the capability of bacteria. Increase in turbidity in the broth meant that the bacteria were growing by utilizing crude oil. The samples were kept in the incubator for about 2 days at 37 ℃ and 200 rmp. Absorbance readings were taken from the 2^{nd} day. The details related to the experiment are shown in the tables below along with graphs.

The 21 isolates that showed growth on oil agar medium showed quite variation in growth patterns. The lowest concentration of crude oil. i.e. 5% was initially used in mineral salts medium (MSM), XYL media and T5 media to check bacterial growth. The isolates S1Y, S2T, S2P, S2E, S1X, S1S, S1F showed high growth on 5% crude oil agar media. The isolates S2S, S2B, S20, S1J, S1I, S2J showed moderate growth on 5% crude oil agar media. While the isolates S1Z, S1T, S1D, S1G, S1Q, S2A, S2D and S1C showed low growth on 5% crude oil agar media.

These isolates were streaked further on 6% crude oil agar media. The isolates S2B, S2S which showed moderate growth on previous media now showed high growth on 6% crude oil agar media along with other isolates i.e, S1X, S1S, S2P, S1Y, S1F and S2T. The isolates S2O, S1J, S1I showed moderate growth as they had shown growth on previous percentage of media. The isolates S1Q, S1G and S2E showed low growth on 6 % crude oil agar media. The most abrupt change was in S2E isolate which had shown high growth on previous percentage of crude oil agar media but on 6 % crude oil it showed low growth. Similarly the isolates S2D, S2J, S2A, S1C and SIZ Showed no growth on 6% crude oil agar media.

These isolates were further streaked on 7 % crude oil agar media. The isolates S2O, S1I and S1J showed moderate growth. The Isolates S1G,S1Q and S2E showed no growth at this percentage. While the Isolates S2B, S1X, S1S, S2S, S2P, S1Y, S1F, S2T showed high growth on this percentage.

Isolates S2O, S1I and S1J showed moderate growth when streaked on 8% crude oil agar media. While the isolates S2B, S1X, S1S, S2S, S2P, S1Y, S1F and S2T showed high growth on 8% crude oil agar media. As these isolates were maintaining their

growths. Again there was no change in isolates growth pattern as compared with previous percentages (5%-6%). Some changes in isolate were seen in 5%-6% crude oil agar media.

4.2.6 Growth in Oil Broth

The isolates that showed growth on concentrations of crude oil ranging from 5% to 8% were selected and analyzed for their crude oil utilizing ability. Spectrophotometer was used to analyze the capability of bacteria. Increase in turbidity in the broth meant that the bacteria were growing by utilizing crude oil. The samples were kept in the incubator for about 2 (48 hours) days at 37 ℃ and 200 rmp. Absorbance readings were taken from the 2^{nd} day. The details related to the experiment are shown in the tables below along with graphs.

Table 4.6: Absorbance Rate With 5% Crude Oil In MSM Broth

Standard	Strain code	Absorbance
1	S1T	0.001
2	SID	0.014
3	S1G	0.035
4	S1Q	0.045
5	S2E	0.69
6	Positive control	0.665
7	Negative control	0

The graph shows (Figure 4.1) the growth of isolates in mineral salt media with 5% crude oil concentration. The low rate of absorbance means low growth of bacteria was in the broth in the presence of crude oil. While high absorbance rate means,the growth of isolates is high. The isolate S2E shows highest absorbance on 5% crude oil agar media with 0.69. S1T shows very low absorbance,which means bacteria growth was very low in this isolate. S1D, S1G and S1Q shows least absorbance. Positive control was *Bacillus megaterium* which was also amended with 5% crude

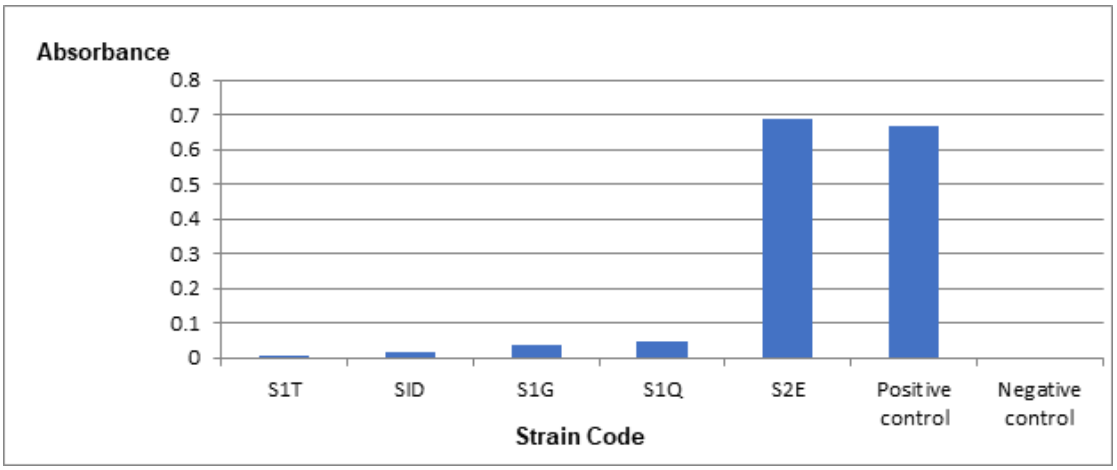


FIGURE 4.1: Growth of Bacterial Isolates in Mineral Salt Media Supplemented With 5% Crude Oil

oil and shows 0.665 absorbance while negative culture was amended with 5% crude oil and has no bacterial culture that is why shows no growth.

TABLE 4.7: Absorbance Rate With 6% Crude Oil In MSM Broth

Standard	Strain code	Absorbance
1	S1Q	0.045
2	S1G	0.045
3	S2E	0.055
4	Positive control	0.665
5	Negative control	0

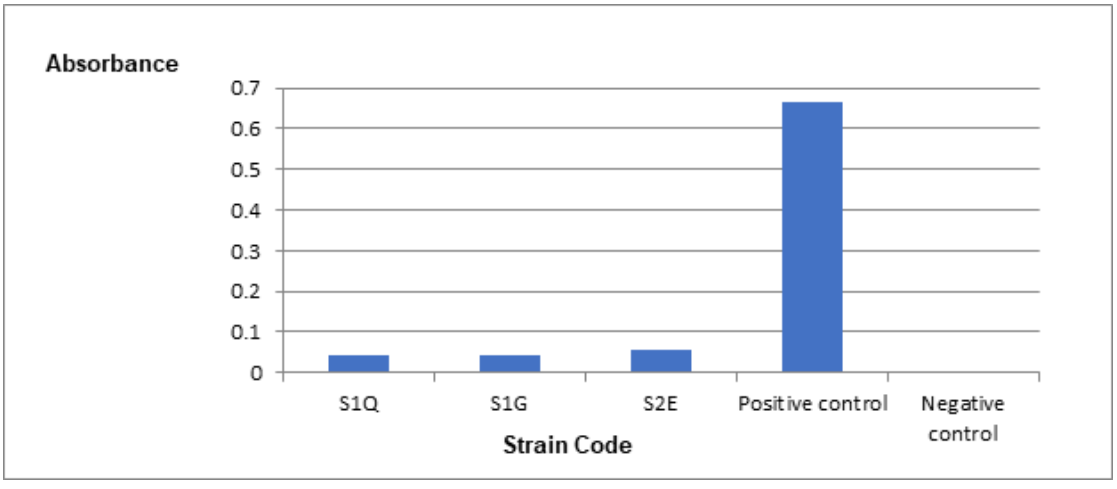


FIGURE 4.2: Growth of Bacterial Isolates In Mineral Salt Media Supplemented With 6% Crude Oil

The graph shows (Figure 4.2) the growth of isolates in mineral salt media with 6% crude oil concentration. The low rate of absorbance means,low growth of bacteria was in the broth in the presence of crude oil. While high absorbance rate means,the growth of isolates is high. This graph shows same absorbance rate in S1Q, S1G and S2E isolates with 0.04. Positive control was *Bacillus megaterium* which was also amended with 6% crude oil and shows 0.665 absorbance which was the same on 5% crude oil concentration. While negative culture was amended with 6% crude oil and has no bacterial culture that is why shows no growth. Highest absorbance rate was seen in only positive strain with 0.665. While an abrupt change was seen in S2E which showed high absorbance on 5% crude oil agar media. But at this stage it showed least growth with absorbance rate of 0.055.

TABLE 4.8: Absorbance Rate With 5% Crude Oil in T5 Broth

Standard	Strain code	Absorbance
1	S2D	0.001
2	S2J	0.01
3	S2A	0.01
4	S20	0.046
5	S1J	0.056
6	S2B	0.5
7	S2S	0.58
8	S1F	0.595
9	S1S	0.676
10	S1X	0.681
11	Positive control	0.473
12	Negative control	0

The graph shows (Figure 4.3) the growth of isolates in T5 media with 5% crude oil concentration. All of the selected isolates had previously exhibited growth on T5 oil agar media till 5%. The negative control was T5 media having 5% of crude oil and no bacterial culture. The positive control on the other hand was

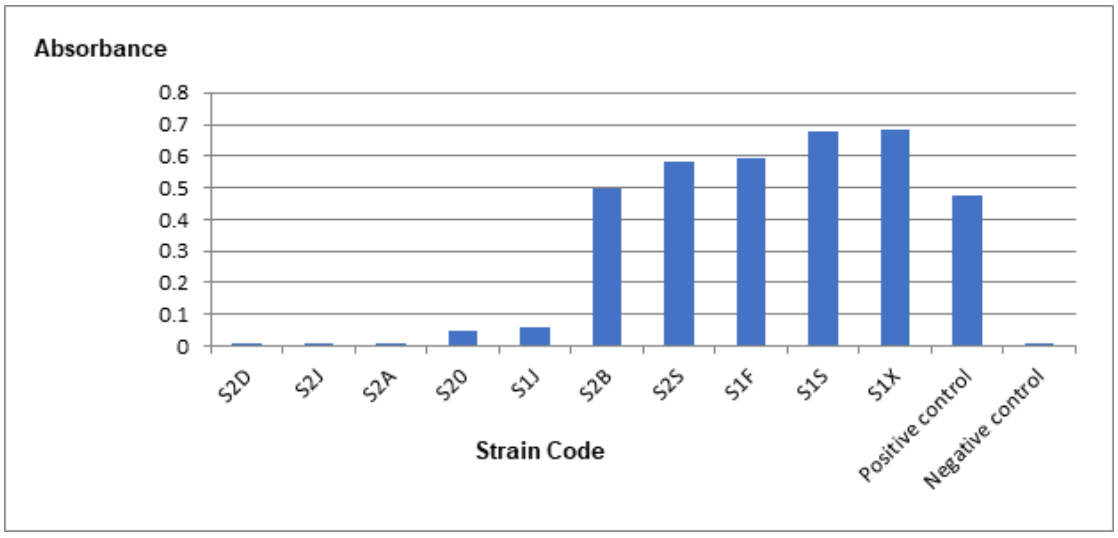


FIGURE 4.3: Growth of Bacterial Isolates In T5 Media Supplemented With 5% Crude Oil

T5 media having 5% of crude oil and *Bacillus megaterium*. The graph clearly shows that the maximum growth was shown by the isolate S1X with the highest absorbance rate as 0.681. The isolate S1S showed second highest growth rate with the absorbance value as 0.681. The isolate S1F showed third highest growth rate with absorbance value as 0.595. S2S showed growth rate on number fourth with absorbance value as 0.58. S2B showed absorbance value as 0.5 and comes in fifth number for the bacterial growth rate. S1J showed absorbance value as 0.056 while the isolate S20 showed absorbance value as 0.046. The isolates S2A and S2J showed absorbance value as 0.01 and S2D showed least absorbance value as 0.001 which shows no growth of bacteria in these isolates. The negative control showed no sign of growth since there was no bacterial culture added in it. The positive control showed absorbance value as 0.473. The low values of absorbance are linked with the low concentration of crude oil which was about 5% for which the isolates were analyzed.

TABLE 4.9: Absorbance Rate With 6% Crude Oil In T5 Broth

Standard	Strain code	Absorbance
1	S20	0.056
2	S1J	0.07
3	S2B	0.777

Table 4.9 Continued From Previous Page

Standard	Strain code	Absorbance
4	S1X	0.8
5	S1S	0.9
6	S2S	0.9
7	S1F	1
8	Positive control	0.473
9	Negative control	0

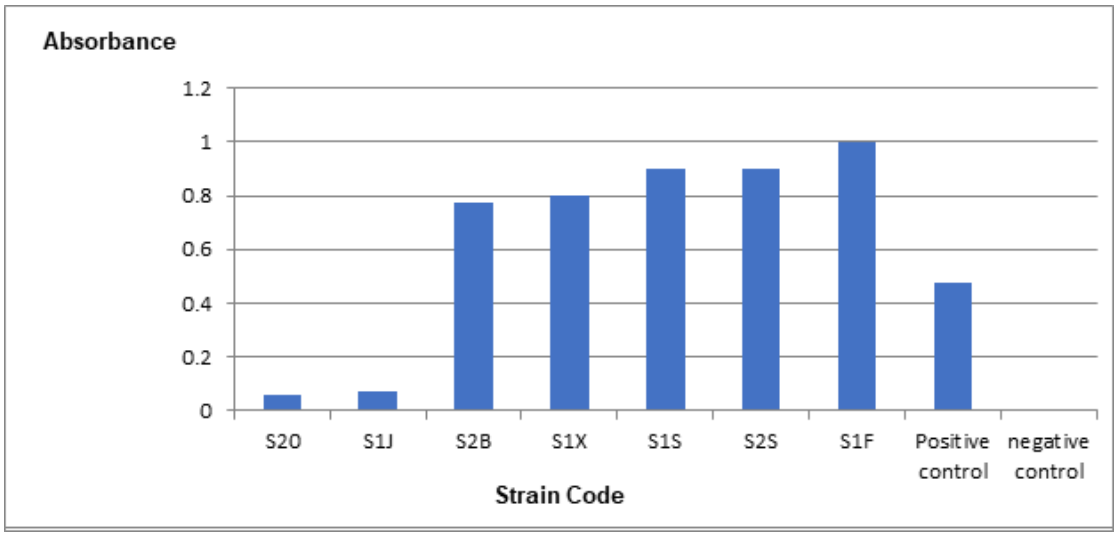


FIGURE 4.4: Growth of Bacterial Isolates In T5 Media Supplemented With 6% Crude Oil

The graph shows (Figure 4.4) the growth of isolates in T5 media with 6% crude oil concentration. All of the selected isolates had previously exhibited growth on T5 oil agar media till 6%. The negative control was T5 media having 6% of crude oil and no bacterial culture. The positive control on the other hand was T5 media having 6% of crude oil and *Bacillus megaterium*. The graph clearly shows that isolate S1F shows highest growth rate with highest absorbance value as 1. This time S2S and S1S both shows second highest growth rate because both showed same absorbance value as 0.9. S1X shows third highest growth rate with absorbance value as 0.8. S2B shows fourth highest growth rate with absorbance value as 0.7. The isolate S1J shows absorbance value as 0.07 and S2O shows least absorbance value as 0.056 which means there was least growth of bacteria. The

negative control showed no sign of growth since there was no bacterial culture added in it. The positive control showed absorbance value as 0.473 which is the same as in 5% crude oil concentration. The low values of absorbance are linked with the low concentration of crude oil which is about 2% for which the isolates were analyzed.

TABLE 4.10: Absorbance Rate With 7% Crude Oil In T5 Broth

Standard	Strain code	Absorbance
1	S20	0.07
2	S1J	0.09
3	S2B	0.9
4	S1X	0.9
5	S2S	1
6	S1S	1.2
7	S1F	1.8
8	Positive control	0.673
9	Negative control	0

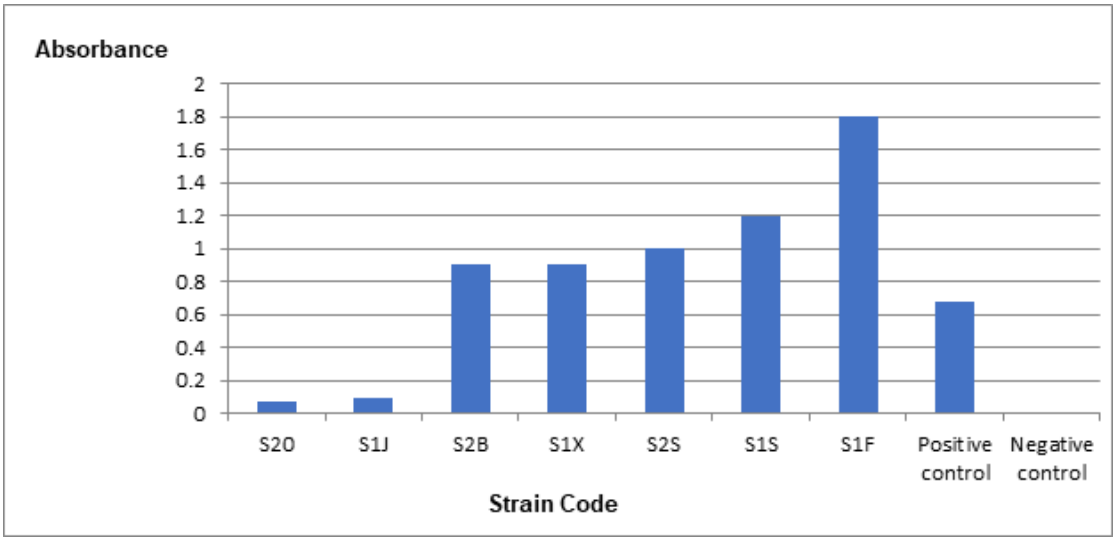


FIGURE 4.5: Growth of Bacterial Isolates In T5 Media Supplemented With 7% Crude Oil

The graph shows (Figure 4.5) the growth of isolates in T5 media with 7% crude oil concentration. All of the selected isolates had previously exhibited growth

on T5 oil agar media till 7%. The negative control was T5 media having 7% of crude oil and no bacterial culture. The positive control on the other hand was T5 media having 7% of crude oil and *Bacillus megaterium*. The graph clearly shows that isolate S1F shows highest growth rate with highest absorbance value as 1.8 as with increasing oil concentration the growth of isolates were increasing and showing biotransformation. S1S shows second absorbance value as 1.2. S2S shows third highest growth rate with absorbance value as 1. S1S and S2B both shows the same growth rate with absorbance values as 0. 9 with fourth highest growth rate. S1J shows absorbance value as 0.09 while S20 shows least growth rate with absorbance value as 0.07. The negative control showed no sign of growth since there was no bacterial culture added in it. The positive control showed absorbance value as 0.673.

Table 4.11: Absorbance Rate with 8% Crude Oil in T5 Broth

Standard	Strain code	Absorbance
1	S1J	0.1
2	S20	0.13
3	S1X	1.2
4	S1S	1.421
5	S2B	1.534
6	S2S	1.714
7	S1F	2.453
8	Positive control	0.773
9	Negative control	0

The graph shows (Figure 4.6) the growth of isolates in T5 media with 8% crude oil concentration. All of the selected isolates had previously exhibited growth on T5 oil agar media till 8%. The negative control was T5 media having 8% of crude oil and no bacterial culture. The positive control on the other hand was T5 media having 8% of crude oil and *Bacillus megaterium*. The graph clearly shows that isolate S1F shows highest growth rate with highest absorbance value as 2.4 which means that this isolate showed highest biotransformation and have high growth

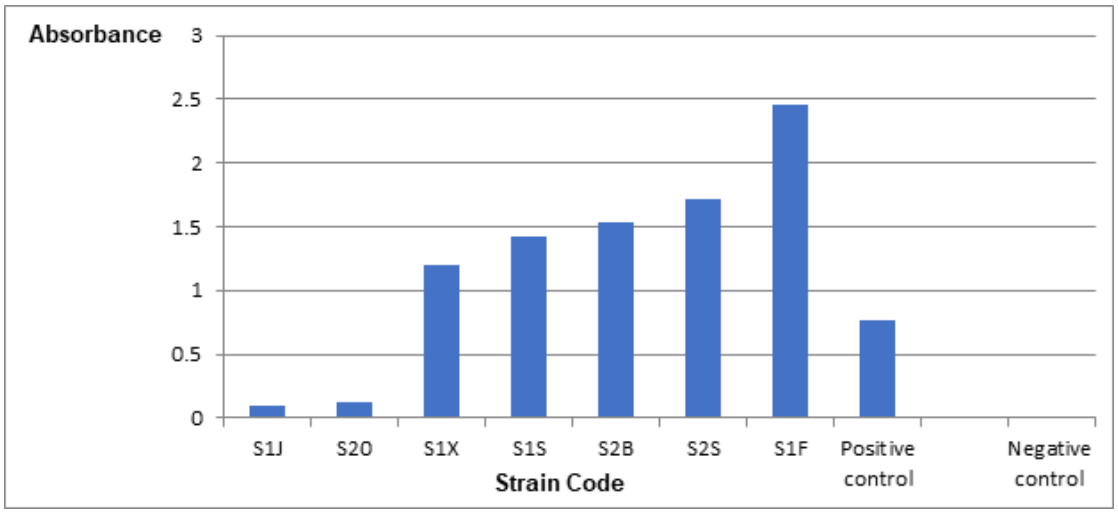


FIGURE 4.6: Growth of Bacterial Isolates In T5 Media Supplemented With 8% Crude Oil

of bacteria. S2S showed second highest growth rate with absorbance value as 1.7. S2B shows third highest growth rate with absorbance value as 1.5. S1S shows fourth highest growth rate of bacteria with absorbance value as 1.4. S1X shows fifth highest growth rate with absorbance value as 1.2. So these five isolates are showing biotransformation with their own absorbance values. While S2O shows absorbance value as 0.13 and S1J shows absorbance value as 0.1 which were least values and shows least growth of bacteria. The negative control showed no sign of growth since there was no bacterial culture added in it. The positive control showed absorbance value as 0.773 which as increased as compared to 7% crude oil concentration. So all the isolates which mentioned above in graph in T5 media with highest absorbance value showed biotransformation to utilize crude oil as sole carbon source.

TABLE 4.12: Absorbance Rate With 5% Crude Oil In XYL Broth

Standard	Strain code	Absorbance
1	S1C	0.001
2	S1Z	0.003
3	S1I	0.07
4	S2P	0.595
5	S1Y	0.649

Table 4.12 Continued From Previous Page

Standard	Strain code	Absorbance
6	S2T	0.665
7	Positive control	0.609
8	Negative control	0

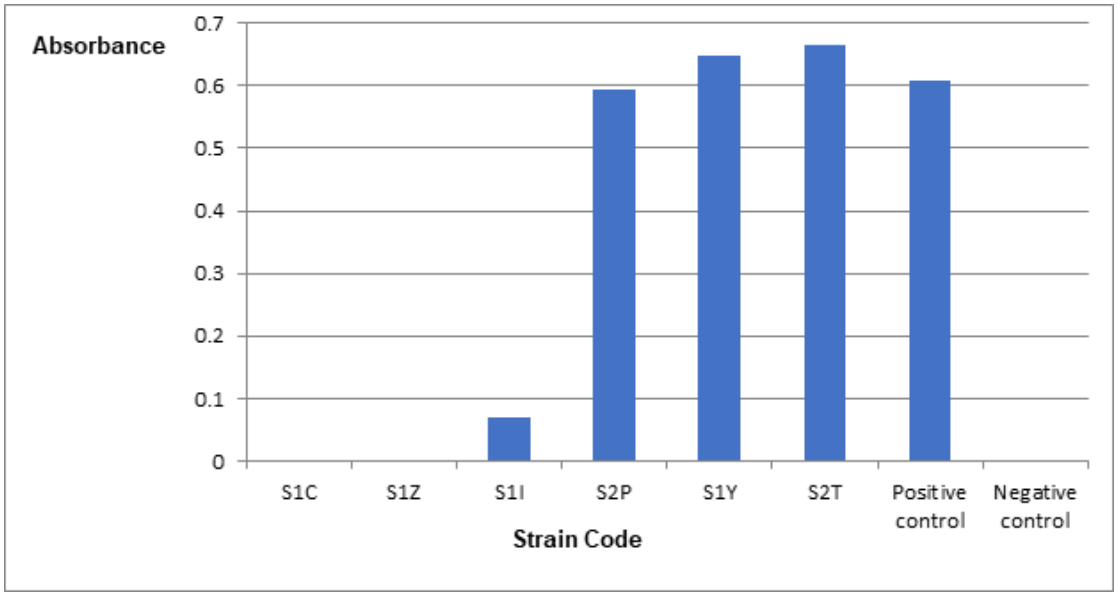


FIGURE 4.7: Growth of Bacterial Isolates In XYL Media Supplemented With 5% Crude Oil

The graph shows (Figure 4.7) the growth of isolates in XYL media with 5% crude oil concentration. All of the selected isolates had previously exhibited growth on XYL oil agar media till 5%. The negative control was XYL media having 5% of crude oil and no bacterial culture. The positive control on the other hand was XYL media having 5% of crude oil and *Bacillus megaterium*. The graph clearly shows that isolate S2T shows highest growth rate with highest absorbance value as 0.665 which means that this isolate showed highest biotransformation and have high growth of bacteria. S1Y shows second highest growth rate with absorbance value as 0.649. S2P shows third highest growth rate with absorbance value as 0.595. S1I shows absorbance value as 0.07 with least growth of bacteria. S1Z shows absorbance value as 0.003 while S1C shows absorbance value as 0.001. So these isolates are showing no growth of bacteria at this percentage. The negative

control showed no sign of growth since there was no bacterial culture added in it. The positive control showed absorbance value as 0.609.

TABLE 4.13: Absorbance Rate With 6% Crude Oil In XYL Broth

Standard	Strain code	Absorbance
1	S1I	0.08
2	S2T	0.85
3	S1Y	0.9
4	S2P	0.9
5	Positive control	0.709
6	Negative control	0

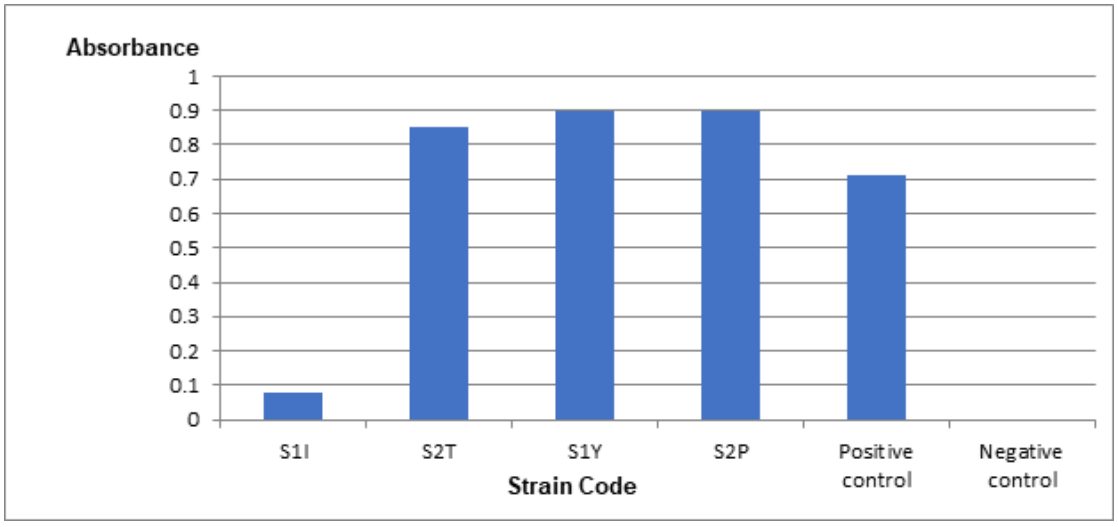


FIGURE 4.8: Growth of Bacterial Isolates In XYL Media Supplemented With 6% Crude Oil

The graph shows (Figure 4.8) the growth of isolates in XYL media with 6% crude oil concentration. All of the selected isolates had previously exhibited growth on XYL oil agar media till 6%. The negative control was XYL media having 6% of crude oil and no bacterial culture. The positive control on the other hand was XYL media having 6% of crude oil and *Bacillus megaterium*. The graph clearly shows that isolate S2P and isolate S1Y both shows highest growth rate with highest absorbance value as 0.9 which means that these isolates showed highest biotransformation and have high growth of bacteria. S2T shows second highest

growth rate with absorbance value as 0.85. S1I shows absorbance value as 0.08 with least growth of bacteria at this percentage. The negative control showed no sign of growth since there was no bacterial culture added in it. The positive control showed absorbance value as 0.709 which was increased as compared to previous percentage of oil.

TABLE 4.14: Absorbance Rate With 7% Crude Oil In XYL Broth

Standard	Strain code	Absorbance
1	S1I	0.09
2	S2T	0.9
3	S1Y	1
4	S2P	1.2
5	Positive control	0.8
6	Negative control	0

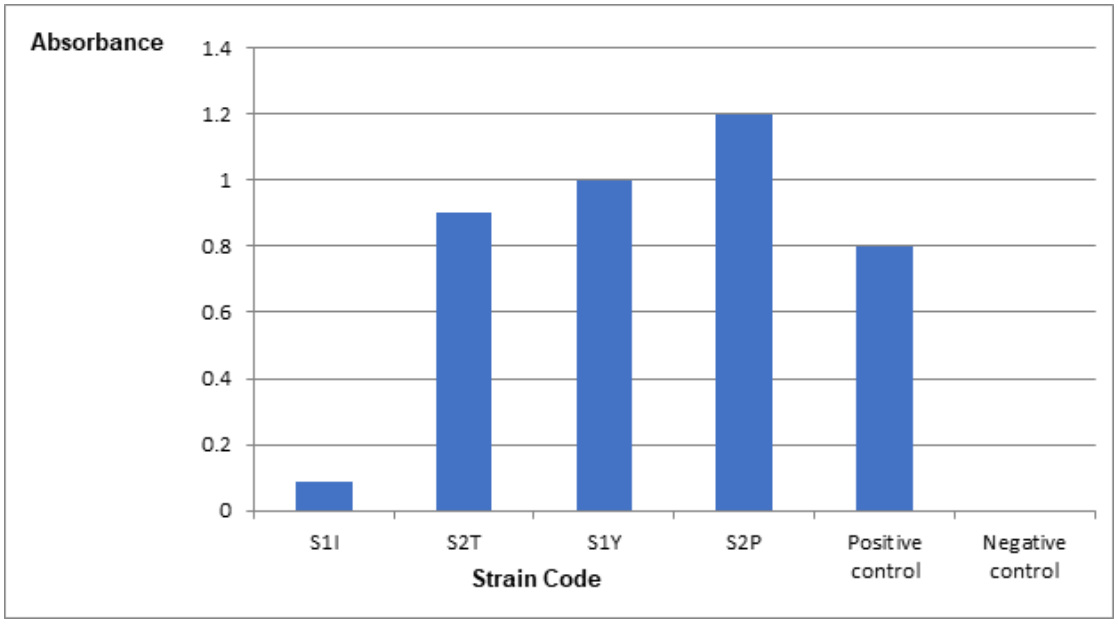


FIGURE 4.9: Growth of Bacterial Isolates In XYL Media Supplemented With 7% Crude Oil

The graph shows (Figure 4.9) the growth of isolates in XYL media with 7% crude oil concentration. All of the selected isolates had previously exhibited growth on XYL oil agar media till 7%. The negative control was XYL media having 7% of crude oil and no bacterial culture. The positive control on the other hand was

XYL media having 7% of crude oil and *Bacillus megaterium*. The graph clearly shows that isolate S2P shows highest growth rate with highest absorbance value as 1.2 which means that this isolates showed highest biotransformation and have high growth of bacteria. S1Y shows second highest growth rate with absorbance value as 1. S2T shows third highest growth rate with absorbance value as 0.9. S1I shows absorbance value as 0.09 with least growth of bacteria at this percentage. The negative control showed no sign of growth since there was no bacterial culture added in it. The positive control showed absorbance value as 0.8 which was increased as compared to previous percentage of crude oil.

TABLE 4.15: Absorbance Rate With 8% Crude Oil In XYL Broth

Standard	Strain code	Absorbance
1	S1I	0.1
2	S1Y	1.2
3	S2P	1.431
4	S2T	1.575
5	Positive control	0.9
6	Negative control	0

The graph shows (Figure 4.10) the growth of isolates in XYL media with 8% crude oil concentration. All of the selected isolates had previously exhibited growth on XYL oil agar media till 8%. The negative control was XYL media having 8% of crude oil and no bacterial culture. The positive control on the other hand was XYL media having 8% of crude oil and *Bacillus megaterium*. The graph clearly shows that isolate S2T shows highest growth rate with highest absorbance value as 1.5 which means that this isolates showed highest biotransformation and have high growth of bacteria. S2P shows second highest growth rate with absorbance value as 1.4. S1Y shows third highest growth rate with absorbance value as 1.2. S1I shows absorbance value as 0.1 with least growth of bacteria at this percentage which means there was no growth of bacteria. The negative control showed no sign of growth since there was no bacterial culture added in it. The positive control showed absorbance value as 0.9 which was increased as compared to previous

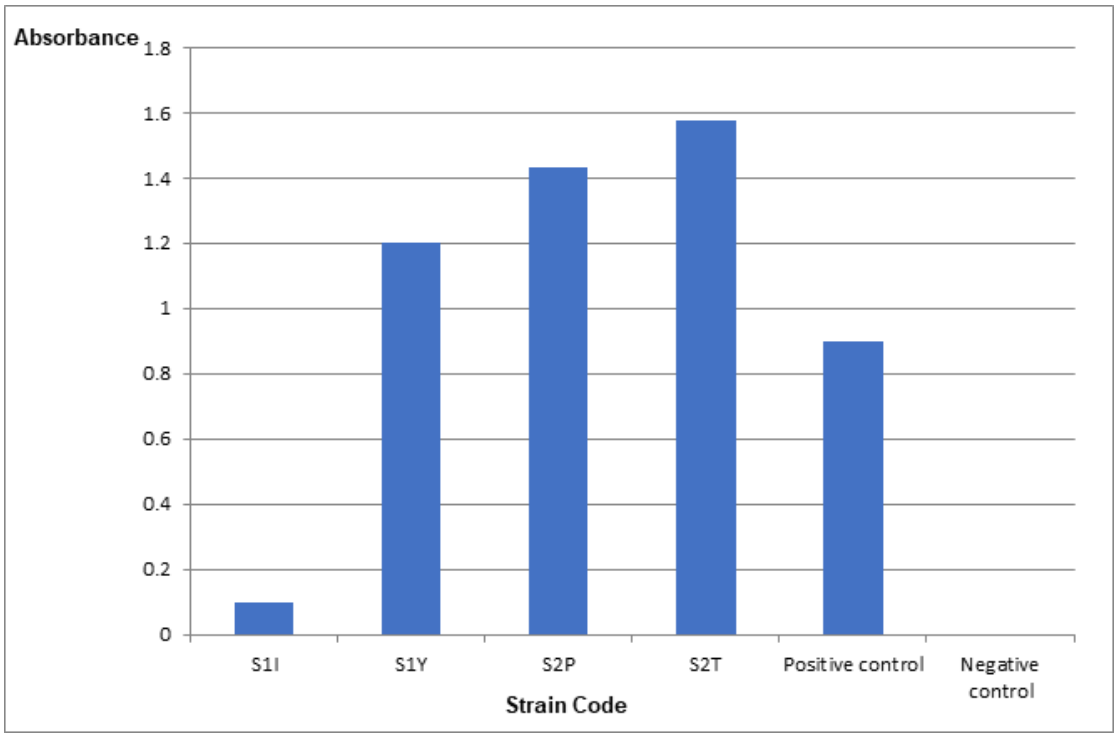


FIGURE 4.10: Growth of Bacterial Isolates In XYL Media Supplemented With 8% Crude Oil

percentage of crude oil. So all the isolates which mentioned above in graph in XYL media with highest absorbance value showed biotransformation to utilize crude oil as sole carbon source.

Heavy crude oil contaminated soil was used to isolate two bacterial strains *B.subtilis* AS2 and *B.licheniformis* AS5 which were able to biotransform effectively within aeorobic and anaerobic conditions. Glucose addition at aerobic conditions was found useful for the growth of bacteria [99]. *B.fimus* and *B.halodurans* have shown biotransformation ability by growing on high concentration of heavy crude oil and converting it in lighter ones by utilizing aromatic compounds. This study indicated that these isolates showed promise for MEOR [100]. It is reported that *Enterobacter cloacea* ERCPPI-1 bacterial strain produced biosurfactant and degraded crude oil by utilizing it as a sole source of carbon after 21 days of incubation with high oil spreading and emulsion properties [101]. *Garciaella petrolearia* TER1G02 has shown preference to reduce thickness of heavy oil by 37%-42% by utilizing asphalt and molasses as a sole carbon sources [102].

4.3 Metal Tolerance Analysis

The rate of biotransformation is greatly affected by heavy metals, these metals interact with the microbial enzymes or their cell walls, interfering with the microbial general metabolism or by interrupting the functioning of the enzymes participating in the degradation of hydrocarbons. The presence of toxic compou- nds is sensed by some bacteria and they produce proteins that either extrude the toxic compounds out the cell [103].

All of the 47 bacterial isolates were grown in (LB) broth that was amended with concentrations of Chromium sulphate salt ranging from 120mgl/L to 180mg/L. Chromium is a metal which exists in two states. It can be beneficial or harmful for living organisms. Cr(III) is a trace element which is essential for appropriate functioning of humans and animals. Its functionality also depends on its concentration. For example it is an important source of balanced human and animal diet at lower concentration while at increased concentration it can interfere with metabolic processes. As chromium is used in industries for several purposes so trivalent and hexavalent chromium disharges from these industries. Cr (III) and Cr(VI) are the most significant. Hexavalent chromium is more dangerous than triv- alent chromium. Along with this reality that, heavy metals are highly lethal to most microbes, there are metal-tolerant bacteria [104]. The graphs and table given below shows the growth rate of bacteria at different concentrations.

Table 4.16: Absorbance Results In Media Containing 0.12g/l Chromium Salt

Standard	Strain codes	Absorbance
1	S1K	0.143
2	S2G	0.147
3	S2I	0.157
4	S1E	0.165
5	S1Q	0.189
6	S2Q	0.191
7	S2C	0.192

Table 4.16 Continued From Previous Page

Standard	Strain codes	Absorbance
8	S2F	0.192
9	S1I	0.206
10	S1O	0.207
11	S20	0.211
12	S2A	0.216
13	S2K	0.223
14	S1M	0.24
15	S1C	0.243
16	S2M	0.27
17	S2U	0.325
18	S1U	0.332
19	S2J	0.38
20	S1A	0.388
21	S2H	0.389
22	S2R	0.403
23	S1J	0.404
24	S1Z	0.406
25	S1N	0.413
26	S1V	0.415
27	S2N	0.419
28	S1P	0.421
29	S1H	0.422
30	S2L	0.424
31	S1R	0.433
32	S1L	0.457
33	S2P	0.459
34	S1W	0.468
35	S1T	0.503

Table 4.16 Continued From Previous Page

Standard	Strain codes	Absorbance
36	S1D	0.513
37	S2E	0.522
38	S2D	0.528
39	S1B	0.538
40	S1Y	0.609
41	S1S	0.619
42	S1X	0.619
43	S2B	0.751
44	S2T	0.752
45	S1G	0.832
46	S2S	0.852
47	S1F	0.891

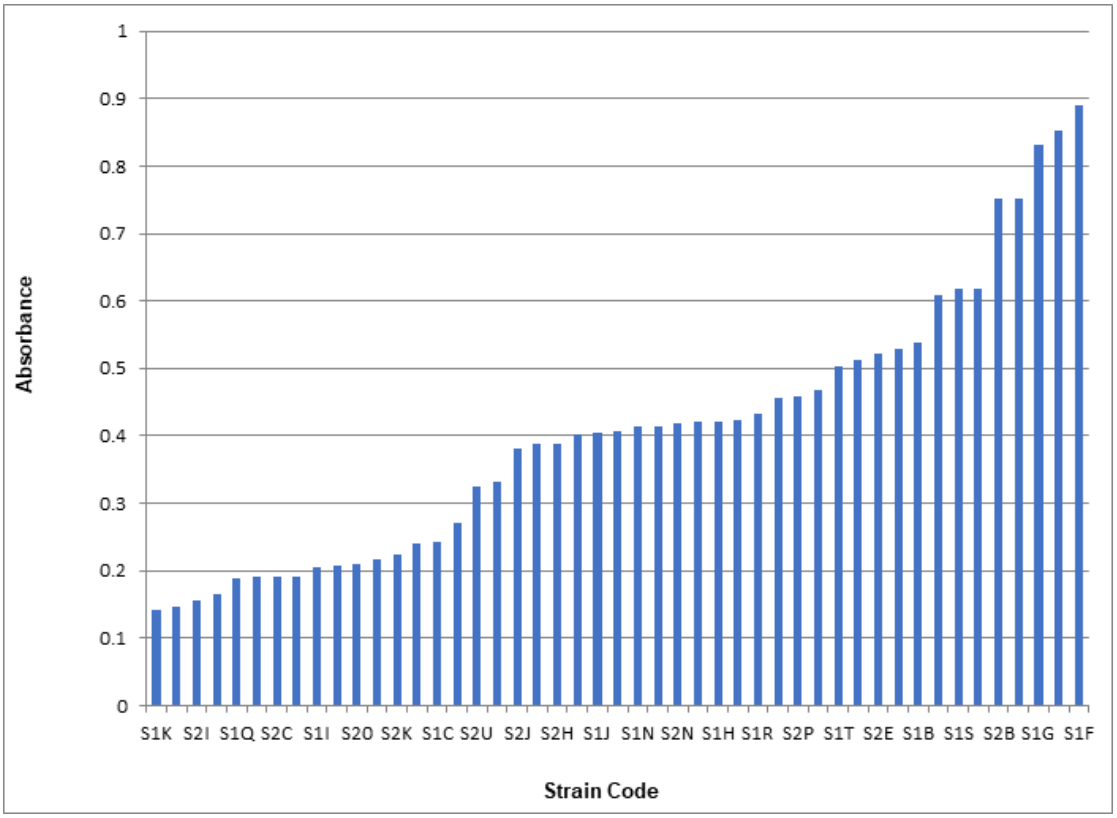


FIGURE 4.11: Growth of Isolates In Media Containing 0.12g/l Chromium Salt

TABLE 4.17: Absorbance Iesults In Media Containing 0.14g/l Chromium Salt

Standard	Strain codes	Absorbance
1	S1E	0
2	S1I	0.003
3	S1K	0.008
4	S1M	0.008
5	S1O	0.013
6	S2C	0.015
7	S1Q	0.016
8	S2A	0.016
9	S1J	0.016
10	S2F	0.017
11	S1U	0.018
12	S2I	0.075
13	S2G	0.075
14	S2Q	0.075
15	S1C	0.1
16	S1H	0.1
17	S2M	0.157
18	S2H	0.157
19	S1N	0.165
20	S2E	0.191
21	S2K	0.191
22	S2O	0.191
23	S1A	0.2
24	S2U	0.211
25	S2J	0.223
26	S1V	0.332
27	S2R	0.332
28	S1W	0.4

Table 4.17 Continued From Previous Page

Standard	Strain codes	Absorbance
29	S1Z	0.403
30	S2N	0.403
31	S1D	0.413
32	S1P	0.413
33	S1R	0.421
34	S2L	0.424
35	S1T	0.433
36	S1B	0.438
37	S1L	0.457
38	S2D	0.522
39	S1Y	0.528
40	S1X	0.602
41	S2B	0.619
42	S2T	0.619
43	S1S	0.619
44	S2P	0.659
45	S1G	0.832
46	S2S	0.852
47	S1F	0.872

TABLE 4.18: Absorbance Results In Media Containing 0.16g/l Chromium Salt

Standard	Strain codes	Absorbance
1	S1A	0
2	S2H	0.001
3	S2E	0.009
4	S1V	0.013
5	S1N	0.015
6	S20	0.075

Table 4.18 Continued From Previous Page

Standard	Strain codes	Absorbance
7	S2M	0.147
8	S2K	0.165
9	S2U	0.191
10	S2J	0.191
11	S2R	0.223
12	S1D	0.3
13	S1Z	0.332
14	S1B	0.338
15	S1P	0.338
16	S2N	0.4
17	S2D	0.403
18	S1R	0.413
19	S1T	0.421
20	S2L	0.424
21	S1W	0.426
22	S1L	0.457
23	S1Y	0.522
24	S1X	0.563
25	S2T	0.602
26	S2B	0.602
27	S1S	0.609
28	S2P	0.759
29	S1G	0.832
30	S2S	0.852
31	S1F	0.871

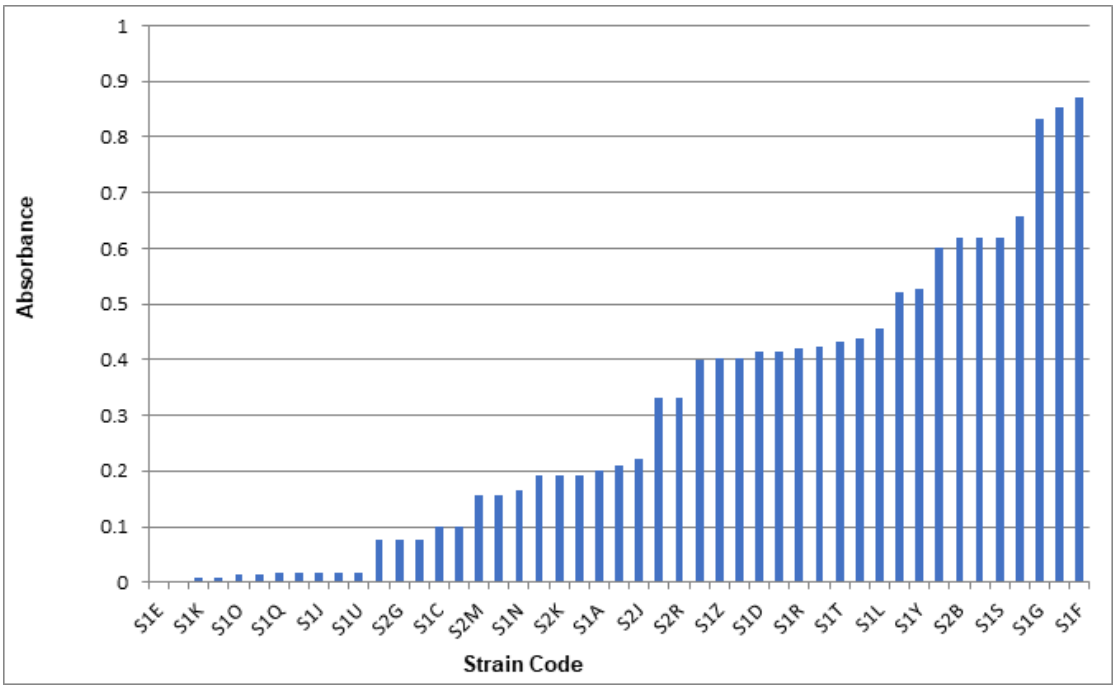


FIGURE 4.12: Growth of Isolates In Media Containing 0.14g/l Chromium Salt

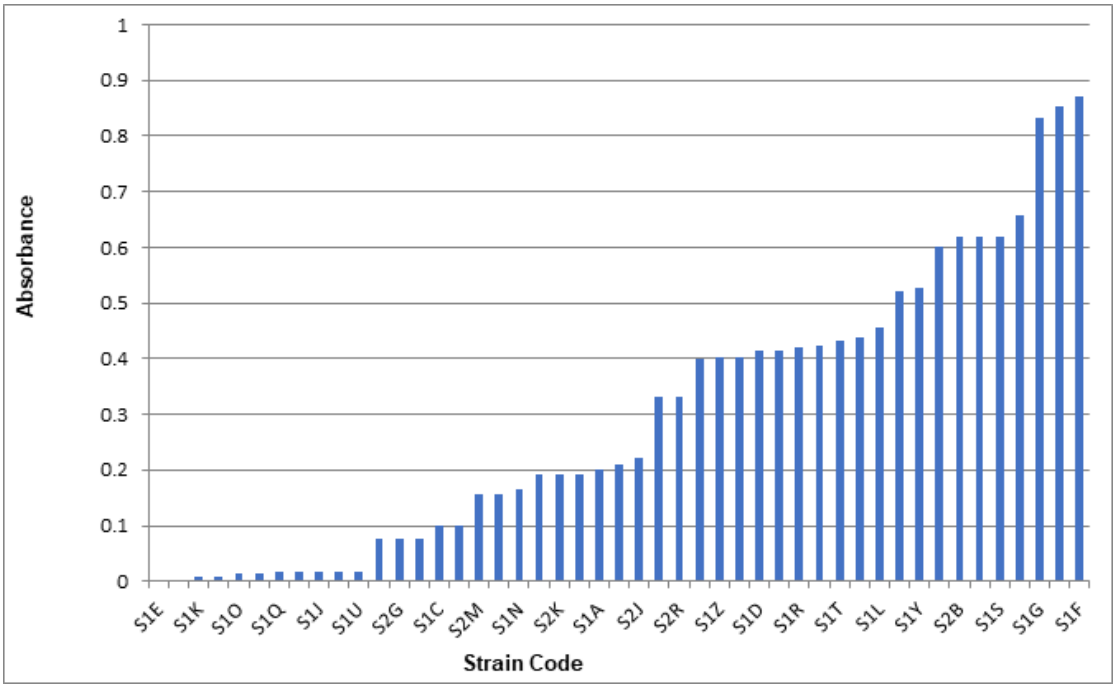


FIGURE 4.13: Growth of Isolates In Media Containing 0.16g/l Chromium Salt

TABLE 4.19: Absorbance Results In Media Containing 0.18g/l Chromium Salt

Standard	Strain Codes	Absorbance
1	S2M	0.143

Table 4.19 Continued From Previous Page

Standard	Strain Codes	Absorbance
2	S2U	0.147
3	S2K	0.157
4	S2J	0.165
5	S2R	0.191
6	S1D	0.2
7	S1B	0.288
8	S2N	0.325
9	S1P	0.325
10	S1Z	0.325
11	S1T	0.388
12	S2D	0.4
13	S1R	0.403
14	S1W	0.403
15	S2L	0.424
16	S1L	0.457
17	S1Y	0.503
18	S1X	0.528
19	S2T	0.601
20	S2B	0.602
21	S1S	0.619
22	S2P	0.759
23	S1G	0.832
24	S2S	0.852
25	S1F	0.87

A steady decline in growth of isolates was observed with the increase in concentration of chromium chloride salt, while a few isolates maintained their growth pattern throughout the study. All the isolates showed a steady decline a growth with the increase in concentration of chromium chloride salt. The isolate S1G

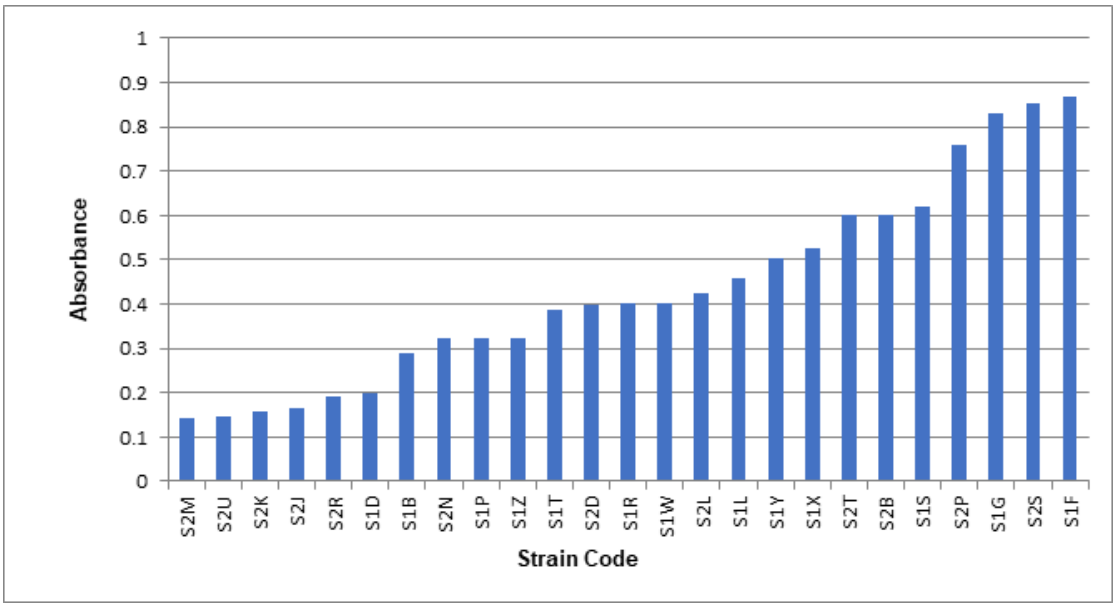


FIGURE 4.14: Growth of Isolates In Media Containing 0.18g/l Chromium Salt

maintained their growth throughtout the study with absorbance value as 0.832. It can be inferred that a few of the isolates that maintained their growth despite the changing concentration of chromium salt have adapted to grow in the presence of chromium salt. The isolate S1F maintained their growth on the last two concentrations of chromium salt and showed highest absorbance value as 0.87 to tolerate high amount of chromium salt.

The concentration of chromium chloride salt was responsible for effecting the growth of isolates as their metal tolerance. The results shows that at different concentrations of chromium salt. The isolates showed varied growth patterns, a few of them maintained their growth at every concentration, while a few showed a steady decrease in growth with increasing salt concentration. Whereas the remaining isolates stopped growing at a very high concentration of chromium salt. Overall it was observed that the increase in chromium salt concentration decreased the growth of isolates.

S2M, S2U, S2K, S2J, S2R, S1D, S1B, S2N, S1P, S1Z, S1T, S2D, S1R, S1W, S2L, S1L, S1Y, S1X, S2T, S2B, S1S, S2P, S1G, S2S, S1F were the isolates showed some growth till the high concentration of chromium salt which was 0.18g/l and from all these isolates S1F showed highest absorbance value as 0.87 which means this

isolate has the ability to tolerate chromium much more as compared to all other isolates.

It is reported that the Maximum Tolerable Concentration (MTC) of heavy metal was chosen as the highest concentration of heavy metals $CdCl_2$, $K_2Cr_2O_7$, $NiSO_4$ and $Co(NO_3)_2$ that allows growth after 2 days i.e., 48 hrs [105].

It was also concluded that *K. variicola* a inhabitant novel strain obsessed significant heavy metal tolerance and bioremediation potential against Ni and Co. It is useful bioremediation mediator to detoxify fabric effluents at industrialized environment [106]. The role of heavy metals MICs (minimal inhibitory concentrations) have been studied for various bacterial strains isolated from numerous habitats like water, soil, manure and sediments [107].

4.4 Fatty Acid Profile by Gas Chromatography

Table 4.20: Fatty Acid Profile of S1F

Fatty Acid	Percent
10:0 iso	0.04
10:00	0.41
10:0 3OH	2.33
12:0 iso	0.03
12:00	2.51
14:1 w5c	0.05
14:00	2.98
15:1 w6c	0.07
16:0 iso	1.15
Sum In Feature 3 (16:1 w7c/16:1 w6c)	35.99
16:1 w5c	0.13
16:00	22.87
17:0 cyclo	3.29

Table 4.20 Continued From Previous Page

Fatty Acid	Percent
17:00	0.26
16:0 3OH	0.04
18:0 iso	0.06
Sum In Feature 8 (18:1 w7c)	26.94
18:1 w5c	0.06
18:00	0.8
Summed Feature 3 (16:1 w6c/16:1 w7c)	35.99
Summed Feature 4.1 (18:1 w6c/ 18:1 w7c)	26.94

Fatty acid profile of S1-F shows that Sum in Feature 3 that was showing peaks for either 16:1 w7c or 16:1 w6c were present in highest concentration (35.99%) that was followd by Summed in Feature 8 (26.94%) which was presenting peaks for 18:1 w7c/18:1 w6c. Other fatty acids which were present in significant amount were anteiso16:0 (22.87%), cyclo 17:0 (3.29%), anteiso 14:0 (2.98%), anteiso 12:0 (2.51%) and anteiso 3OH 10:0 (2.33%). All others which were in less the 1% concentration were ignored (Table 4.20).

TABLE 4.21: Fatty Acid Profile of S1S

Fatty Acid	Percent
10:00	0.11
10:0 3OH	0.5
12:00	2.21
12:0 2OH	0.12
12:0 3OH	0.04
14:1 w5c	0.08
14:00	0.81
15:1 w6c	0.06
14:0 2OH	2.4
16:0 iso	0.56

Table 4.21 Continued From Previous Page

Fatty Acid	Percent
Sum in feature 3 (16:1 w7c/16:1 w6c)	39.03
16:1 w5c	0.22
16:00	21.61
17:0 cyclo	2.17
17:00	0.1
16:0 3OH	0.05
Sum In Feature 8 (18:1 w7c)	29
18:1 w5c	0.11
18:00	0.75
19:0 iso	0.06
Summed Feature 3 (16:1 w7c/16:1 w6c)	39.03
Summed Feature 8 (18:1w6c/18:1 w7c)	29

Fatty acid profile of S1-S shows that Sum in Feature 3 that was showing peaks for either 16:1 w7c or 16:1 w6c were present in highest concentration (39.03%) that was followd by Summed in Feature 8 (29.00%) which was presenting peaks for 18:1 w7c/18:1 w6c. Other fatty acids which were present in significant amount were anteiso 16:0 (21.61%), cyclo 17:0 (2.17%), anteiso 14:0 2OH (2.40%), anteiso 12:0 (2.21%). All others which were in less the 1% concentration were ignored (Table 4.21).

Table 4.22: Fatty Acid Profile of S1X

Fatty Acid	Percent
13:1 at 12-13	0.13
14:00	0.16
Sum in Feature 2 (14:0 3OH/16:1 iso I)	0.45
Sum in Feature 3 (16:1 w7c/16:1 w6c)	0.22
16:00	17.19

Table 4.22 Continued From Previous Page

Fatty Acid	Percent
17:00	0.74
16:0 2OH	0.42
Sum in Feature 8 (18:1 w7c)	17.06
18:00	17.2
18:1 w7c 11-methyl	0.83
19:0 cyclo w8c	43.08
18:0 2OH	0.23
18:0 3OH	0.69
20:2 w6,9c	0.49
20:1 w7c	0.39
20:00	0.72
Sum in Feature 2 (14:0 3OH/16:1 iso I)	0.45
Sum in Feature 3 (16:1 w6c/16:1 w7c)	0.22
Sum in Feature 8 (18:1 w6c/ 18:1w7c)	17.06
19:0 iso	0.04
Summed Feature 3 (16:1 w6c/16:1 w7c)	36.44
Summed Feature 8 (18:1 w6c/18:1 w6c)	28.59

Fatty acid profile of S1-X shows that anteiso 19:0 cyclo w8c that was showing peaks for w8c was present in highest concentration (43.08%) that was followd by anteiso 18:0 (17.20%). Anteiso 16:0 showing peaks (17.19%) which was followed Sum in Feature 8 (18:1 w6c/ 18:1 w7c) which was showing peaks for either 18:1 w6c and 18:1 w7c. All others which were in less the 1% concentration were ignored (Table 4.22).

Table 4.23: Fatty Acid Profile of S1Y

Fatty Acid	Percent
10:00	0.06
10:0 3OH	0.29

Table 4.23 Continued From Previous Page

Fatty Acid	Percent
12:0 iso	0.03
12:00	1.78
14:1 w5c	0.07
14:00	2.72
15:1 w6c	0.1
16:0 iso	1.15
Sum In Feature 3 (16:1 w7c/16:1 w6c)	36.44
16:1 w5c	0.14
16:00	25.12
17:1 w8c	0.09
17:0 cyclo	1.9
17:00	0.42
18:0 iso	0.05
18:1 w9c	0.07
Sum In Feature 8 (18:1 w7c)	28.59
18:1 w5c	0.07
18:00	0.87
19:0 iso	0.04
Summed Feature 3 (16:1 w6c/16:1 w7c)	36.44
Summed Feature 8 (18:1 w6c/18:1 w6c)	28.59

Fatty acid profile of S1-Y shows that Sum in Feature 3 that was showing peaks for either 16:1 w7c or 16:1 w6c were present in highest concentration (36.44%) that was followd by Summed in Feature 8 (28.59%) which was presenting peaks for 18:1 w7c/18:1 w6c. Other fatty acids which were present in significant amount were anteiso 16:0 (25.12%), cyclo 17:0 (1.90%), anteiso 14:0 (2.72%), anteiso 12:0 (1.78%) and 16:0 iso (1.15%). All others which were in less the 1% concentration were ignored (Table 4.23).

TABLE 4.24: Fatty Acid Profile of S2B

Fatty Acid	Percent
10:00	0.06
10:0 3OH	0.73
12:00	2.3
14:1 w5c	0.06
14:00	3.46
16:0 iso	0.12
Sum In Feature 3 (16:1 w7c/16:1 w6c)	28.38
16:1 w5c	0.23
16:00	26.73
17:1 w7c	0.12
17:0 cyclo	17.49
17:00	0.04
16:0 3OH	0.06
Sum In Feature 8 (18:1 w7c)	18.52
18:1 w5c	0.06
18:00	1.57
19:0 cyclo w8c	0.05
20:1 w7c	0.02
Summed Feature 3 (16:1 w7c/16:1 w6c)	28.38
Summed Feature 8 (18:1 w6c/18:1 w7c)	18.52

Fatty acid profile of S2-B shows that Sum in Feature 3 that was showing peaks for either 16:1 w7c or 16:1 w6c were present in highest concentration (28.38%) that was followd by anteiso 16:0 (26.73%). Other fatty acids which were present in significant amount were Summed in Feature 8 (18.52%) which was presenting peaks for either 18:1 w7c/18:1 w6c, cyclo 17:0 (17.49%), anteiso 14:0 (3.46%),

anteiso 12:0 (2.30%) and anteiso 18:0 (1.57%). All others which were in less the 1% concentration were ignored (Table 4.24).

TABLE 4.25: Fatty Acid Profile of S2P

Fatty Acid	Percent
10:0 3OH	1.81
12:00	2.03
12:0 2OH	0.14
14:00	0.89
14:0 2OH	2.71
16:0 iso	0.66
Sum In Feature 3 (16:1 w7c/16:1 w6c)	38.08
16:1 w5c	0.23
16:00	20.99
17:0 cyclo	3.71
Sum In Feature 8 (18:1 w7c)	28.09
18:00	0.68
Summed Feature 3 (16:1 w6c/16:1 w7c)	38.08
Summed Feature 8 (18:1 w6c/18:1 w7c)	28.09

Fatty acid profile of S2-P shows that Sum in Feature 3 that was showing peaks for either 16:1 w7c or 16:1 w6c were present in highest concentration (38.08%) that was followd by Summed in Feature 8 (28.09%) which was presenting peaks for 18:1 w7c/18:1 w6c. Other fatty acids which were present in significant amount were anteiso16:0 (20.99%), cyclo 17:0 (3.71%), anteiso 14:0 2 OH (2.71%), anteiso 12:0 (2.03%) and anteiso 3OH 10:0 (1.81%). All others which were in less the 1% concentration were ignored (Table 4.25).

TABLE 4.26: Fatty Acid Profile of S2S

Fatty Acid	Percent
10:0 3OH	0.39

Table 4.26 Continued From Previous Page

Fatty Acid	Percent
12:00	2.02
14:1 w5c	0.12
14:00	0.8
14:0 2OH	1.75
16:0 iso	0.55
Sum In Feature 3 (16:1 w7c/16:1 w6c)	39.78
16:00	21.84
17:0 cyclo	1.72
Sum In Feature 8 (18:1 w7c)	30.19
18:00	0.84
Summed Feature 3 (16:1 w6c/16:1 w7c)	39.78
Summed Feature 8 (18:1 w6c/18:1 w7c)	30.19

Fatty acid profile of S2-S shows that Sum in Feature 3 that was showing peaks for either 16:1 w7c or 16:1 w6c were present in highest concentration (39.78%) that was followd by Summed in Feature 8 (30.19%) which was presenting peaks for 18:1 w7c/18:1 w6c. Other fatty acids which were present in significant amount were anteiso 16:0 (21.84%), cyclo 17:0 (1.72%), anteiso 14:0 2OH (1.75%), anteiso 12:0 (2.02%). All others which were in less the 1% concentration were ignored (Table 4.26).

Table 4.27: Fatty Acid Profile of S2T

Fatty Acid	Percent
Sum In Feature 2	0.04
11:00	0.09
12:00	0.1
13:0 iso	0.26
13:0 anteiso	0.19
14:0 iso	3.41

Table 4.27 Continued From Previous Page

Fatty Acid	Percent
14:1 w5c	0.05
14:00	0.54
13:0 iso 3OH	0.06
13:0 2OH	0.11
15:0 iso	7.95
15:0 anteiso	38.23
15:1 w6c	0.07
14:0 iso 3OH	0.42
16:1 w7c alcohol	0.52
16:1 iso H	0.8
16:0 iso	12.55
16:0 anteiso	0.37
16:1 w11c	0.3
Sum In Feature 3 (16:1 w6c/16:1 w7c)	0.87
16:00	3.28
15:0 iso 3OH	0.29
15:0 2OH	1.07
17:1 iso w10c	0.23
Sum In Feature 9 (16:0 10-methyl)	0.39
Sum In Feature 4 (17:1 iso I/anteiso B)	7.86
17:0 iso	3.66
17:0 anteiso	13.73
17:1 w8c	0.04
17:1 w6c	1.13
17:00	0.18
16:0 iso 3OH	0.11
18:0 iso	0.25
Sum In Feature 5 (18:2 w6, 9c/18:0 ante)	0.19

Table 4.27 Continued From Previous Page

Fatty Acid	Percent
18:1 w9c	0.14
17:0 iso 3OH	0.09
17:0 2OH	0.2
19:0 anteiso	0.1
20:00	0.15
Summed Feature 2 (14:0 3OH/16:1 iso I)	0.04
Summed Feature 3 (16:1 w6c/16:1 w7c)	0.87
Summed Feature 4 (17:1 anteiso B/iso I)	7.86
Summed Feature 5 (18:0 ante/18:2 w6,9c)	0.19
Summed Feature 9 (16:0 10-methyl/17:1 iso w9c)	0.39

Fatty acid profile of S2-T shows that 15:0 anteiso that was showing peak was present in highest concentration (38.23%) that was followd by 17:0 anteiso (13.73%). Other fatty acids which were present in significant amount were 16:0 iso (12.55%), Summed Feature 4 (17:1 showing peaks for either iso I/anteiso B (7.86%), iso 17:0 (3.66%), anteiso 17:1 w6c (1.13%) , anteiso 15:0 2OH (1.07%), iso 15:0 (7.95%), iso 14:0 (3.41%). All others which were in less than 1% concentration were ignored (Table 4.27).

Above mentioned fatty acids of bacterial strains have increased surfactant activity of bacteria to biotransform them. Fatty acid profile of S1-X shows that anteiso 19:0 cyclo w8c was present in highest concentration (43.08%) in all eight strains.

In addition to plants and animals, microorganisms are also produced biosurfactants that are fit for bringing down surface or potentially interfacial pressure by apportioning at the water-air and water-oil interfaces [108]. They can have an assortment of structures, including unsaturated fats, impartial lipids, phospholipids, glycolipids, and lipopeptides. Biosurfactants help in the tertiary phase of oil recuperation from low-creation oil supplies by discharging oil trapped by capillary pressure. The movement of biosurfactants relies upon their basic parts, e.g.,

the sorts of hydrophilic and hydrophobic gatherings and their spatial direction [109]. An enormous group of research has been done on surf- actants produced by microorganisms, yeast, and fungi. Microbial surfactants have an incredibly wide scope of compound structures and each seems to assume various roles in the life cycle of producing microorganisms [110]. Biosurfactants of microorganisms regulate their cell surface features by attaching or detaching from surfaces according to require. Decomposition of materials is restrained at the point when organic particles are bound irreversibly to surfaces [111]. Bound substrates are desorbed from surfaces or their water solubility is increased by biosurfactants to improve the growth [112]. Improving oil recovery by bio- surfactant production is cost effective approach. During a study eight lipopeptide biosurfactants were isolated with different specific activities by a variety of Bacillus species. These eight lipopeptides were the same in their amino acid composition (Glu/Gln: Asp/Asn: Val:Leu, 1:1:1:4), but they were differed in the fatty acid composition. Specific biosurfactant activity depended on the ratios of both iso to normal even-numbered fatty acids and anteiso to iso odd-numbered fatty acids revealed by multiple regression analysis. The fatty acid portions of the biosurfactants contained 3-hydroxy tridecanoate (3-OH-C_{13}), tetradecanoate (3-OH-C_{14}), pentadecanoate (3-OH-C_{15}), and hexadecanoate (3-OH-C_{16}). The 3-OH-C_{13} and 3-OH-C_{15} fatty acids were present as mixtures of iso and anteiso isomers, while 3-OH-C_{14} was comprised of normal and iso isomers. The 3-OH-C_{16} fatty acid contained only the normal isomer. In some cases, the 3-OH-C_{14} and 3-OH-C_{15} fatty acids together constituted the majority of the fatty acids of the lipopeptide. However, in other cases, 3-OH-C_{14} alone was the major fatty acid isomer [113]. Yakimov et al. found that an increase in the percentage of branched-chain fatty acids in lichenysin A BAS50 of *Bacillus licheniformis* strain decreased the surface activity and that an increase in the percentage of straight-chain 3-hydroxytetradecanoate (n-3OH-C_{14}) increased the surface activity which proves that fatty acid composition also affects its activity [114].

Chapter 5

Conclusions and Recommendations

In this study soil samples of OGDL oil refinery were used to screen bacteria for their surfactant activity. Four samples were used for the isolation of bacterial strains. Total 47 isolates were purified after experimentation. These isolates were further screened out to check their biotransformation ability. For this analysis crude oil was added from 5% to 8% onwards until unless cells stop showing any signs of growth. Growth curves were drawn for absorbance to observe increase in growth and biotransformation of strains was determined by observing change in absorbance for 2 days. The crude oil utilizing ability of bacteria was evaluated by growth of the isolates in oil agar media. Total 21 isolates out of 47 showed growth in crude oil agar media. The isolates that showed growth on concentrations of crude oil ranging from 5% to 8% were selected and analyzed for their crude oil utilizing ability. Spectrophotometer was used to analyze the capability of bacteria. Eight bacterial strains S1F, S1S, S2S, S1X, S2B, S2P, S2T and S1Y had shown ability to biotransform with high absorbance value after utilizing crude oil as a sole carbon source.

Metal tolerance of the bacterial strains was estimated against CrSO4.7H20 from 120mg/L to onwards 180mg/L. Measurement of optical density at wavelenghth

600 nm by a spectrophotometer was used for determining the growth of bacteria after 24 hours. Few strains had shown ability to tolerate metal from which S1F showed greater ability of tolerance with 0.87 absorbance value. GC-MS of bacterial strains has shown fatty acid profile which were reported to be involved in enhanced surfactant activity for biotransformation. The fatty acid analysis of the isolates possibly predicts the presence of phopholipid biosurfactants. They increase the yield of petroleum from the depleted reservoir, by reducing the capillary forces that may hinder the movement of oil through rock pores and by reducing the interfacial tension. They also help in enhancing the formation of stable oil-water emulsions and breaking down the oil film present in the rocks that are vital for the maximum extraction of oil and extends the lifetime of the reservoir.

Microbial Enhanced Oil Recovery (MEOR) is a useful process to increase oil recovery from a reservoir. Microorganisms can raise the fluidity and decrease the capillary forces which retain the oil into the reservoir. Low energy requirement and independence of the price of crude oil are advantages of MEOR over conventional EOR. Glycolipids, Lipopeptides, Lipoproteins and Polymeric Biosurfactants can also be explored in future.

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