To Evaluate the Potential of Isolated Lipolytic Bacterial Strains for Bioremediation of Vegetable Oil Industry Wastewater and for Biodiesel Production

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Dedication

This thesis is dedicated to all the torch bearers and seekers of knowledge who are consistently and continuously trying to breach the barriers of unknown for the betterment of this world.

Declaration

The material and information contained in this thesis is my original work. I have not previously presented any part of this work elsewhere for any other degree.

Qurrat Ul Ain Rana

Certificate

This thesis submitted by ***Qurrat Ul Ain Rana*** is accepted in its present form by the Department of Microbiology, Quaid-i-Azam University, and Islamabad, Pakistan; as satisfying the thesis requirements for the degree of Master of Philosophy in Microbiology.

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List of Acronyms

° C	Degree Celsius
µl	Microlitre
APHA	American public Health Association
BOD	Biochemical Oxygen Demand
CFU	Colony Forming Units
COD	Chemical Oxygen Demand
CTAB	Cetyltrimethylammonium Bromide
$CuSO_4$	Copper Sulfate
DEE	Diesel Engine Exhaust
EDTA	Ethylenediaminetetraacetic acid
FAME	FattyAcid Methyl Esters
FFA	Free Fatty Acid
FOGs	Fats, Oils and Grease
FTIR	Fourier Transform Infrared Spectrometer
GAC	Granular Activated Carbon
GHG	Green House Gas
H_2SO_4	Sulfuric Acid
HCL	Hydrochloric Acid
IEA	International Energy Agency
IR	Infrared
$(NH_4)_2SO_4$	Ammonium Sulfate
K_2HPO_4	Dipotassium Hydrogen Phosphate
KH_2PO_4	Potassium Dihydrogen Phosphate
LB	Lauria-Bertani
M	Mo;ar
mg	Milligram
$MgSO_4$	Magnesium Sulfate
ml	Milliliter
MRVP	Methyl Red Vogues Proskaeur
Na_2SO_4	Sodium Sulfate
NaCl	Sodium Chloride
NaK-Tartrate	Sodium Potassium Tartrate
NaOH	Sodium Hydroxide
nm	Nanometre
OD	Optical Density
PAC	Polyaromatic Hydrocarbon
PNP	Para-nitro Phenol
PNPL	Para-nitro Phenyl Laurate
RPM	Revolution Per minute
TE	Tris-EDTA
TS	Total Solids

TSI	Triple Sugar Iron
TSS	Total Suspended Solids
UV	Ultra Violet
VSS	Volatile Suspended Solid
ZnSe	Zinc Selenide

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ABSTRACT

Bioremediation and biodiesel production are two of the best solutions to eliminate oil pollution in naturally occurring waterbodies and to overcome energy crisis respectively. In this study the application feasibility of lipase producing bacterial strains was evaluated for simultaneous bioremediation of oil contaminated wastewater and biodiesel production from non-edible oil. Efficient lipase producing bacteria were isolated from the wastewater of a local vegetable oil industry. These strains were then investigated for their potential to remediate (COD reduction) oil containing wastewater. These isolated strains were then further evaluated for their potential to synthesize biodiesel from mustard, soybean, jatropha and taramira oils utilizing methanol for the transesterification of oils. Selected strain was then identified using 16s rRNA sequencing to be *Bacillus subtilis* strain Q1 and optimized for biodiesel production from non-edible taramira oil. Finally, simultaneous bioremediation of vegetable oil contaminated wastewater and microbial production of biodiesel from non-edible taramira oil was carried out using efficient lipase producer *Bacillus subtilis* strain Q1.

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ABSTRACT

Vegetable oil is an essential food commodity which is used in every single household. The use of vegetable oil by human beings dates back to antiquity. Physical and chemical properties of vegetable oils render them useful in cooking processes. With the increase in population the demand for this important food item is increasing day by day. This increase in demand of vegetable oils has led to intensification of vegetable oil industries all around the globe. According to world's statistics portal, world's vegetable oil production amounted to about 179 million metric tons in 2015/2016.

The major risk posed by these vegetable oil industries to environment is release of their untreated oil contaminated wastewater in fresh water streams, lakes or other aquatic bodies. The effluent from these industries contains large quantities of organic matter in the form of total suspended solids (TSS), volatile suspended solids (VSS), total solids (TS), fats, oil and grease (FOGs) that increase biochemical oxygen demand (BOD) and chemical oxygen demand (COD) of water in which they are spilled. Once in aquatic environment they cause odor and fouling of water. Moreover, the oils present in this wastewater forms film over the top of water thus decreasing its dissolved oxygen content which leads to fatal circumstances for both aquatic animals and plants. Once in sewer pipelines fats, oils and grease present in wastewater clogs pipes leading to blockage and sewers outbursts (Kacheing'a and Momba, 2015).

Physicochemical treatment methods are applied to treat wastewater emerging out from vegetable oil industries but these methods are not only energy and cost intensive but also instead of completely eradicating oil contaminant, concentrate it and this concentrated FOG still needs treatment (Paraskeva and Diamadopoulos, 2006).

Alternative to physicochemical treatment of wastewater released by vegetable oil industries is biological treatment. Bioremediation is the process where microorganisms or their enzymes are applied to biologically degrade the contaminant in question. Depending upon the type of microflora employed for bioremediation process, bioremediation can be in situ or ex situ. Biological process holds a lot of advantages over physicochemical process as it is naturally occurring, cost and energy efficient, have high feasibility percentage and it completely degrades the pollutant instead of concentrating it (Chiacchierini et al., 2004).

The enzymes that play a vital role in degrading fats, oil and grease contaminating water are lipases which are ubiquitously produced by animals, plants and microorganisms. However, microbial lipases are the ones which are applied in bioremediation studies. Microorganisms produce these enzymes when they come in environment containing fats or oils as carbon source.

Pakistan, being an agriculture country, plants crops with seeds containing high amount of oils. Numerous industries are present in the country which extracts vegetable oils from these oil containing plant seeds. A lot of revenue is generated every year by exporting these vegetable oils. In Pakistan, most of the vegetable oil producing industries relies on the process of filtration to treat their wastewater but filtration does not completely remove FOGs. Thus an efficient system needs to be acquired by these industries for complete removal of FOGs and bioremediation is one of the best option.

Apart from environmental pollution caused by vegetable oil industries, another crisis currently faced by the world is depletion of fossil fuels. In order to maintain high living standards and economic growth, energy is a crucial factor. An estimated 50% increased energy demand by 2030 than today is reported by International Energy Agency (IEA). Transportation sector has undergone an exponential growth in the past 30 years due to increase in automobiles, not only in developed countries but also in developing countries. According to U.S. Energy Information Administration 1.8% increase in global transportation energy is expected per year from 2005 – 2035. Oil and gas are the energy sources required by transportation factor but if the present trend in their utilization was continued they will exhaust in next 41 to 63 years (Statistical Review of World Energy). In Pakistan alone, the total 48% of petroleum consumption is by transportation section and 40% by power section (Shahid and Jamal, 2011). This situation points out to finding a new fuel source for transport sector. Biodiesel seems to be a promising option.

Mono alkyl esters of long chain fatty acids derived from vegetable or animal fat oils are termed as biodiesel. The prefix bio emphasizes its biological origin. As biodiesel is produced from vegetable oils so, unlike fossil fuels, biodiesel is renewable source of

energy with no risk of its depletion. Apart from being non-renewable fossil fuels are also related to many environmental problems. The diesel engine exhaust DEE of automobiles that run on fossil fuels is reported to be carcinogenic specifically lined to lung cancer (Bünger et al., 1998). GHG emission of petro-diesel is quite high and is directly linked with global climatic shifts and global warming. As compared to petro diesel, biodiesel shows remarkably low GHG emission. Its combustion alone gives about 75-90% reduction in emission of poly aromatic hydrocarbon and a total 90% reduction in unburnt hydrocarbon. Moreover, biodiesel is nontoxic and biodegradable with 4 times faster rate of degradation than petro diesel (Demirbas, 2007). Due to higher flash point of biodiesel its storage and transportation is safe. With high combustion efficiency biodiesel can be run on conventional diesel engines without extensive modification. Vegetable oils can be converted into biodiesel through three methods namely micro emulsions, thermal cracking (pyrolysis) and transesterification (alcoholysis). Out of these three transesterification reaction is most widely employed. Transesterification can be carried out chemically or biologically. Chemical transesterification employees an acid or a base as a catalyst to convert fatty acids and alcohol into their corresponding alkyl esters. Biological transesterification reaction utilizes whole cells or enzymes for the purpose of conversion. The enzymes used to carry out transesterification reactions are lipases which in absence of water and presence of an alcohol carry out transesterification reaction instead of hydrolysis. Biological synthesis of biodiesel is advantageous over chemical synthesis as chemical synthesis of biodiesel is energy intensive, generates acid and alkali containing wastewater which requires treatment, has low selectivity which leads to unwanted side reactions, leads to soap formation and makes it difficult to remove glycerol (Noureddini et al., 2005).

The feedstock for biodiesel production is both edible and non-edible oils but maximum production of biodiesel overall the world is through edible oils. For the crops to be suitable for biodiesel production must have high yield, their seeds must have high oil content and their oils have fatty acids composition which yields biodiesel whose properties are comparable to or better than petro diesel (Gui et al., 2008).

As world's 95% biodiesel is generated from edible oil which means we are converting food resource into energy resource. This will not only lead to imbalance in food supply and demand but also deforestation and destruction of ecosystem to attain land for planting of crops which yield oil as a feedstock for biodiesel production. To eradicate this upsetting situation researchers are proposing alternative oil sources also known as greener oil resources which are actually non edible oils for biodiesel production. These non-edible oil producing crops are usually desiccation resistant and can be grown on lands which do not support growth of edible crops.

In this study oil containing wastewater sample was collected from a local Pakistani vegetable oil producing industry. Bacterial strains which were efficient in lipase production were then isolated from this oil containing wastewater. The isolated bacterial lipolytic strains were enriched and subjected to bioremediation of the same wastewater. In addition to this, these lipolytic isolates were enriched and after harvesting their cells, these strains were also tested for biological biodiesel production. Four oils namely mustard, soybean, jatropha and taramira oil were selected as feedstocks for biodiesel production. Out of these oils mustard and soybean oil were edible oils whereas jatropha and taramira oils are non-edible. The isolated bacterial strain which had highest potential of COD reduction during bioremediation process was selected for simultaneous bioremediation and biodiesel production. Bioremediation process not only reduced COD but oil present in wastewater also served as an excellent substrate for growth of lipase producing bacteria. Non-edible taramira oil was selected for simultaneous reaction. The parameters for biodiesel production were first optimized for the selected strain. Then after carrying out the bioremediation reaction through continuous culture with the selected strain, cells were harvested from the bioremediation reaction and utilized for biodiesel production with taramira oil at optimized conditions. Biological synthesis of biodiesel from taramira oil and mustard oil obtained from *Brassica campestris* and simultaneous bioremediation and biodiesel production by transesterification of non-edible taramira oil for biodiesel production have not been reported up till now.

Aim of The Study:

To investigate the potential of indigenous lipase producing bacterial strains isolated from vegetable oil industry wastewater, for the bioremediation of water contaminated with oil and for biodiesel production from both edible and non-edible oils.

Objectives:

- To isolate efficient lipase producing bacteria from vegetable oil industry wastewater.
- To employ these isolated lipolytic bacterial strains for bioremediation of vegetable oil industry wastewater.
- To biologically synthesize biodiesel from edible and non-edible oils using these isolated lipolytic bacterial isolates
- To simultaneously bio remediate vegetable oil industry wastewater and produce biodiesel from non-edible oil using an isolated lipolytic bacterial strain

2.1 Bioremediation

2.1.1 Vegetable Oils:

Oils that are obtained from different oil containing vegetables, fruits and nuts are known as vegetable oils. Oils from these oil containing sources are obtained via seed pressing, solvent extraction or via combination of both these methods in vegetable oil industries and refineries. The crude oils extracted through these methods are further subjected to several refining processes which render these oils edible. The most popular vegetable oils include mustard oil, canola oil, soya bean oil, olive oil, sunflower oil, coconut oil and peanut oil. Depending upon the saturation level of these oils they are either liquid or solid at room temperature. Higher saturation state yields solid state at room temperature (Aluyor et al., 2009).

2.1.2 Vegetable Oil Industry Wastewater:

With the increase in demand of food there has been observed an increase in the demand of edible oils in past few decades. This increase is followed by an increase in vegetable oil industries. These oil industries process vegetable oil to make it suitable for human consumption. These industries utilize the processes of extraction, refining and transportation of vegetable oils to bring their product in market. During all these processes they generate effluents which contain fats, oil and grease. This fats, oil and grease containing wastewater is considered to be a hazardous pollutant especially if it gets injected into aquatic environment. These industries are responsible for polluting environment by means of oily effluent discharge directly into fresh water bodies, oil spills and discharging untreated sludge containing fats, oil and grease directly into environment (Jameel et al., 2011).

2.1.3 Fats, Oils and Grease:

Fats, oils and grease are the fundamental constituents of wastewater emerging out from any vegetable oil industry and they are responsible for generating environmental pollution associated with these industries. In terms of wastewater fats, oil and grease are considered as a group of related materials rather than a specific chemical compound

which can be extracted by organic solvents like hexane and methanol. They are non-polar and hydrophobic in nature (Travis et al., 2008).

2.1.4 Environmental Impact of Fats, Oil and Grease:

The wastewater from vegetable oil industries containing fats, oil and grease when released into freshwater reservoirs, streams or lakes, results into formation of oil in water emulsions or formation of oil films on the surface of water. These oil films and oil water emulsions inhibits oxygen present in air to diffuse into water leading to decrease in amount of dissolved oxygen in water. This decrease in dissolved oxygen adversely affects the aquatic ecosystem. Low oxygen level in water results in death of aquatic animals including fish. Aquatic plants also suffer effects due to decreased oxygen level in water (Kacheing'a and Momba, 2015). Moreover, fats, oils and grease present in the wastewater streams also clogs the sewer pipelines which effectively reduce the diameter of pipes and can block the pipes completely which leads to sewer overflows (Williams et al., 2012).

2.1.5 Physicochemical Treatment Methods for Vegetable Oil Industry Wastewater and their Demerits:

A number of physicochemical treatment methods are available for the treatment of wastewater generated by vegetable oil industries. Application of simple physical methods like dilution, filtration, sedimentation, centrifugation and evaporation have been tried but all these methods are unable to completely remove fats, oil and grease from wastewater. All these methods can concentrate the contaminants but these concentrated contaminants still need further treatment. Adsorption technologies have also been employed. Most commonly used adsorption methods are adsorption on activated clay and adsorption on granular activated carbon (GAC). However, these methods are expensive, require highly upgraded and maintained facilities and also entails the possible risk of emitting the contaminants back into environment (Paraskeva and Diamadopoulos, 2006).

2.1.6 Bioremediation:

Bioremediation is the often used biological method for the treatment of vegetable oil industry wastewater. It is the process by which microorganisms break down the contaminant into its simpler non-hazardous constituents by means of producing contaminant degrading enzymes. The term bioremediation is often used with respect to ecology, waste management and environmental radiation. Biological agents that can degrade contaminants can either do it aerobically or anaerobically. Biomineralisation is another term associated with bioremediation which implies to the phenomenon where biological agents convert the organic matter into its constituent minerals. Thus, by definition bioremediation is the process by which organisms chemically transform the hazardous compounds present in environment into their non-toxic forms (Díaz, 2008).

2.1.7 Advantages of Bioremediation over Physicochemical Treatments:

Generally, bioremediation includes all those processes and actions that are required to biotransform an environment, which is altered by pollutants, into its original state. In order to achieve anticipated results, the processes that are used may vary but principle remains same i.e., the use of microorganisms or enzyme to degrade the pollutant. The process of bioremediation holds several advantages over physicochemical treatment methods as:

- It employs the already occurring biological process in nature
- It either completely degrades the pollutant or immobilizes it rather than transferring it from one to another environmental medium
- It is inexpensive and financially feasible as it requires less clean up time and expenditures than other treatment methods
- It can often be done at the site of contamination without transporting pollutants to another site which poses a serious threat to human health and environment (Chiacchierini et al., 2004).

2.1.8 Types of Bioremediation:

Based upon the site where the process is being carried out bioremediation is divided into two types:

1. **In situ Bioremediation**
2. **Ex situ Bioremediation**

In situ Bioremediation:

As the name indicates in situ bioremediation is the process which is carried out at the site of contamination. There is no need for the transportation of the polluted soil or water to another place for carrying out biodegradation. Hence, it is a safer method with no risk of transferring contaminant from one environmental medium to another. In situ bioremediation can be done for both contaminated soil and water. In this process indigenous microorganisms of the contaminated environment are provided with inorganic nutrients and oxygen to enhance their growth and they utilize the contaminant as carbon source thus degrading it to non-hazardous compounds.

Types of in situ Bioremediation:

Intrinsic bioremediation or Biostimulation: This process involves microorganisms which are already present at the site of contamination known as indigenous microorganisms. The metabolic growth of these indigenous microorganisms is enhanced by providing them with nutrients and higher oxygen supply and they take up the pollutant as organic carbon source thus degrading it.

Engineered in situ Bioremediation: This approach involves the engineering of environmental conditions present at the site of contamination. When the site conditions are not favorable to support microbial growth they are engineered to make them positive for growth of microorganisms by adjusting their pH, nutrient content and oxygen level and then exogenous microorganisms are inoculated at the site for degradation of contaminant.

Biosparging: Biosparging involves the injection of pressurized air below the soil into ground water where it accelerates biodegradation by enhancing the growth of naturally occurring bacteria in the contaminated environment.

Bioventing: This treatment method involves the injection of air and nutrient by means of well into the contaminated soil which enhances the growth of indigenous microorganisms thus rapidly degrading the pollutant. This type of in situ remediation works well for soil contaminated deep under surface.

Bioaugmentation: This treatment method utilizes exogenous microorganisms which are not native to the polluted site for biodegradation. However, this method has low success rates as most of the times exogenous microorganisms are unable to compete with indigenous microflora of the site.

Ex situ Bioremediation:

Ex situ bioremediation approach requires the contaminated soil to be excavated from the site or contaminated water to be transported from the site to another site where bioremediation can be carried out. Thus, this method of bioremediation has more disadvantages than advantages. It entails the hazard of transferring contaminant from one environmental medium to another.

Types of ex situ Bioremediation:

1. **Solid Phase System**
2. **Slurry Phase System**

Solid Phase System: It is used for the treatment of contaminated soil and includes organic wastes like agricultural waste, sewerage waste and municipal waste. Solid phase system includes the following processes:

1. Land Farming: In this process the contaminated soil is excavated and then spread over a prepared bed. The spread soil is periodically tilled to increase aeration. The basic idea is to provide indigenous microbes with nutrients and oxygen to stimulate their degradative tendency.

2. Composting: The contaminated soil is mixed with non-hazardous organic components like agricultural waste to enhance microbial growth which also causes an increase in temperature which kills pathogens if any, present in the contaminated soil.
3. Biopiles: this technique is actually a hybrid of both land farming and composting. Especially engineered cells are utilized as aerated compost piles. This treatment method is used for remediation of relatively resistant pollutants like hydrocarbons.

Slurry Phase System:

It is the most rapid treatment method out of all other remediation methods. Contaminated soil is mixed with water containing nutrients to bring it to a slurry phase. it is then put into a reactor where all environmental conditions are optimized like pH, temperature and oxygen content. This enhances the growth of indigenous microorganisms and keeps them in contact with the pollutant which increases the rate of bioremediation. Bioreactors are example of slurry phase system. Bioreactors produce a three phase i.e., solid, liquid and gas mixing environment and the rate and extent of bioremediation in a bioreactor is always higher than the in situ systems. But for bioreactor the contaminated soil need to be physically pretreated (washing) before adding it to the reactor (Kumar et al., 2011).

2.1.9 Bioremediation of Vegetable Oils:

Role of Lipases:

Lipases are the enzymes which play a major role in the bioremediation of wastewater contaminated with any type of oil. Lipases are basically glycerol ester hydrolyases. They have the ability to act upon the acyl glycerol to yield fatty acids and glycerol. Lipases are water soluble enzymes and hence can breakdown water insoluble lipid contaminants by catalyzing the hydrolysis of their ester bonds (Iqbal and Rehman, 2015).

Lipases are ubiquitously produced by animal, plants and microorganisms but it is the microbial lipases which are preferred the most for bioremediation of oils. Microbial lipases are extracellular enzymes which are produced by microorganisms when they come in contact with environment containing lipids (Sugahara and Varéa, 2014).

For the degradation of vegetable based oils, the first step is to cleave their ester bonds and convert them to fatty acids. This step is catalyzed by enzymes like lipases and esterases. Lipases are proteins which are hydrophobic in nature and they hydrolyze the carboxyl ester bonds present in mono, di and tri acyl glycerol which are the chief constituents of microbial, plants and animal fats and oils. Because of their high degradation potential, lipases are employed in degradation efforts of wastes which are rich in oils (Saifuddin and Chua, 2006).

2.1.10 Factors Affecting the Bioremediation Rate of Vegetable oils:

Main factors that influence the bacterial bioremediation of vegetable oils are temperature, pH, oxygen supply and nutrients.

Temperature affects the bioremediation of vegetable oils by affecting microbial metabolism and enzyme activity. Decrease in temperature decreases the rate of bioremediation by slowing down the enzyme activity and oil uptake by microorganism. By increasing temperature enzyme activity and oil metabolism increases especially from 30° C to 40° C after which the rate slows down due to denaturation of cell proteins and enzymes.

pH for the optimum growth of majority of bacteria is near neutral thus, the bioremediation rate drastically increases by neutralizing the pH. However, reduction in bioremediation rate is observed by both increase and decrease in pH.

Oxygen is another important factor affection the rate of vegetable oil bioremediation. The initial step in the catabolism of oils is their oxidation which requires oxygen. Oxygen is considered to be rate limiting factor in all types of aerobic bioremediation. Anaerobic bioremediation of oils has also been reported but due to its extremely slow rate it is insignificant as compared to aerobic degradation. Oxygen supply to microorganisms is increased by increasing the aeration in bioremediation setup.

Inorganic Nutrients like nitrogen and phosphorous also limits the bioremediation if unavailable to microorganisms. These can be provided to microorganism by addition of nitrogen and phosphorous salts in minimal amount to the wastewater which needs to be

treated. Addition of nitrogen and phosphorous stimulates the bioremediation of oil contaminating water (Leahy and Colwell, 1990).

Table 2.1: Different bacterial species used for bioremediation of water contaminated with oil

Bacteria	Type of contaminant oil	References
Pseudomonas diminuta	Vegetable oil and grease	(El-Masry et al., 2004)
Serratia marcescens	Vegetable oil and grease	(Affandi et al., 2014)
Aeromonas hydrophila	Vegetable oil and grease	(Affandi et al., 2014)
Bascillus cereus	Vegetable oil and grease	(Affandi et al., 2014)
Bacillus subtilis	Vegetable oil and grease	(Bala et al., 2015)
Micrococcus luteus	Vegetable oil and grease	(Bala et al., 2015)
Stenotrophomonas maltophilia	Vegetable oil and grease	(Bala et al., 2015)

2.2 Biodiesel

Increase in utilization of automobiles not only in developed countries but also in the developing countries has led to depletion of fossil fuels and the availability of fossil fuels is decreasing day by day. As, this situation is worsening there is a need of finding an

alternative energy fuel source to save ourselves from facing this threatening energy crisis situation in near future. At this time, biodiesel is considered to be the best alternative energy fuel which can replace fossil fuel and fulfill the world's energy demand.

Chemically, biodiesel is mono alkyl esters of long chain fatty acids derived from vegetable or animal fat oils (Olguín, 2012). Its biological origin is indicated by the prefix bio added to the conventional term diesel.

2.2.1 Advantages of Biodiesel over Fossil Fuel:

Among non-renewable sources of energy fossil fuels are the first on the list. These fossil fuels are associated with the production of pollutants and greenhouse gas (GHG) emissions that are directly linked to climatic changes, global warming and diseases (Aranisola e al., 2014). The diesel engine exhaust (DEE) of an automobile running on fossil fuel is considered to be cytotoxic and mutagenic and has been proved to be carcinogenic on experimental animals. Different studies have shown 1.5 Risk factor of developing lung cancer in humans after long term exposure to DEE. DEE has a carbon core which can easily absorbs onto it different organic pollutants like poly aromatic compounds (PACs) which are both carcinogenic and mutagenic. The size of these particles is small enough (0.1 to 0.3 µm) that they can be inhaled easily and about 10% of them are retained in the alveoli of lungs after inhalation causing lung cancer. Various studies have shown the mutagenic effect of DEE on bacterial and mammalian in vitro cell assays (Bünger et al., 1998).

Biodiesel has multiple advantages over the fossil fuels. It is renewable alternate source of energy which can be made easily available. The biodiesel-petro diesel blends can easily be run on conventional diesel engines and do not require any extensive modification. it can easily be stored in the way petro diesel is stored. Transporting, storing and handling risks of biodiesel are much less as compared to petro diesel and it can easily be produced from domestic renewable oil crops like soybean, rapeseed and sunflower. It can be used alone or as a blend like B20 which contains 20% biodiesel and 80% diesel.

Biodiesel possesses higher combustion efficiency than petro diesel. This is due to the oxygen content of biodiesel. Its oxygen content is such that it decreases its oxidation

potential and homogeneity of oxygen with fuel at the time of combustion increases its combustion efficiency.

Biodiesel shows remarkably low emissions of GHGs as compared to petro diesel. Its combustion alone gives about 75% to 90% reduction in emission of poly aromatic hydrocarbon and a total 90% reduction in unburnt hydrocarbon. It further reduces the emission of carbon monoxide and particulates. Sulfur content of biodiesel is 20 to 50 times less than petro diesel which reduces the emission of So_x. Biodiesel contains a very little amount of nitrogen and as N_2O emission depends upon initial concentration of nitrogen, biodiesel drastically reduces the emission of N_2O.

Another advantageous property of biodiesel is that it is nontoxic and its degradation rate is 4 times faster than petro diesel. Its oxygen content is responsible for quick degradation. Studies show that even in aquatic environment biodiesel is 77% to 89% degraded in 28 days while petro diesel was only 18% degraded (Demirbas, 2007).

2.2.2 Disadvantages of Biodiesel:

Some of biodiesel's drawbacks include viscosity, pour point and cloud point being higher, a lower energy content engine power and speed, higher cost, engine cocking and engine incompatibility.

2.2.3 Biodiesel Production:

Initially, in 1980 the idea of direct use of vegetable oils as biodiesel was pursued but the results came out to be impractical and unsatisfactory. The reasons behind this failure were high viscosity of these oils as well as these oils contained high acid content. Furthermore, gum formation during storage and combustion was another problem which needed to be dealt with. Direct use of oil also led to carbon depositing on engine and thickening of lubricating oil. Thus, there arose a need to transform these vegetable oils into more practically implementable form. The three well established methods for the conversion of vegetable oils into biodiesel are

- Micro emulsions
- Thermal cracking (Pyrolysis)

- Transesterification (Alcoholysis) (Aransiola et al., 2014).

Micro emulsions:

In order to overcome the problem of high viscosity of vegetable oils micro emulsion were used. Micro emulsions were formed by mixing of vegetable oils with polar solvents like methanol or ethanol. A micro emulsion is defined as a mixture of ionic and nonionic or polar and nonpolar components aided with surfactant and the mixture is isotropic, thermally stable and homogenous macroscopically.

Thermal Cracking (Pyrolysis):

Pyrolysis is defined as the thermal breakdown of a compound into its constituents and it involves the heating of compound in the absence of oxygen which cleaves the chemical bonds and yields small molecules. The pyrolysis products of vegetable oils are usually small alkanes and alkenes. A small amount of carboxylic acids is also resent in the product. The disadvantages of this method include expensive equipment, costly in terms of heat energy and production of low value materials and more gasoline than fossil diesel.

Transesterification (Alcoholysis):

Transesterification or alcoholysis is the reaction between fats or oils and alcohol which results in the production of Fatty Acid Methyl Esters (FAMEs) and glycerol. Figure 1 shows how the transesterification reaction is carried out. To increase the yield of desired product which is FAMEs and reaction rate catalyst is used. Enzymes, alkalis or acids are used to catalyze the reaction. As this reaction is reversible, in order to shift the equilibrium towards the product side, an excess of alcohol is used. Ethanol, methanol, propanol, butanol and amyl alcohols are among the priority candidates for transesterification reactions. Out of these five ethanol and methanol are the most widely used alcohols for biodiesel production. Although ethanol is more environmental friendly, can be produced from agricultural waste, renewable and less objectionable biologically but methanol is more widely used due to its low cost. A stoichiometric molar ratio of 1:3 for oil and alcohol is required for complete conversion of the substrates but practical

implication requires higher molar ratio to drive the equilibrium toward higher yield of biodiesel (Ma and Hanna, 1999).

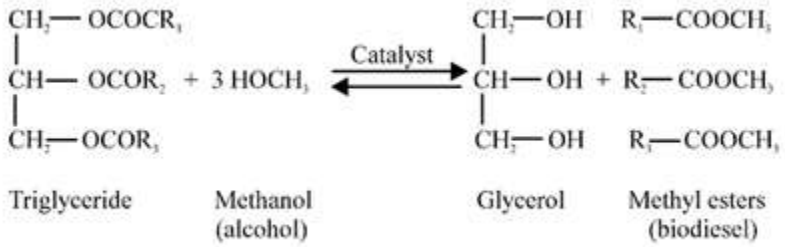


Figure 2.1. Transesterification reaction for biodiesel production (Ma and Hanna, 1999).

2.2.4 Chemical Synthesis of Biodiesel:

Transesterification of vegetable oils for production of biodiesel via chemical method employee's chemical catalysts which are mainly acids and bases. Chemical transesterification reaction involves three main steps:

1. First step is an intermediate tetrahedral formation
2. In the second step, this unstable intermediate tetrahedral breaks down into fatty acid ester and diglyceride ion.
3. The last step involves the recovery of catalyst by means of proton transfer

All these steps are repeated for every fatty acid present in the oil and the end result is the formation of fatty acid esters (biodiesel) and glycerol (Demirbas, 2008).

Acid catalyzed transesterification method is useful for low quality cheap feedstocks which are high in Free Fatty Acids (FFAs). The commonly used acid catalysts are sulfuric acid, phosphoric acid, hydrochloric acid and sulfonic acids. This method is water sensitive and presence of water leads to incompletion and further inhibition of the reaction. The reaction is carried out at a higher alcohol to oil molar ratio, a large amount of acid catalyst and at low to moderate temperature (Helwani et al, 2009).

Alkali catalyzed transesterification is one of the most common method used in laboratories, pilot scale studies and in industry for the production of biodiesel. The alkali catalysts used are metal hydroxides usually sodium hydroxide and potassium hydroxide and alkoxides. Sometimes potassium and sodium carbonate are also used. The advantage of this method over acid catalysis is the low production cost of catalyst but the feedstock for this process must contain very low amount of FFAs and no moisture content as this leads to soap formation instead of complete conversion of triglycerides into fatty acid esters (Cetinkaya and Karaosmanoglu, 2004).

Chemical synthesis of biodiesel has a variety of disadvantages which include:

- These methods are energy intensive
- Recovery of glycerol after the completion of reaction is difficult
- Removal of catalyst from the product
- The process generates alkali or acid wastewater depending upon the catalyst, which requires treatment.
- Free Fatty Acids and water leads to soap formation
- Unwanted side reactions take place due to low selectivity of these methods (Noureddini et al., 2005).

2.2.5 Biological Synthesis of Biodiesel:

All the challenges that are faced during chemical synthesis of biodiesel are alleviated by biological synthesis. It is an environmental friendly approach. Biological synthesis of biodiesel is carried out by enzyme catalyzed transesterification reaction. The group of enzymes which catalyze the conversion of fatty acids into their corresponding esters are lipases. Lipases are produced ubiquitously by animals, plants and microorganisms but the most widely used lipases for the purpose of biodiesel production are microbial lipases. Both fungal and bacterial lipases have been reported for the production of biodiesel. Lipases can either be used directly via immobilization for the production of biodiesel or whole cell approach can be used in which free or immobilized lipase producing microbial cells are used for the production of biodiesel. Immobilization approach is utilized to increase both the stability and reusability of biological catalyst.

In nature lipase (triglycerols ester hydrolases EC 3.1.1.3) are hydrolytic enzymes that breakdown long chain triacylglycerols (TAG) into their corresponding fatty acids. However, lipases remain remarkably active and stable in non-aqueous environments. In the absence of water and presence of another organic solvent lipases activity is shifted from hydrolysis to synthesis. Thus, in the presence of oil and alcohol instead of water they carry out transesterification reaction instead of hydrolysis.

The transesterification reaction is dependent upon the catalytic amino acid charge-relay triad Asp-His-Ser present at the catalytic side of enzyme. These three amino acids are hydrogen bonded to each other. In the first step histidine completely pulls the protons from serine alcohol itself, making serine alcohol an active nucleophile. This nucleophilic serine alcohol then attacks the carbonyl carbon of substrate forming a tetrahedral intermediate 1. In the next step the protons previously pulled by histidine are transferred to the diglyceride which is released and the electrons are transferred back to carbonyl carbon. In the next step the serine ester formed by the release of diglyceride from tetrahedral intermediate 1 reacts with alcohol forming tetrahedral intermediate 2. Again the electrons are pushed back to carbonyl group and the fatty acid ester is released. Serine takes backs its protons from histidine reforming serine alcohol. The function of aspartic acid is to pull positive charge from histidine when histidine gets completely protonated (Jegannathan et al., 2008).

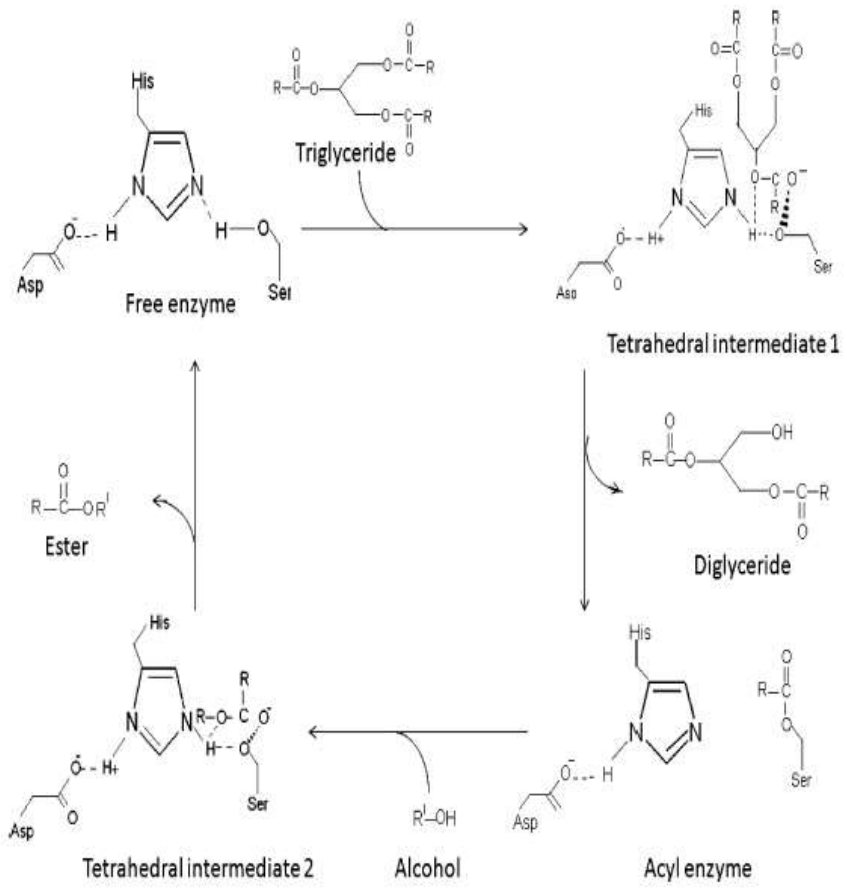


Figure 2.2. Mechanism of lipase catalyzed transesterification (Jegannathan et al., 2008).

Enzymes are known for their high specificity and efficiency thus, transesterification of oils with lipases do not lead to any kind of side reactions, a disadvantage faced in chemical synthesis. Enzyme catalyzed transesterification gives higher yield of methyl esters. Unlike chemical synthesis biological synthesis does not get affected by the presence of Free Fatty Acids in the feed stock with the working range of about 0.5% to 80% FFAs in the feed stock. Furthermore, glycerol recovery after completion of reaction

is easier and of high grade as compared to chemical synthesis. Biological synthesis is also carried out at temperature (30°C to 40°C) lower than chemical synthesis. However, enzymatic synthesis requires solvents like n-hexane, n-heptane and cyclohexane to increase their efficiency and require longer incubation time for completion of reaction (Aranisola et al, 2014).

Table 2.2: List of Microorganisms and plant oils utilized for biodiesel production.

Microorganism	Type of Oil	Reference
Pseudomonas cepacia	Soybean oil	Noureddini et al., 2005
Burkholderia cepacia	Jatropha oil	Shah and Gupta, 2008
Enterobacter aerogenes	Jatropha oil	Kumari et al., 2009
Thermomyces lanuginosa	Soybean oil	Du et al., 2005
Bascillus subtilis	Waste cooking oil	Ying and Chen, 2007
Pseudomonas fluorescens	Soybean oil	Zhao et al.,2007
Mucor miehei	Tallow	Nelson et al., 1996

2.2.6 Feedstock for Biodiesel Production:

Oils from both edible and non-edible plants serve as a feedstock for biodiesel production but currently maximum production of biodiesel throughout the world is from edible oils. About 84% of net biodiesel production overall in the world is from rapeseed oil, 13 %

from sunflower oil, 1% from palm oil and 2% from soybean and other oils. In order to be the suitable feedstock for biodiesel production crops need to meet certain criteria (Gui et al., 2008).

Oil yield from crops is one of the crucial factor which determines the crop's suitability for biodiesel production. The higher the yield of oil from a crop, the more it is preferred for biodiesel production. Higher oil yields help in the reduction of production cost of biodiesel as about 70% to 80% of the biodiesel cost depends upon the type of feedstock used for its production.

Oil composition is another key factor that plays role in determining the suitability of crop as a raw material for biodiesel production. The composition of oil from feedstock determines the properties of biodiesel that is produced from it. Biodiesel properties differ according to the type of fatty acids present in the oil from which it is produced. While generating biodiesel it must be kept in mind that the properties of generated biodiesel should be comparable to or better than petro diesel so that it can be used in diesel engine directly without requiring any modifications (Gui et al., 2008).

2.2.7 Edible vs Non-Edible Oils as Feedstock:

Nearly 95% of biodiesel around the world is produced from edible oils. In this way we are converting edible oil which is actually a food resource into automobile energy fuels. A global imbalance between food supply and demand is expected if we keep on using edible oil for biodiesel production. This will also lead to deforestation and ecosystem destruction as more land will be subjected to the growth of plants producing oils that are suitable for biodiesel production (Luskin and Potts, 2011). Deforestation has already started in countries like Brazil, Indonesia and Malaysia for the purpose of oil crop plantation to increase their yield of biodiesel. Moreover, with the increase in demand of biodiesel as an alternative to petro diesel the land which was previously used for food crop production will also be used for fuel crop production and more edible oil will be subjected to biodiesel production which will lead to a worldwide depletion of edible oil as food source (Monbiot, 2004)

In order to surmount this threatening and upsetting situation researchers are trying to utilize alternative oil resources also known as greener oil resources like non-edible oils for the production of biodiesel. Non-edible oil plants which are suitable for biodiesel production are luckily exclusive in the habitats and botanical requirements. They can be cultivated on lands which are not deemed suitable for the growth of food crops. Usually these plants can be grown in drought stricken areas with little water requirement. Their growth does not require intensive care and efforts. Some of these plants can also grow on saline and sandy soils. Most commonly used non-edible oils for biodiesel production are jatropha oil and castor oil (Kumar and Sharma et al., 2011).

2.2.8 Oils used in study:

Four oils are used in this study. Mustard oil and soybean oil are edible and jatropha oil and taramira oil are non-edible.

Jatropha oil is obtained from *Jatropha curcas* plant which belongs to Euphorbiaceae family. It is in the form of large shrub or tree with the height of about 5m to 7m. it is drought resistant with the ability to grow in semi-arid and arid conditions. The life of plant is 30 to 50 years. It starts producing seeds after 12 months and maximum productivity is attained at the age of 5 years. The plant's range of seed production is from 0.1 to 8 t ha^{-1} yr^{-1}. Its seeds are hydrocarbon rich containing about 43% to 59% oil. *Jatropha curcas* oil is rich in oleic acid and the fatty acid composition of jatropha oil is in harmony for the production of high grade biodiesel. The anti-nutritional factor of jatropha oil is phorbol esters which also increase its shelf life protecting it from insect attacks (Kumar and Sharma et al, 2009).

Table 2.3 Fatty acid composition of Jatropha oil

Fatty acids	**Percentage (w/w)**
Oleic acid	42%
Linoleic acid	35%
Palmitic acid	14%
Stearic acid	6%
Others	1%

Taramira oil obtained from *Eruca sativa* plant belonging to Brassicaceae family. It is grown in Pakistan for preparing traditional medications, as a minor oil yielding crop. It is drought resistant and also shows some degree of salt resistance. Its cultivation does not require any sort of intensive maintenance and it can grow in barren lands with low soil fertility and rain fall. Its seeds contain up to 35% oil. The anti-nutritional factors of taramira oil includes its strictly pungent smell, skin irritation and high amount of erucic acid which not only renders it unpalatable but is also related to certain cardiac problems (Chakarbarti and Ahmad, 2009).

Table 2.4 Fatty acid composition of Taramira oil

Fatty acids	**Percentage (w/w)**
Palmitic acid	10.2%
Stearic acid	1.6%
Oleic acid	22.8%
Linoleic acid	6.4%
Linolenic acid	11.9%
Eicosenoic acid	6.4%
Erucic acid	40.8%

Soybean oil is derived from the seeds of soybean or soya bean plant scientifically known as *Glycine* sp. belonging to the family Fabaceae. Soybean plant is an annual twinning vine with purple flowers and small round dark brown seeds produced inside pods which drops down at maturity. The soybean seed contains about 18% to 24% oil and 35% to 40% protein content (Khan et al., 2014). As compared to jatropha and taramira, soybean seeds and oil have high nutritional values. Soybean seeds have high protein content and these proteins amino acids which are essential for human growth and health at all developmental stages. Moreover, soybean oil contains high percentage of linoleic acid which is a member of omega-6 fatty acids, an essential fatty acid group which is an important component of all mammal's diet (Gui et al., 2008).

Table 2.5 Fatty acid composition of Soybean oil (Lee et al., 1998)

Fatty acids	**Percentage (w/w)**
Palmitic acid	11.67%
Stearic acid	4.96%
Oleic acid	36%
Linoleic acid	47.31%
Linolenic acid	0.33%

Mustard oil used in this study was obtained from the oil containing seeds of *Brassica campestris* L. This plant belongs to the family Brassicaceae. It is an annual crop which is cultivated in semi-arid to arid lands which are well irrigated. Its cultivation requires some degree of care and maintenance and unlike non-edible oil crops mentioned it is not drought resistant. Its seeds are about 1.5 mm in diameter and contain about 30% to 46% oil (Ahmad et al., 2012). Although this oil is used for the purpose of cooking and preservation of pickles but it is high in erucic acid which as mentioned earlier is linked to cardiac diseases like myocardial lesions due to fat deposits on heart tissue. Mustard oil contains allyl isothiocyanate which is responsible for its pungent smell. This oil is also used for massage purpose (Swati and Das, 2015).

Table 2.6 Fatty acid composition of Mustard oil (Swati and Das, 2015).

Fatty acid	**Percentage (w/w)**
Palmitic acid	4.5%
Stearic acid	2.78%
Oleic acid	38.21%
Linoleic acid	25.31%
Linolenic acid	11.3%
Arachidonic acid	10.86%
Erucic acid	11.35%

In order to determine the potential of isolated lipolytic bacteria for bioremediation and for biodiesel production following materials and methods were utilized.

3.1 Sample Collection and Storage:

Oil contaminated wastewater sample was collected from a local vegetable oil and ghee industry in Hattar, Pakistan. Sample was collected in 10-liter water can and brought to laboratory. In laboratory, prior to analysis, sample was kept at 4° C to cease any microbial activity which may lead to biodegradation (APHA, 2005).

3.2 Physicochemical Parameter Analysis of Sample:

Physicochemical parametres like pH, O&G content and COD were determined first. pH of the sample was measured by taking about 20 ml sample in a 50 ml Erlenmeyer flask and placing the electrode of pH meter in it till the reading is stabilized on the display screen.

Oil and Grease content was estimated by using the Partition-Gravimetric Method as described in 21st edition of APHA Standard methods for the examination of water and wastewater.

Reagents: n-Hexane, Sodium Sulfate Na_2SO_4 and 1M Sulfuric acid.

Procedure: At first pH of the sample (100 ml) which is taken for oil and grease estimation is adjusted to 2 using 1M acid. The weight of rotary distillation flask was measured. In a separatory funnel took 100 ml of the sample and 30 ml of pure n-hexane. Shook it vigorously for about 5 minutes. Allowed the layers to separate. Drained out the aqueous layer into a beaker and organic layer into the distillation flask of rotary distillatory. Poured the aqueous layer again into separatory funnel along with fresh batch of 30 ml n-hexane n shook for 5 minutes before letting the layers to separate. Drained the aqueous layer into beaker and organic phase into the distillation flask of rotary distillatory containing 30 ml n-hexane from previous separation. With the aqueous layer again repeated the separation step. The 90 ml n-hexane obtained after the separation procedure was filtered through the filter paper sprinkled with 10 grams of Na_2SO_4 to remove any moisture present in it. Now set the temperature of rotary distillatory at 85°C,

placed the distillation flask at its assigned position and run rotary distillatory till all of the n-hexane was evaporated and collected in the round bottom flask present on the other end of condenser. Again measure the distillation flask. Calculate the amount of oil and grease by the formula

mg of oil and grease/liter = (A-B) x 1000/ml of sample used.

A = initial weight of the distillation flask

B = final weight of distillation flask after evaporation of n-hexane (APHA, 2005).

COD measurement: For COD measurement digestion solution and H_2SO_4 reagent were first prepared.

Digestion solution:

Components	**Quantity g or ml/L**
$K_2Cr_2O_7$	10.216 g
H_2SO_4	167 ml
$HgSO_4$	33.3 g

H_2SO_4 Reagent: Dissolved 5.5 g Ag_2SO_4 in 1 liter H_2SO_4.

Procedure: Sample for the purpose of COD measurement was first filtered. For COD measurement 2.5 ml of H_2SO_4 reagent, 3 ml digestion solution and 2 ml sample were added in screw capped glass vials and placed in digester for 2 hours at 150°C. After 2-hour glass vials were taken out, allowed to cool down to room temperature and COD was measured using Spectroquant.

3.4 Isolation and Screening of Lipolytic Bacterial Isolates:

Tween-20 Agar:

Tween-20 was the screening media used for the isolation of lipase producing bacteria from wastewater sample. The composition of Tween-20 media is shown in Table 3.1 and its pH was kept at 7.5.

Table 3.1 Tween-20 Media composition

Media Components	Concentration (g/L)
Peptone	10
NaCl	5
$CaCl_2.2H_2O$	0.1
Agar	20
Tween 20	1 %

Serial dilutions of sample wastewater from 10^{-1} to 10^{-9} were made in distill water. About 100 µl of each dilution was spread on Tween-20 plates with the help of glass spreader. The inoculated plates were then wrapped in cling film and incubated at 37°C. Results (colonies giving white hallows) were observed after 24 hours (Le, 2008).

Phenol Red Olive Oil Agar:

To confirm lipase production, selected colonies from Tween-20 plates were further streaked on Phenol-Red agar plates. The composition of Phenol-Red media is described in table 3.2. Media's pH was adjusted to 7.5. After inoculation plates were wrapped in cling film and kept in incubator of 37°C. Results were observed after 24 hours of incubation (Lee et al., 2015).

Table 3.2 Phenol Red Olive Oil Agar Composition (Lee et al., 2015).

Media Components	Concentration
Phenol-Red	0.01% w/v
Olive oil	0.1% v/v
$CaCl_2$	0.1% w/v
Agar	2% w/v

Rhodamine B Olive Oil Agar:

Selected colonies from Tween-20 agar were also streaked on Rhodamine B agar. The composition of Rhodamine B Agar is shown in table 3.3. Media's pH was adjusted to 7.

Table 3.3 Rhodamine B Olive Oil Agar Composition.

Media Components	Concentration
Nutrient Broth	0.8% w/v
NaCl	0.4% w/v
Rhodamine B	0.001% w/v
Olive oil	2% v/v
Yeast Extract	0.05% w/v
Agar	2% w/v

After inoculation plates were wrapped in cling foil and kept at 37°C for 24 hours after which results were noted (Kumar et al., 2012).

3.5 Characterization of Isolates:

Gram staining:

For the purpose of gram staining, bacterial smear was first made on slide by taking a drop of normal saline on glass slide and then adding half a loop of culture to it. Mixed and let it dry. Afterwards, the smear was heat fixed. A drop of Crystal violet was added to the heat fixed smear and washed after 30 seconds. Gram's Iodine was then applied to smear for 30 seconds and rinsed with water. 95% ethanol was applied as a decolorizer for a few seconds rinsed with water and counter stain safranin was applied for 30 seconds. Again rinsed with water air dried and observed under microscope.

3.6 Biochemical Testing:

Selected bacterial isolates were subjected to biochemical testing for identification, in accordance with the Bergey's Manual of Determinative bacteriology (9^{th} Edition). For this purpose, tests mentioned below were carried out.

- Catalase Test

- Nitrate Test
- Citrate Utilization Test
- Triple Sugar Iron (TSI) Test
- Oxidase Test
- MRVP Test
- Urease Test

Catalase Test:

A drop of hydrogen peroxide was placed in the centre of an aseptic glass slide and with the help of sterile loop culture was transferred to that drop. Catalase positive colonies formed bubbles while others did not.

Nitrate Reduction Test:

Nitrate reduction test was carried out in broth. Composition of nitrate reduction broth is mentioned below.

Solution A

Reagent	**Quantity (ml)**
30% acetic acid (5M)	1000
Sulphuric Acid	8

Solution B

Reagent	**Quantity**
Acetic Acid (5M)	1000ml
Alpha-napythylamine	5g

Procedure:

For nitrate reduction test about 5 to 8 ml of nitrate reduction broth was added to sterile test tubes and inoculated. After inoculation tubes were incubated at 37°C for 24-48 hours. After incubation solution A and B were added. if cherry red colour is observed, results are considered positive and if cherry red colour is observed after the addition of zinc dust, results are considered negative. This test shows the potential of isolates to convert NO^{-3} to NO^{-2}.

Citrate Utilization Test:

For citrate utilization test slants of Simon citrate agar were prepared and inoculated with the bacterial isolates. After inoculation slants were incubated for about 24 to 48 hours at 37°C. results were considered positive if the colour of slants changed from Prussian blue to green along with growth. This test depicted the potential of microbes to utilize citrate as sole carbon source.

Triple Sugar Iron Test:

Slants of TSI were first made and then inoculated with bacterial isolates in such a way that slants were first stabbed and then streaked on the surface with inoculating needle. After incubation slants were incubated at 37°C for 24 to 48 hours. Alkaline reaction gave red colour while acidic fermentation converted the slant's colour from red to yellow. Colour change in butt represents glucose fermentation and colour change in slant represents lactose fermentation.

Oxidase Test:

1g of Tetramethyl-p-phenylenediamine·2HCl was added to 1000 ml of distilled water to prepare oxidase reagent. On a filter paper 2 to 3 drops of oxidase reagent were placed and bacterial colony was transferred to it. Formation of dark blue colour depicted positive result.

Methyl Red Vogues Proskaeur Test:

MR-VP broth was prepared, autoclaved and 5 ml was poured into each sterile glass test tube. It was then inoculated and incubated at 37°C for 24 to 48 hours. Afterwards the test tubes were divided into two sets for performing MRVP test.

Barritt's Reagent:

Barritt's reagent comprises of solution A & B

Solution A

Reagent	Quantity
Alpha-naphthol	5gm
Ethyl alcohol	100ml

Solution B:

Reagent	Quantity
Potassium hydroxide	40g
Deionozed Water	100ml

MR-VP Broth:

Methyl Red Indicator:

Reagent	**Quantity**
Methyl Red	0.1g
95% Ethanol	300ml
Distill Water	2000ml

Procedure:

For Methyl Red test two drops of indicator were added to the test tubes. If red colour appeared results were considered positive. For VP test 0.6ml of a-naphthol and 0.8ml of KOH were added to the test tubes and shaken for 5 minutes. Appearance of red colour was taken as positive result.

Urease Test:

Urease broth was prepared, autoclaved, transferred to sterile test tubes, inoculated and incubated at 37°C for 24 to 48 hours. Appearance of light pink colour confirmed that the inoculated microbe produces urease.

3.7 Molecular Identification of Strains:

For the purpose of molecular identification of strains DNA was first extracted and then sent to MACREOGEN, Korea for sequencing.

DNA Extraction:

Reagents: 1X TE buffer, Proteinase K, RNAse A, CTAB, Sodium Dodecyl Sulfate (SDS) 10%, 5M NaCl, phenol: chloroform: isoamylalcohol in the ratio of 25:24:1, isopropanol and 3M sodium acetate.

Procedure: For DNA extraction CTAB method was followed. Bacterial culture was first enriched in nutrient broth. About 10 ml of this enriched nutrient broth was centrifuged at 10000 RPM for 15 mins. Supernatant was discarded and pellet was dissolved in 1ml 1X TE buffer via vortexing. Again centrifuged TE buffer discarded supernatant and re-dissolved pellet in 600 µl of 1X TE buffer by vortexing. Added to this solution, 3 µl proteinase K, 40 µl of RNAse A and 30 µl 10% SDS, shook gently and incubated at 37°C for 1 hour. After incubation added to this solution 100 µl of CTAB and 80 µl of 5M NaCl and again incubated for 10 mins at 65°C. after incubation added to this solution, 600 µl phenol: chloroform: isoamyl-alcohol in the ratio of 25:24:1. Centrifuged for 15 mins at 10000 RPM and carefully picked upper layer and placed it in new Eppendorf. Repeated this step again. Now to the upper layer added 600 µl of isopropanol and 3M sodium acetate 60 µl. shook slowly and centrifuged at 10000 RPM for 6 mins. Slowly discarded

supernatant and washed the pellet with 70% ethanol. Dried tube and dissolved in 100 µl of TE buffer (Wilson, 1987).

3.8 Gel Electrophoresis:

To confirm DNA extraction gel electrophoresis was performed. 10X TAE buffer was prepared by dissolving 48.4 g of Tris base [tris (hydroxymethyl) aminomethane], 11.4 mL of glacial acetic acid (17.4 M) and 3.7 g of EDTA in 1000 ml of deionized water. Gel was then prepared by dissolving 1% agarose in 10X TAE buffer and heating in microwave for two minutes. After 2 minutes, gel was removed from microwave and 3 µl ethidium bromide was added to it. Gel was then poured in tray and comb was kept to form wells. After solidification of gel 3 µl of sample along with loading dye was loaded into the wells and gel was ran for 30 mins at 110 volts and 400 mA. Gel was then observed under UV light.

3.9 16S Ribosomal RNA Sequencing:

16S rRNA sequencing for bacterial identification was commercially performed by Macrogen Standard Custom DNA Sequencing Services (Macrogen Inc. Seoul, Korea).

3.10 Culture Enrichment:

Culture enrichment was done by inoculating 20 ml of autoclaved nutrient broth in 100 ml of Erlenmeyer flask with two loops full of culture under sterile condition and kept for 24 hours at 37°C and 150 RPM.

3.11 Production of Lipase through Submerged Fermentation:

The composition of enzyme production media is described in table 3.4. Bacterial cultures were first enriched in nutrient broth for 24 hrs. 5% 24 hours fresh enriched culture was then inoculated in 50 ml of production media contained in 250 ml of Erlenmeyer flask and incubated at 37 °C and 150 RPM. After 24 and 48 hrs of fermentation 5 ml of media was taken out from the flask and centrifuged at 10000 RPM for 10 mins. Pellet was discarded and supernatant was stored at -20°C.

Table 3.4 Composition of Enzyme Production Media.

Media Components	Percentage
Peptone	0.2% w/v
KH_2PO_4	0.1% w/v
NaCl	0.25% w/v
$MgSO_4.7H_2O$	0.04% w/v
$CaCl_{2.}2H_2O$	0.04% w/v
Olive Oil	2% v/v
Tween 20	1-2 drops

3.12 Lipase Assay:

Lipase activity was assayed using the method described by Kumar et al., 2005 with a few modifications.

Reagents: Tris-Hcl Buffer at pH 7, Acetonitrile, Isopropanol, para-nitro phenyl Laurate (PNP-L) and para-nitro phenol (PNP).

Standard Assay Conditions:

Temperature 37°C

pH 7

Procedure: 20mM of PNP-L solution was prepared in 1:1 isopropanol and acetonitrile. This is called the substrate solution. The supernatant collected from enzyme production media is called enzyme solution. Added 75 µl of substrate solution to 25 µl of enzyme solution and made final volume of the solution 3 ml by adding tris-HCl buffer at pH 7. This solution mixture was incubated at 37°C for 30 minutes. After 30 mins the solution was chilled at -20°C for 10 mins to stop enzyme activity and centrifuged for 5 mins at 10000 RPM to remove the precipitates formed. The absorbance of released PNP was then

noted at 410 nm. The unknown concentration of PNP released was measured from standard curve.

Negative control containing heat inactivated enzyme was used as blank.

Standard curve for PNP was developed by making 10-100 µM solution of PNP in tris-HCl buffer at pH 7 and recorded absorbance at 410 nm.

One unit of enzyme activity is defined as enzyme catalysing the release of 1 µM of para-nitro phenol per minute per ml under standard assay conditions (Temperature 37°C and pH 7).

3.13 Protein Estimation:

Reagents:

Solution A: 4g NaOH + 20g Na_2CO_3 per liter distilled water

Solution B1: 10g $CuSO_4.5H_2O$ per liter distilled water

Solution B2: 20g NaK-Tartrate per liter distilled water

Solution C: Solution A, B1 and B2 were mixed in a ratio of 10:0.1:0.1 respectively. It was prepared fresh.

Solution D: It was formed by mixing Folin Phenol with water in a ratio of 1:1. It is freshly prepared and is light sensitive. It cannot be stored.

Standard Stock Solution of Bovine Serum Albumin

Procedure:

Standard Lowery Method was used for protein estimation. The method is based on Biuret reaction, in which the peptide bonds of proteins react with copper under alkaline conditions to produce Cu+, which reacts with the folin reagent and strong blue color appears. 1ml of test sample (supernatant) was mixed with 1ml of solution C, shook well and was incubated for 10 minutes at room temperature. Then 0.1ml of solution D was added to reaction tube in dark and after shaking it was incubated for 30 minutes in dark. Absorbance was checked via spectrophotometer at 650nm (Classics Lowry et al., 1951).

3.14 Methanol sensitivity testing:

Methanol sensitivity test was performed on the isolated bacterial strains as methanol will be used directly along with the cells for the production of biodiesel. Methanol was added to autoclaved nutrient broth in 5%, 10% and 15% concentration. 5% of 24 hrs fresh culture enriched in nutrient broth was added to the methanol containing nutrient broth and incubated at 37°C and 150 RPM. Growth OD was noted after 24, 48 and 72 hours at 600 nm wavelength. Experiment was carried out in 100 ml of Erlenmeyer flasks containing 30 ml of nutrient broth containing methanol. Mouth of flasks were tightly closed with rubber stoppers and further sealed with paper tape to avoid methanol evaporation from media.

3.15 Oil Toxicity test:

Two of the oils taramira and jatropha, used in this study are reported to have toxic effect on microorganisms. As for biodiesel production, these oils will also be used directly along with cells so their effect on growth of isolated bacterial strains were tested beforehand. The composition of media for toxicity testing is shown in table 3.5. Media contained oils as carbon source to check whether the bacterial isolates get inhibited by oils or are able to breakdown triglycerides into fatty acids via their extracellular lipase and grow using them as carbon source. 50 ml of media was taken in 250 ml Erlenmeyer flaks and inoculated with 5% of 24-hour fresh culture enriched in nutrient broth. After inoculation flasks were incubated at 37°C and 150 RPM. Optical Density for growth was recorded after 24, 48 and 72 hours at 600 nm wavelength. Four sets of experiment were conducted for each isolate each set containing different type of oil. Along with taramira and jatropha, olive oil and cooking oil were also used as reference oils to check and relate the growth of strains.

Table 3.5 Composition of Media Prepared for Toxicity Testing.

Media Components	**Percentage**
Peptone	0.2% w/v
KH_2PO_4	0.1% w/v
NaCl	0.25% w/v
$MgSO_4.7H_2O$	0.04% w/v
$CaCl_2.2H_2O$	0.04% w/v
Oils (Taramira, jatropha, Olive, Cooking oil)	2% v/v
Tween 20	1-2 drops

3.16 Biodegradation Study of Oil and Grease from Wastewater:

For bioremediation of oil and grease from wastewater using isolated lipolytic strains batch system was used.

Batch System:

The Minimum Salt Media (MSM) used for biodegradation study is described in table 3.6. MSM was prepared by autoclaving each salt separately and then mixing in sterile condition. Wastewater used for this experiment was also autoclaved. 50 ml of MSM and 50 ml of Wastewater was mixed in 250 ml Erlenmeyer flask. 5% 24-hour fresh cultures enriched in nutrient broth were inoculated into the MSM-Wastewater solution. About 5 ml of this solution was taken out in a test tube for COD measurement. The inoculated media was then incubated at 37°C and 150 RPM. Each day 5ml sample was taken out from the reactor for COD measurement and the experiment was carried out till no more COD reduction occurred. For COD measurement the sample taken out was always

filtered or centrifuged to remove biomass (cells) from it. Percentage COD reduction was calculated from the following formula

%age COD Reduction = $[(COD_{initial} - COD_{final}) / COD_{initial}] \times 100$

Table 3.6 Composition of MSM (Lee, 2008)

Media Components	**Concentration (g/L)**
K_2HPO_4	1.12
KH_2PO_4	0.48
NaCl	5
$MgSO_4.7H_2O$	0.1
$(NH_4)_2SO_4$	2
EDTA	0.001

3.17 Biodiesel Production:

Following methods were used in order to determine the potential of isolated lipolytic strains for biodiesel production.

Preparation of cells:

As the biodiesel production technique in this study involves whole-cell harvesting approach for lipase production, cell mass is first generated by growing the strains in LB broth.

Table 3.7 LB broth composition

Media Components	**Concentration (g/L)**
Peptone	10
NaCl	5
Yeast Extract	5

Two loops full of cultures were inoculated into 100 ml LB broth contained in 250 ml Erlenmeyer flasks under sterile conditions. Inoculated media was then incubated at 37°C and 150 RPM for 48 hours to achieve maximum biomass production. After 48 hours, media was centrifuged at 4000 RPM for 15 minutes. RPM was kept low so that cells do not disrupt during centrifugation. After centrifugation supernatant was discarded and cell pellet was stored in normal saline at 4°C.

Microbial synthesis of Biodiesel (Transesterification):

About 1 ml of harvested cells from LB broth, were added to 1:6 ratios of oil and methanol mixture for every oil (taramira, jatropha, mustard and soybean oil). At first oil was added to 50 ml Erlenmeyer flask and 1 ml cell pellet was added and mobilized in the oil layer. In this way cells remain protected from direct methanol exposure which may lead to cell inhibition. A large inoculum size was used so that in case if some of the cells get inhibited by methanol other remain active. Methanol is added afterwards. Mouths of the flasks were closed tightly with rubber corks and further sealed with tapes so that methanol does not evaporate. 300 µl n-hexane was added as solvent and emulsifier to increase the surface contact area of oil and methanol which gives lipase an easy access to both the components for transesterification reaction (Devanesan et al., 2007). Reaction mixture was then incubated at 37°C and 150 RPM for 48 hours. After 48 hours, flask's contents were shifted to separatory funnel which was kept static for 24 hours to allow the layers to separate. After complete settling and layer formation the upper yellow layer of biodiesel was collected in screw cap glass vials.

Biodiesel Purification (Methanol separation):

For separation of excess methanol and purification of biodiesel distillation was carried out. For this purpose, biodiesel was poured in the distillation round bottom flask of distillation apparatus and the temperature was adjusted to 65°C which is the boiling point of methanol. Distillation apparatus was stopped when there was no more methanol collection in the flask on the other side of distillation tube. Volume of pure biodiesel was noted and it was stored in screw capped glass vials and kept in dark.

Percentage volumetric yield:

It was calculated by using formula

%age yield of biodiesel (volume) =

[amount of biodiesel produced (ml)/ initial amount of oil used (ml)] x 100

Fourier Transform Infrared Spectrometer:

Fourier Transform Infrared Spectrometer (FTIR) was carried out for all the initially produced biodiesel samples. Initially produced biodiesel samples included samples from all the 24 reactions in which each of the six bacterial isolated strains were used for the production of biodiesel from all four oils. FTIR was used to analyse whether the samples contain ester peaks which are characteristic of biodiesel. FTIR was done by Tensor 27 (Bruker), FTIR spectrophotometer equipped with ZnSe ATR. Liquid sample was loaded in Sample assembly designed for liquids and the analysis was performed. A total of 16 scans were carried out and the average was represented in the form of a spectrum showing different peaks at different ranges. The software that was used in the analysis is Opus65.

3.18 Optimization of Parameters for Biodiesel Production using Plackett-Burman Design:

For the purpose of optimization of parameters to attain highest percentage volumetric yield of biodiesel with one selected isolate and taramira oil, Plackett-Burman design was used through Stat-Ease Design Expert Software version 7.0. Plackett-Burman design gives information about the effect of single factors on response i.e. %age yield.

Five factors namely temperature, agitation speed, oil: methanol ratio, n-hexane percentage with respect to oil and inoculum size were selected for optimization and entered into the design table and 3 central points were selected. The design then gave 15 runs and 15 experiments were conducted in accordance with the factor values specified by the design in 15 runs.

Table 3.8 Runs given by Plackett-Burman design for experiment.

Runs	Factor 1 A: Molar ratio	Factor2 B: Agitation RPM	Factor 3 C: Inoculum size %	Factor 4 D: Temperature Celsius	Factor 5 E: n-hexane %
1	9.00	150.00	10%	37.00	10.00
2	9.00	300.00	10%	37.00	6.00
3	9.00	150.00	30%	55.00	6.00
4	3.00	150.00	10%	37.00	6.00
5	6.00	225.00	20%	46.00	8.00
6	3.00	150.00	30%	37.00	10.00
7	9.00	150.00	30%	55.00	10.00
8	3.00	150.00	10%	55.00	6.00
9	6.00	225.00	20%	46.00	8.00
10	3.00	300.00	30%	37.00	10.00
11	9.00	300.00	10%	55.00	10.00
12	6.00	225.00	20%	46.00	8.00
13	3.00	300.00	30%	55.00	6.00
14	9.00	300.00	30%	37.00	6.00
15	3.00	300.00	10%	55.00	10.00

For each experiment conditions for each factor was kept as given by the design. Incubation time was kept constant at 48 hours. 50 ml Erlenmeyer flasks were used to

contain oil, inoculum and methanol for each experiment. Flask's mouth was tightly closed with rubber cork and further sealed with paper tape so that methanol does not evaporate. Flasks were kept in incubator at temperature and agitation speed given by the design for 48 hours. After 48 hours, biodiesel was separated from glycerol layer by using separatory funnel and layers were allowed to separate for 24 hours before separation. Biodiesel was purified and methanol was recovered by using distillation apparatus.

3.19 Simultaneous Bioremediation of Oil Contaminated Wastewater and Biodiesel Production from Non-Edible Taramira oil:

Following strategy was carried out for simultaneous Bioremediation of Oil Contaminated Wastewater and Biodiesel Production from Non-Edible Taramira oil using *Bacillus subtilis* strain Q1.

Bioremediation experiment (Continuous System):

The selected bacterial strain Q1 was first enriched in nutrient broth for 24 hours by inoculation 20 ml nutrient broth in 100 ml Erlenmeyer flask with two loops full of the culture under sterile condition. MSM was prepared as described in table 3.6. 50 ml of MSM and 50 ml of autoclaved wastewater sample were mixed in 250 ml of Erlenmeyer flask under sterile condition. 5 ml of this mixture was taken out and stored for COD measurement. 5% inoculum was then added to MSM plus wastewater solution and incubated at 37°C and 150 RPM. After every 24 hours, flask was taken out and about 33.3 ml of incubated mixture of MSM and wastewater was taken out and 33.3 ml of fresh autoclaved MSM and wastewater mixture in 1:1 ratio was added to the flask. The mixture which was taken out was centrifuged to remove bacterial biomass. Pellet was discarded and supernatant was decanted in 15 ml falcon, shaken well to mix the oil and water layers which were separated due to centrifugation and then its COD was measured.

Flow rate, Organic Loading Rate and Organic Removal Rate were calculated from the following equation:

Flow Rate = Volume of the Reactor / Retention Time

Organic Loading Rate = [$COD_{initial}$ x Flow Rate] / Volume of the Reactor

Organic Removal Rate = [ΔCOD x Flow Rate] / Volume of the Reactor

Harvesting of Cells:

As in continuous reactors cells remain active and alive at the end of the reaction the alive and actively lipase producing cells were harvested by means of centrifugation. All the MSM and wastewater solution that came out from the flask after 3 days was centrifuged at 4000 RPM for 15 minutes. Supernatant was discarded and pellet was stored at 4°C in normal saline.

Methanolysis of Taramira oil by cells harvested from bioremediation experiment:

Oil was poured into a 50 ml Erlenmeyer flask and cell pellet obtained from bioremediation reaction was added and mixed shaken well so that the cells get mobilized in oil layer. Methanol was then added to this oil and cells mixture. Mobilizing the cells in oil layer also protects cells from coming into direct contact with methanol and protects them from methanol inhibition. N-hexane was then added to the reaction mixture. Mouth of flask was tightly closed with rubber cork and further sealed with paper tape to avoid evaporation of methanol. The conditions for all the factors was kept in accordance with the run of the design which gave the highest volumetric yield of biodiesel. Flask was incubated in shaker incubator for 48 hours. After 48 hours, biodiesel was separated from glycerol layer by using separatory funnel and layers were allowed to separate for 24 hours before separation. Biodiesel was purified and methanol was recovered by using distillation apparatus.

4.1 Physicochemical parameter analysis of Vegetable oil industry wastewater.

Physicochemical characteristics of Wastewater collected from Vegetable Oil Industry are given in table 4.1.

Table 4.1 Physicochemical characteristics of Wastewater collected from Vegetable Oil Industry.

Parameters	**Vegetable Oil Industry Wastewater**
Fats, oil and grease content	1500 mg/L
pH	7.2
Original COD	1046 mg/L
Enhanced COD (1ml oil/100ml sample)	3798 mg/L

4.2 Isolation and Screening of Lipolytic Isolates from Vegetable Oil Industry Wastewater:

Inoculation on Tween-20 Agar Plates:

Dilutions of wastewater sample were made from 10^{-1} to 10^{-10} and 0.1 ml of each dilution was spread on Twee-20 agar plates. Countable range of colonies came in plates inoculated with dilutions 10^{-6} and 10^{-7} with 112 and 55 colonies per plate respectively as shown in Figure 4.1 and 4.2. CFU/ml for plate inoculated with 10^{-6} dilution was calculated to be 1.12×10^{9} and CFU/ml for the plate inoculated with 10^{-7} dilution came out to be 5.5×10^{9}. Those colonies which showed the largest white hallow around them were further streaked on Tween-20, Phenol-Red Olive Oil and Rhodamine B Olive oil plates to confirm their lipolytic activity.

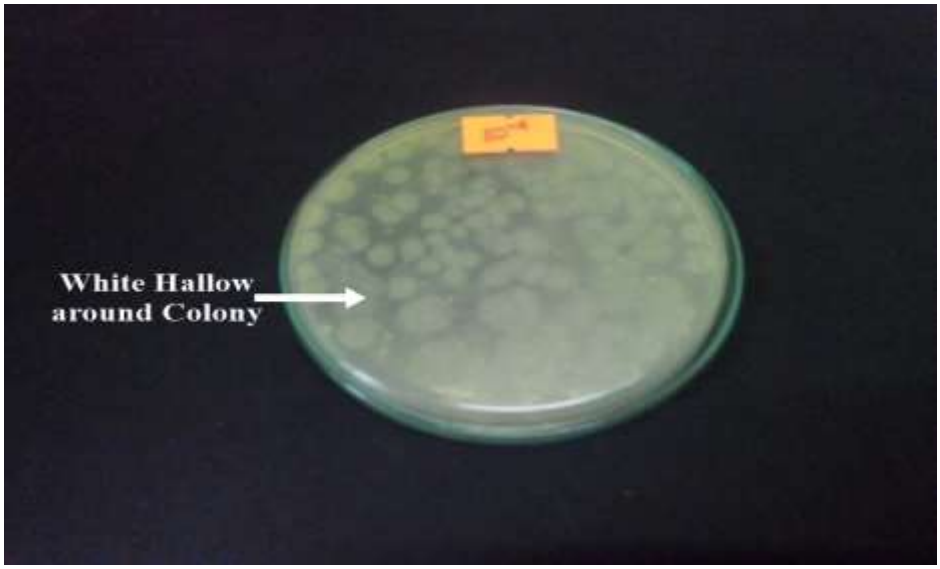


Figure 4.1 Colonies on Tween-20 Agar Plates inoculated with 10^{-6} dilution of sample wastewater.

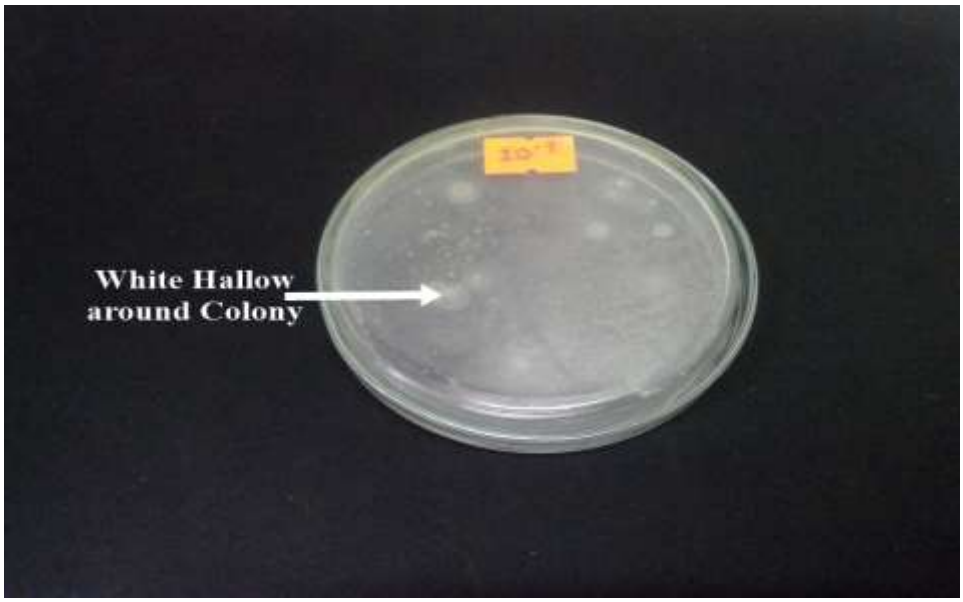


Figure 4.2 Colonies on Tween-20 Agar Plates inoculated with 10^{-7} dilution of sample wastewater

Figures 4.3, 4.4, 4.5 and 4.6 shows selected strains individually streaked on Tween-20 Agar plates having while hallows

Figure 4.3 Strain Q1 on Tween-20 Agar plate

Figure 4.4 Strain Q5 on Tween-20 Agar plate

Figure 4.5 Strain Q6 on Tween-20 Agar plate

Figure 4.6 Strain Q8 on Tween-20 Agar

Inoculation on Phenol-Red Olive Oil Agar Plates:

In order to confirm the lipolytic activity, selected strains were further inoculated on Phenol-Red Olive Oil Agar Plates. Lipase positive strains changed the color of plates from red to yellow by changing the pH of from basic to acidic.

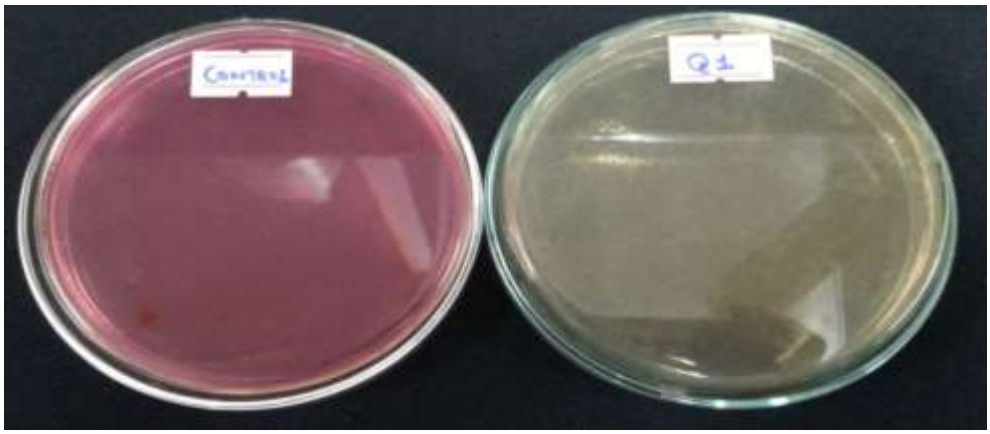


Figure 4.7 Strain Q1 on Phenol-Red Agar Plate.

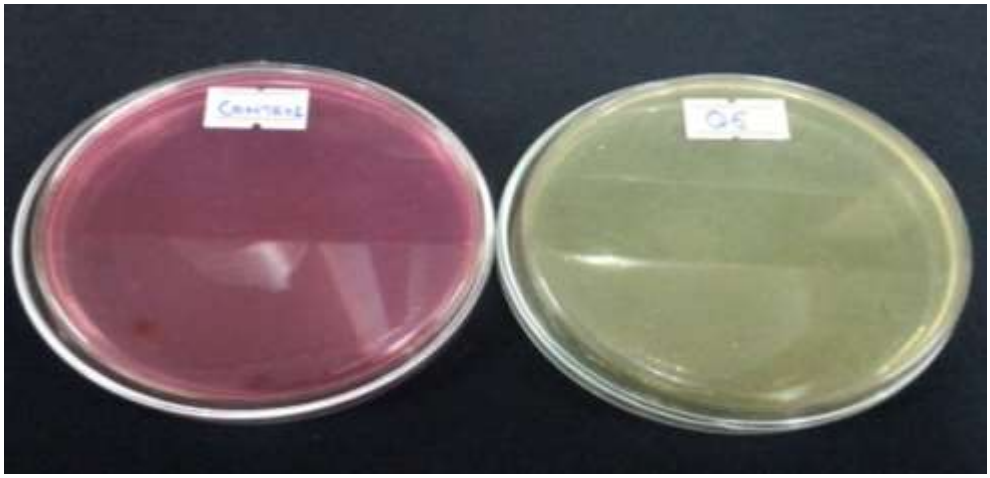


Figure 4.8. Strain Q5 on Phenol-Red Agar Plate.

Figure 4.9 Strain Q6 on Phenol-Red Agar Plate.

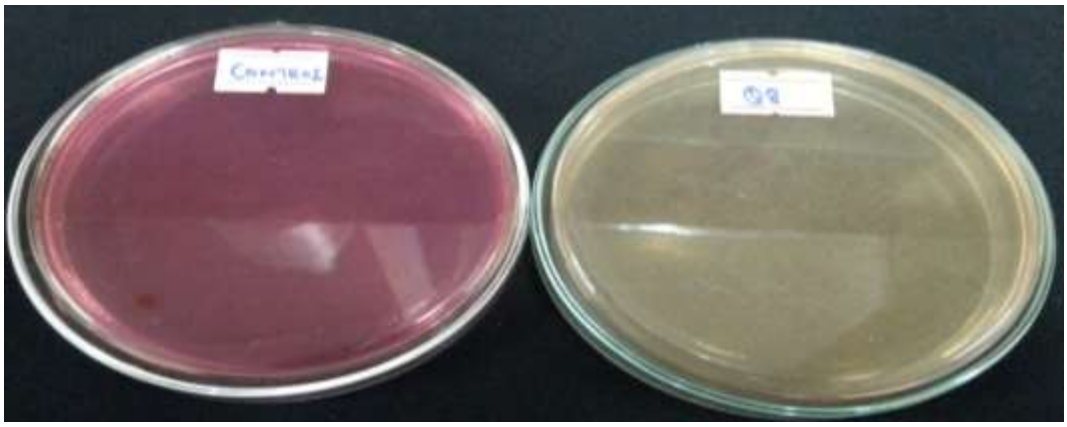


Figure 4.10 Strain Q8 on Phenol-Red Agar Plate.

Figure 4.11 Strain FH1 on Phenol-Red Agar Plate.

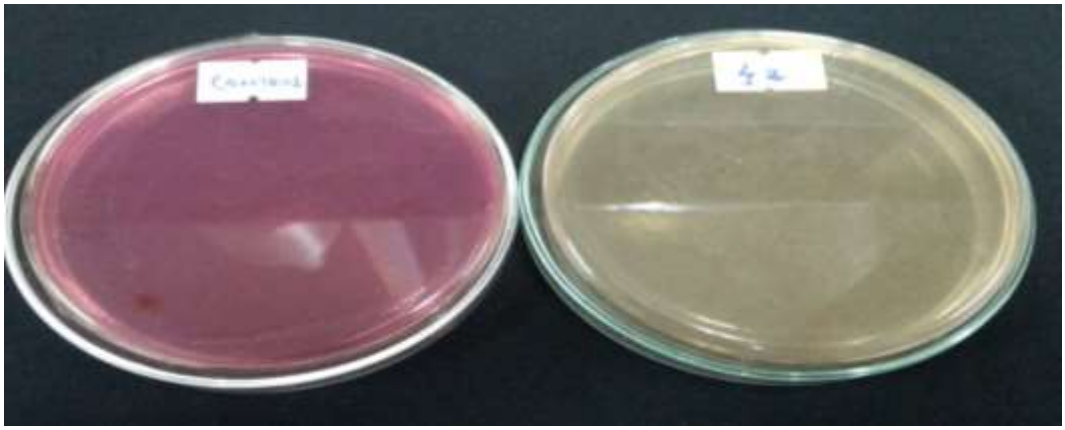


Figure 4.12 Strain *S. aureus* ATCC 6538 on Phenol-Red Agar Plate.

Inoculation on Rhodamine B Olive Oil Agar Plates:

Selected isolated bacterial strains were also streaked on Rhodamine B Olive Oil Agar Plates. After growth lipase positive strains glowed fluorescent orange when observed under UV light.

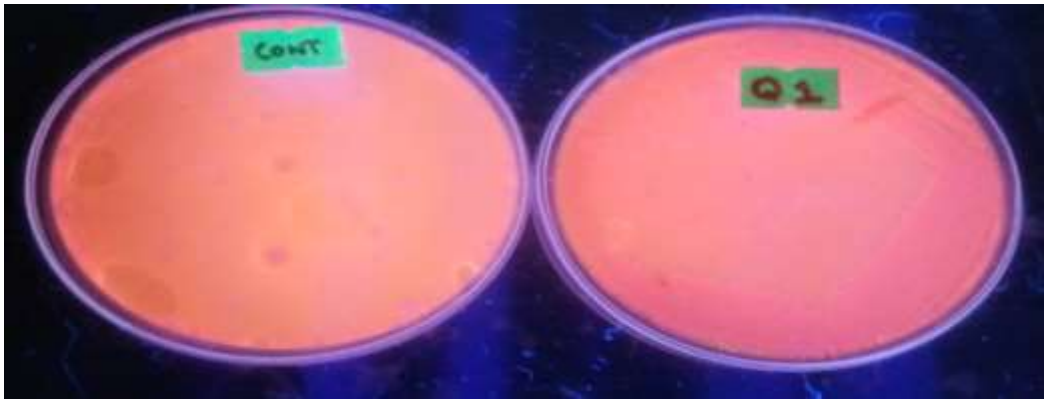


Figure 4.13 Strain Q1 on Rhodamine B Olive Oil Agar Plates.

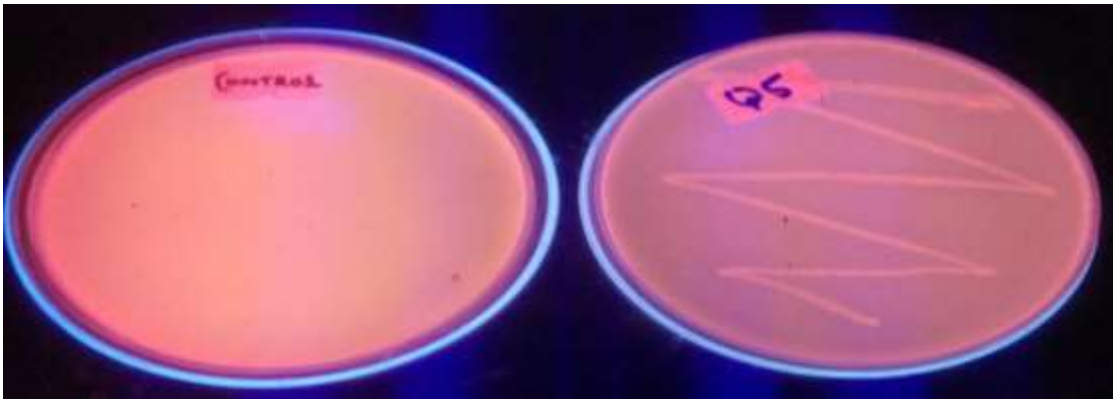


Figure 4.14 Strain Q5 on Rhodamine B Olive Oil Agar Plates.

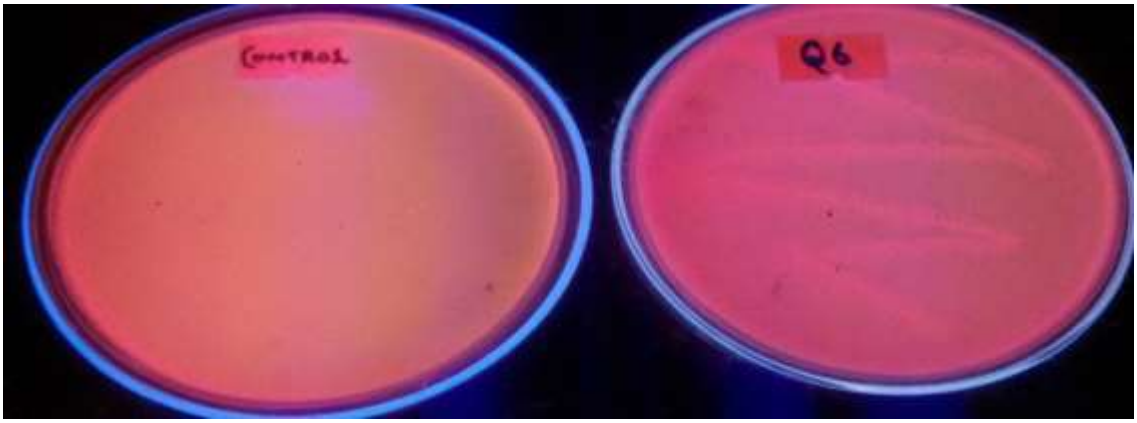


Figure 4.15 Strain Q6 on Rhodamine B Olive Oil Agar Plates.

Figure 4.16 Strain Q8 on Rhodamine B Olive Oil Agar Plates.

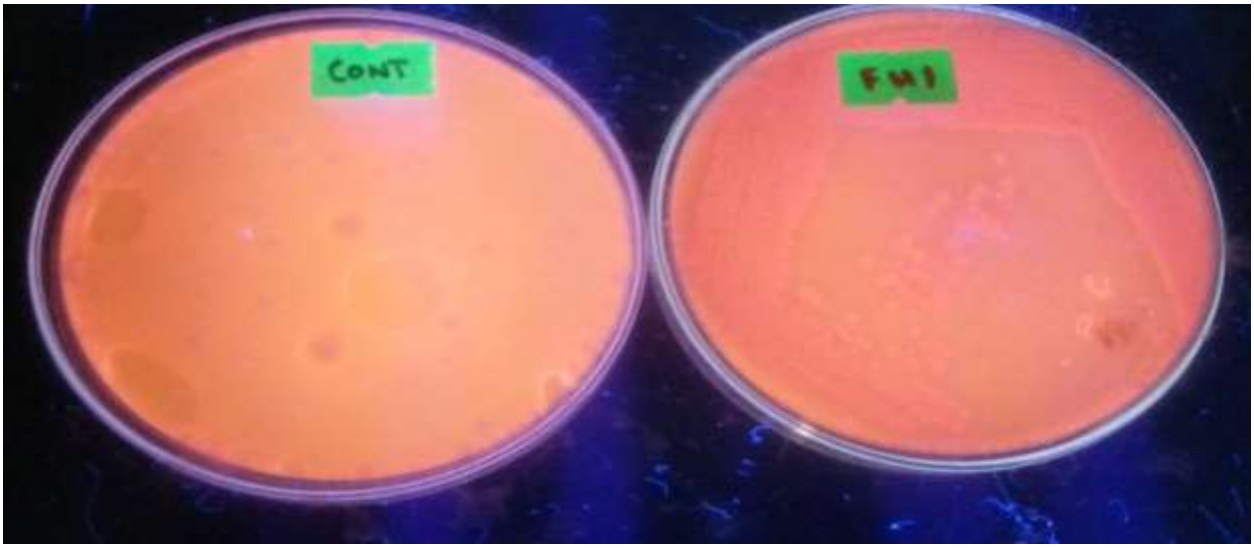


Figure 4.17 Strain FH1 on Rhodamine B Olive Oil Agar Plates.

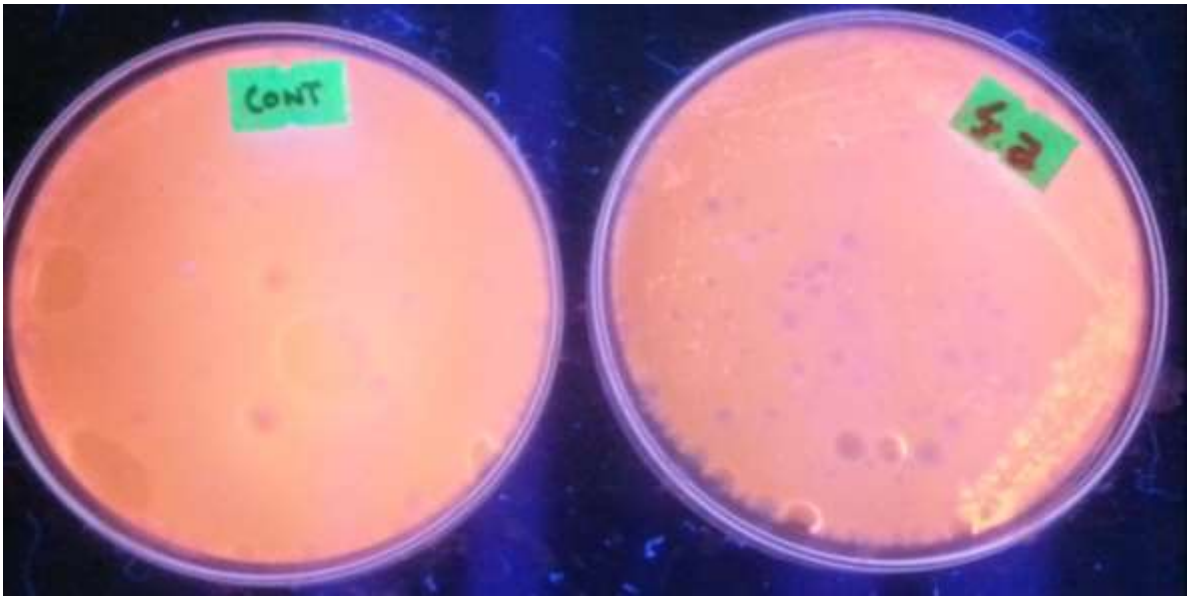


Figure 4.18 *S. aureus* ATCC 6538 on Rhodamine B Olive Oil Agar Plates.

4.3 Gram staining:

Gram staining was performed to differentiate between gram positive and gram negative strains and its results are shown in table 4.2.

Table 4.2 Gram staining results of bacterial strains.

Bacterial Strains	**Gram Positive/Negative**
Q1	Gram positive
Q5	Gram positive
Q6	Gram positive
Q8	Gram negative
FH1	Gram positive
S. aureus ATCC 6538	Gram positive

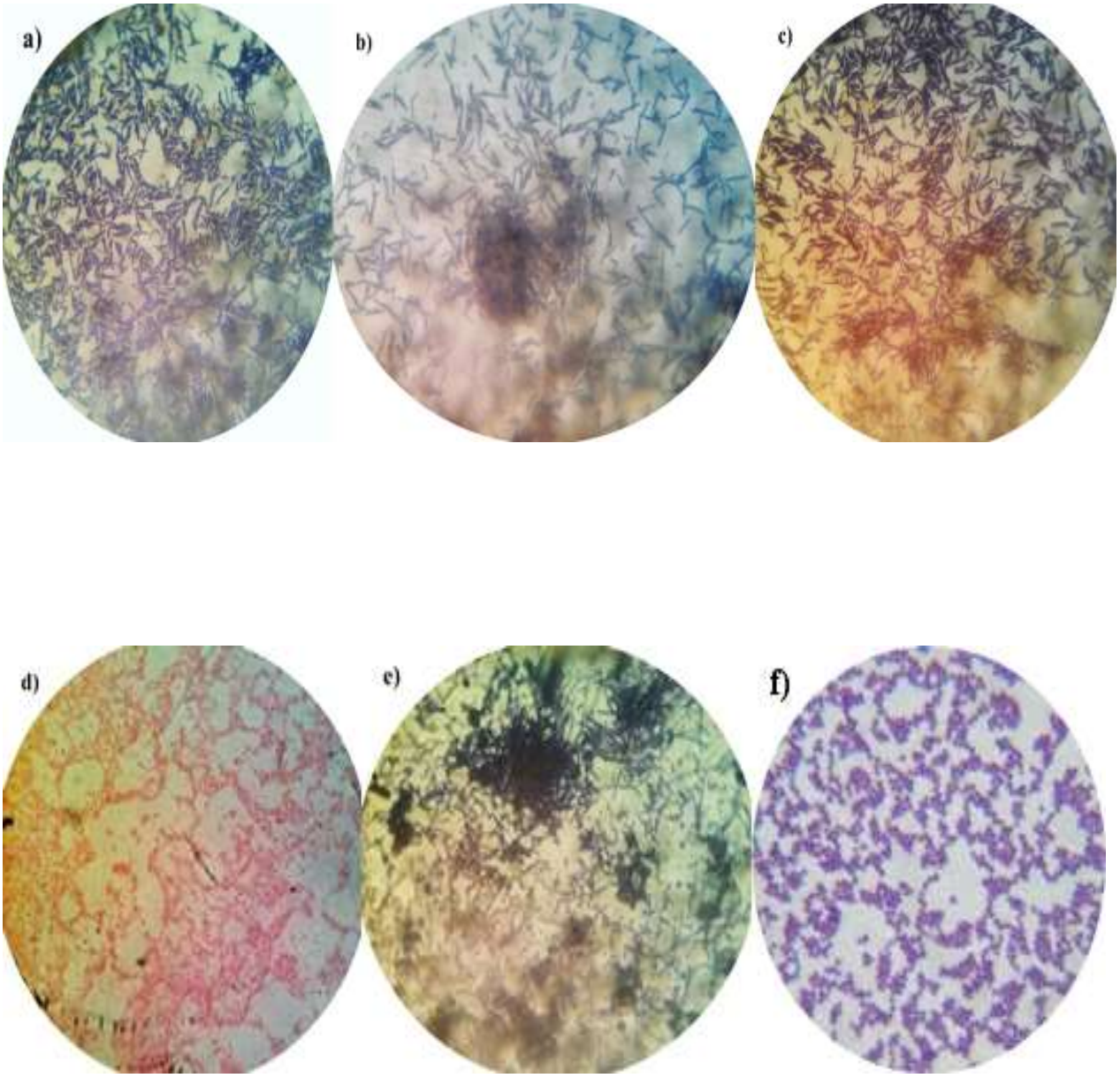


Figure 4.19 Gram staining of a) Q1, b) Q5, c) Q6, d) Q8, e) FH1, f) *S. aureus* ATCC 6538

4.4 Biochemical Testing:

Biochemical tests in accordance with Bergey's Manual of Determinative Bacteriology 9th Edition were carried out for the identification of isolated bacterial strains. Results for biochemical identification are given in table 4.3.

Table 4.3 Results for Biochemical Testing of Isolates.

Isolates	Catalase	Nitrate	Citrate Utilization	TSI	Urease	Oxidase	Methyl Red	Vogues Proskauer	Identification
Q1	+	+	+	**A/A**	-	+	-	+	***Bacillus* sp.**
Q5	+	+	+	**A/A**	-	+	-	+	***Bacillus* sp.**
Q6	+	-	+	**K/A**	-	+	-	+	***Bacillus* sp.**
Q8	+	-	+	**K/K**	+	+	+	+	***Pseudomonas* sp.**
FH1	+	+	+	**A/A**	-	+	-	+	***Bacillus* sp.**

4.5 DNA extraction and Gel Electrophoresis:

After extraction of DNA via CTAB method, 1% agarose gel electrophoresis was performed on the sample to confirm the presence of DNA. After running the sample in gel for 30 minutes, gel was observed under UV light to examine DNA bands.

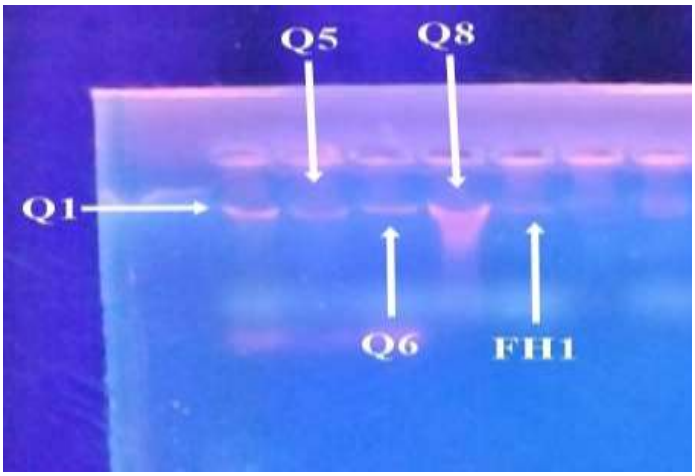


Figure 4.20 DNA bands in 1% Agarose Gel under UV.

4.6 16S rRNA Sequencing and Phylogenetic Tree:

Sequencing was commercially performed by Macrogen Standard Custom DNA Sequencing Service Korea. Two selected strains Q1 and Q5 were sent for sequencing and the received sequences were subjected to phylogenetic analysis. The phylogenetic analysis of the 16S rRNA sequence showed that both the strains belonged to genus Bacillus. Strain Q1 showed 99% similarity with *Bacillus subtilis* strain OPTB45 (KP225289) followed by *Bacillus* sp. WDM2 (KT735241) hence it was designated as *Bacillus subtilis* Q1. Strain Q5 was found to be 99% similar to *Bacillus subtilis* C17 (KU681037) and so was designated as *Bacillus subtilis* Q5. The phylogenetic tree for strains Q1 and Q5 constructed by using neighbor joining (NJ) method is shown in figure 4.2.1.

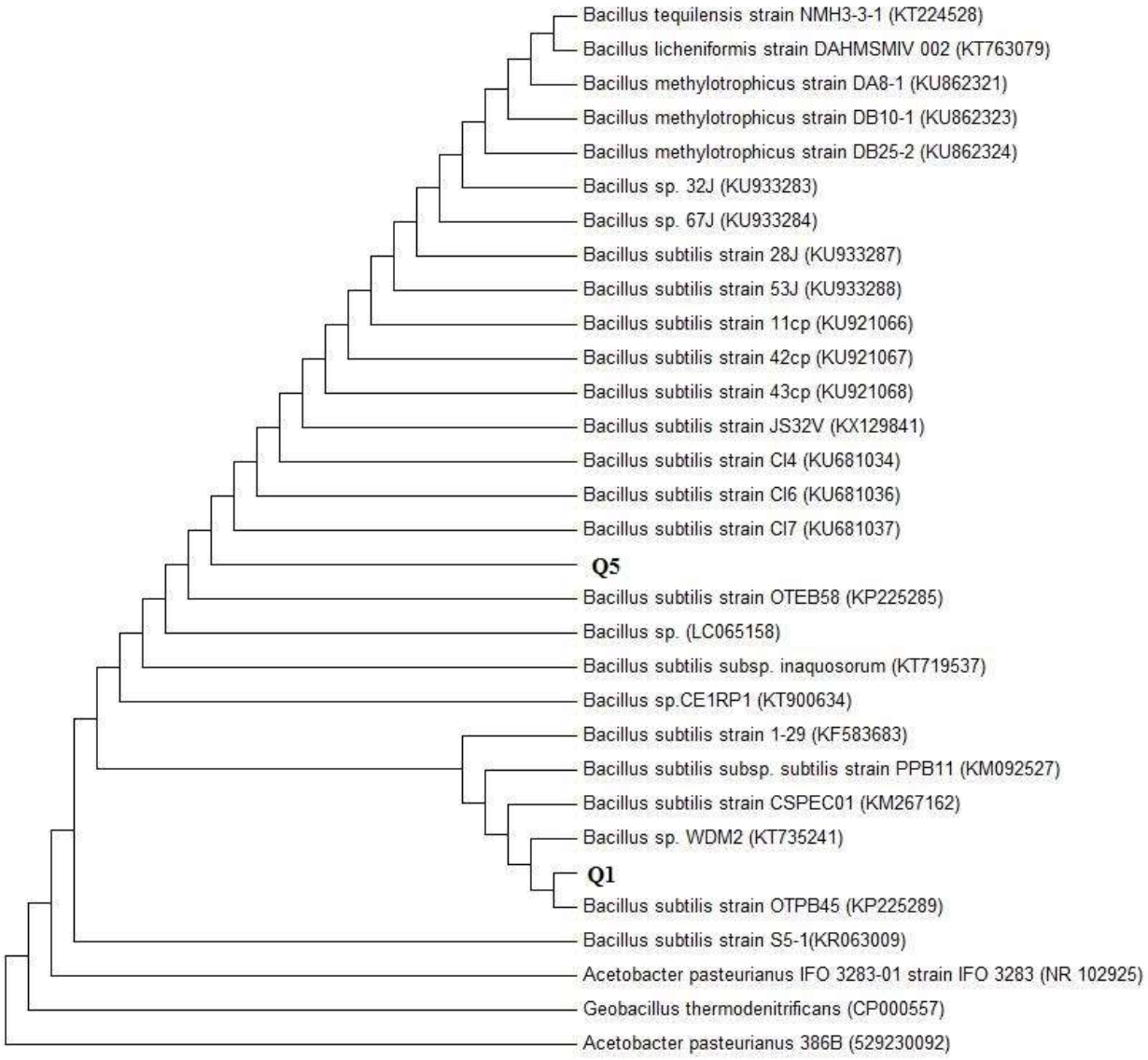


Figure 4.21 Phylogenetic tree for partial 16S rRNA gene sequences from isolates Q1 and Q5 showing relationships between presented strains and related sequences.

4.7 Enzyme Activity:

Both crude enzyme activity and specific activity of lipase produced by bacterial isolates and *S. aureus* ATCC 6538 was estimated through lipase assay and protein estimation and

by estimating the unknown amount of para-nitro phenol through standard curve of PNP in tris-Hcl buffer. Unknown amount of protein produced by microorganisms was estimated through standard curve of bovine-serum albumin. One-unit enzyme activity was defined as amount of enzyme catalyzing the release of 1 µM of para-nitro phenol per minute at 37°C and pH 7 using para-nitro phenyl laurate as substrate.

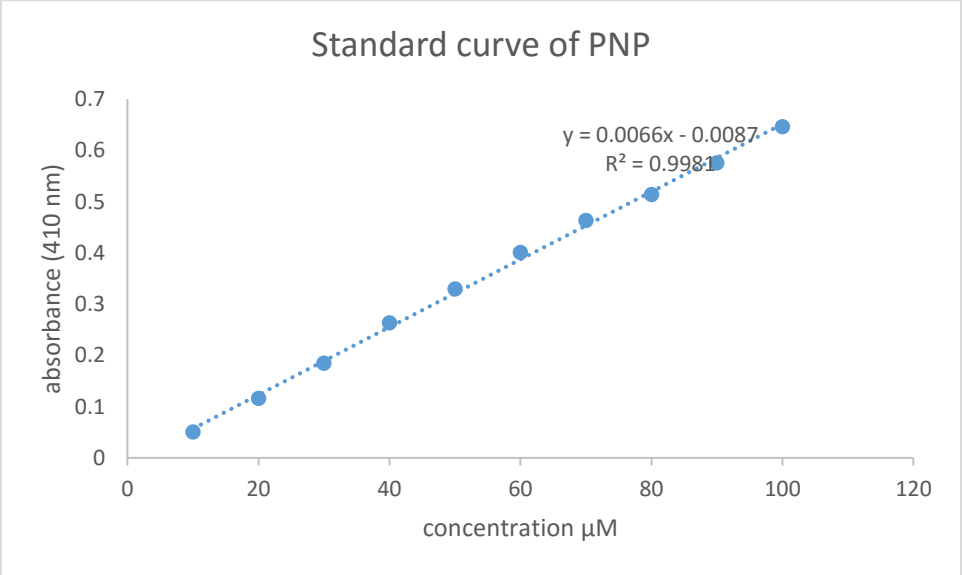


Figure 4.22 Standard curve of para-Nitro phenol.

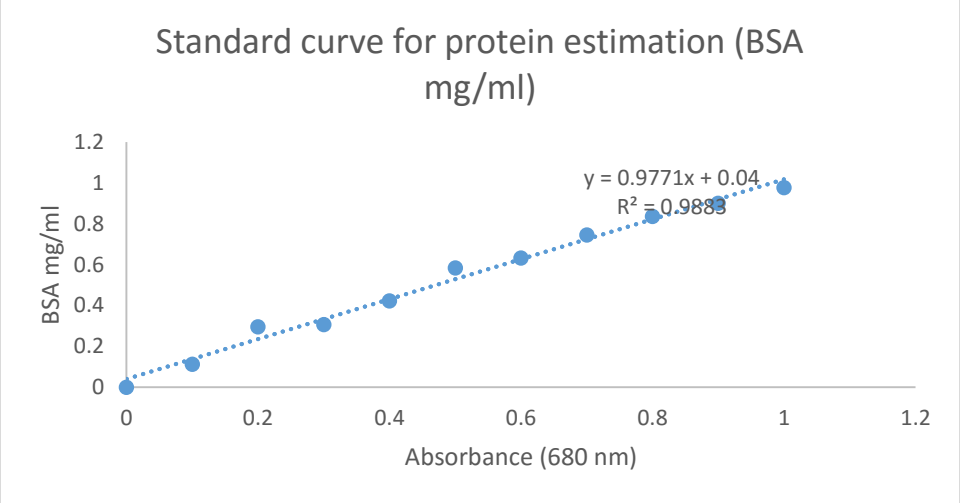


Figure 4.23 Standard curve of Bovine-Serum Albumin.

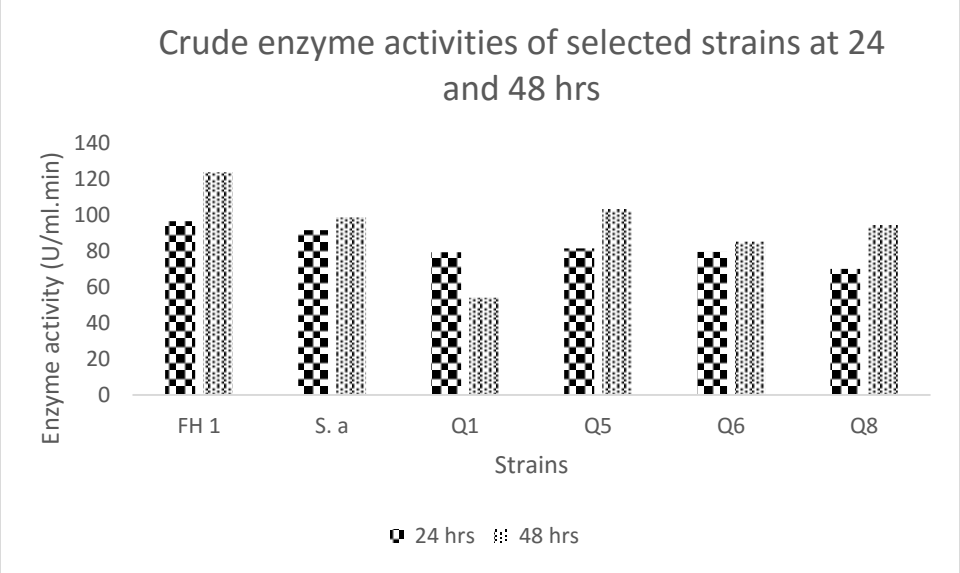


Figure 4.24 Crude Enzyme Activity of Lipase produced by selected lipolytic bacterial strains during 24 and 48 hours.

Highest enzyme activity at both 24 and 48 hours was given by strin FH1.

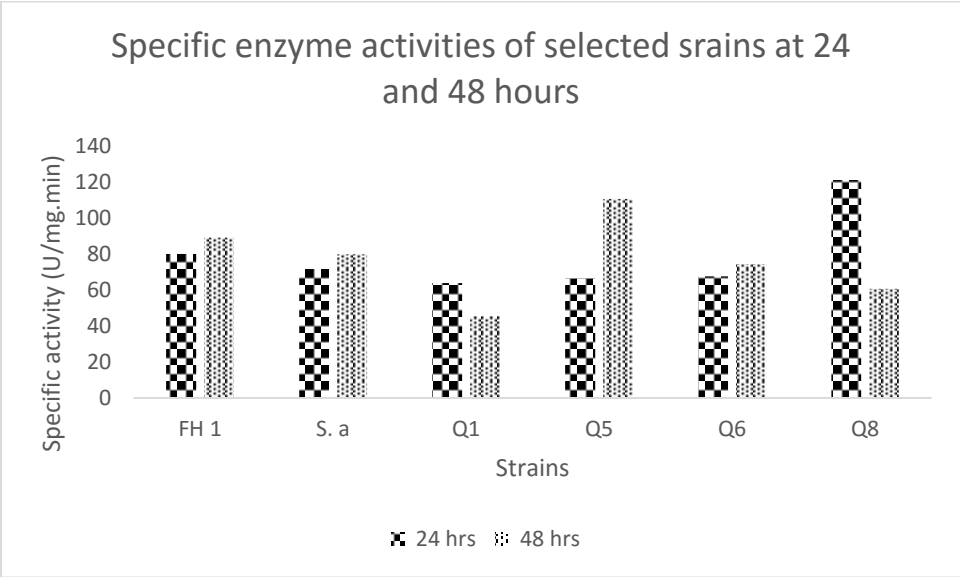


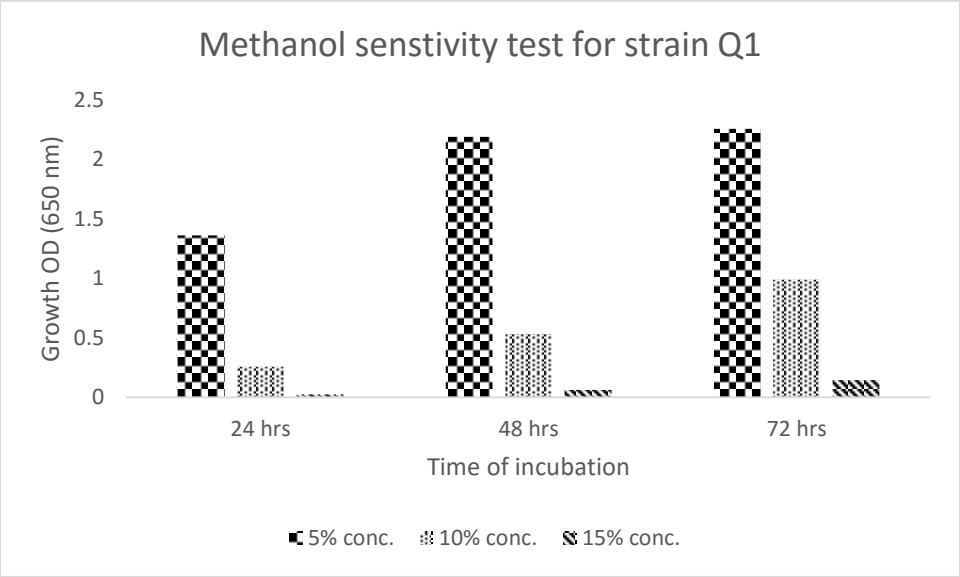
Figure 4.25 Specific Enzyme Activity of lipase produced by lipolytic bacterial strains at 24 and 48 hours.

Q8 gave highest specific activity at 24 hours whereas highest specific activity at 48 hours was observed with strain Q5.

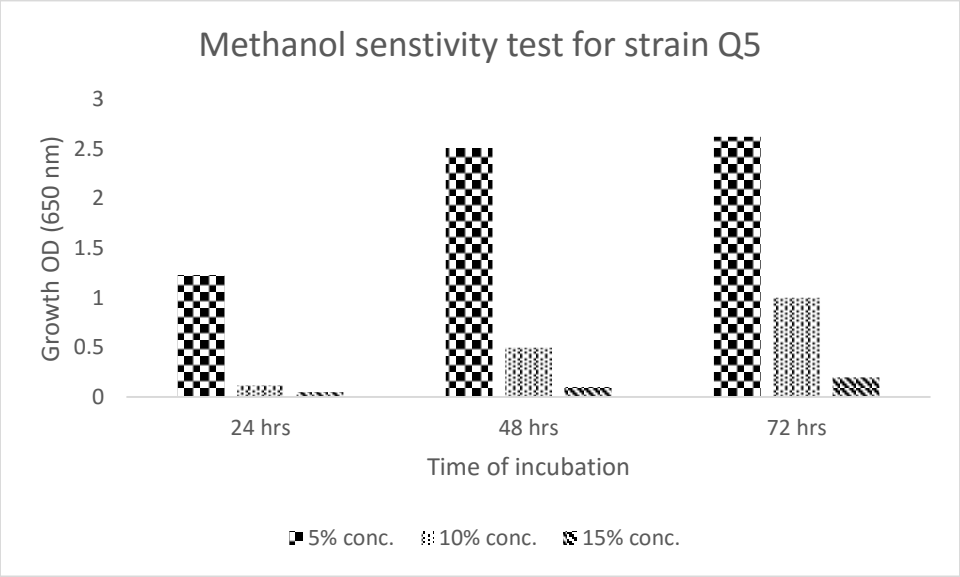
4.8 Methanol Senstivity Test:

Methanol senstivity test was performed to checkmethanol tolerance of strains. All the strains showed good growth at 5% of methanol concentration in 24 hrs, 48 and 72 hours. Low growth was observed at 10% concentration of methanol at 24, 48 and 72 hours for all the strains and a very low growth OD was obtained for all the strains at 15% concentratin of methanol even after 72 hours of incubation.

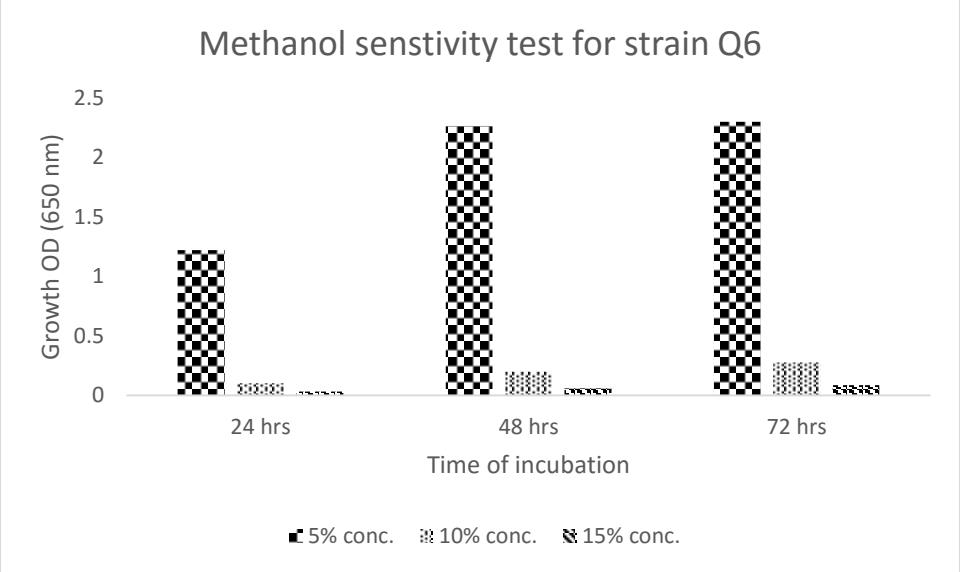
a)



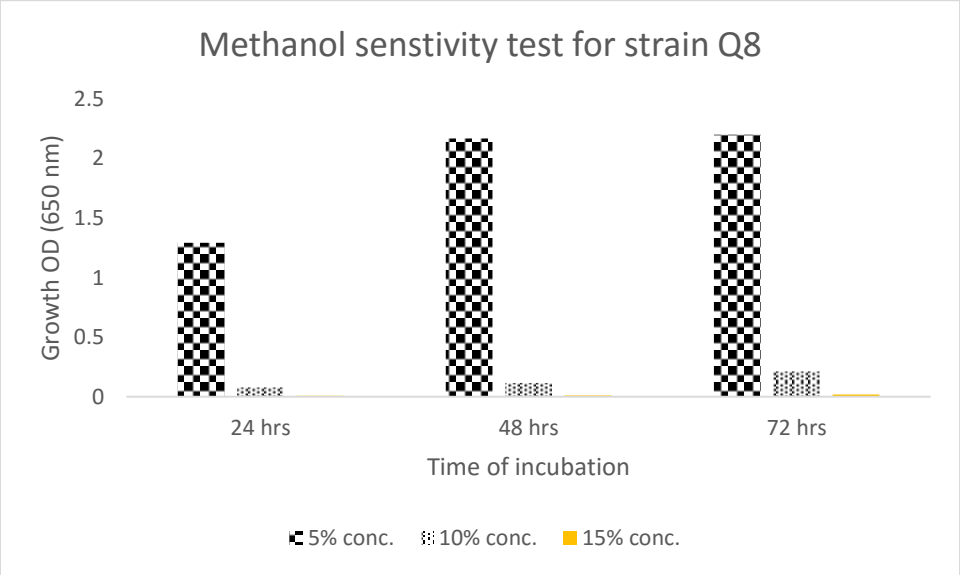
b)


Methanol senstivity test for strain Q5
Growth OD (650 nm)
3
2.5
2
1.5
1
0.5
0
24 hrs
48 hrs
72 hrs
Time of incubation
5% conc.
10% conc.
15% conc.

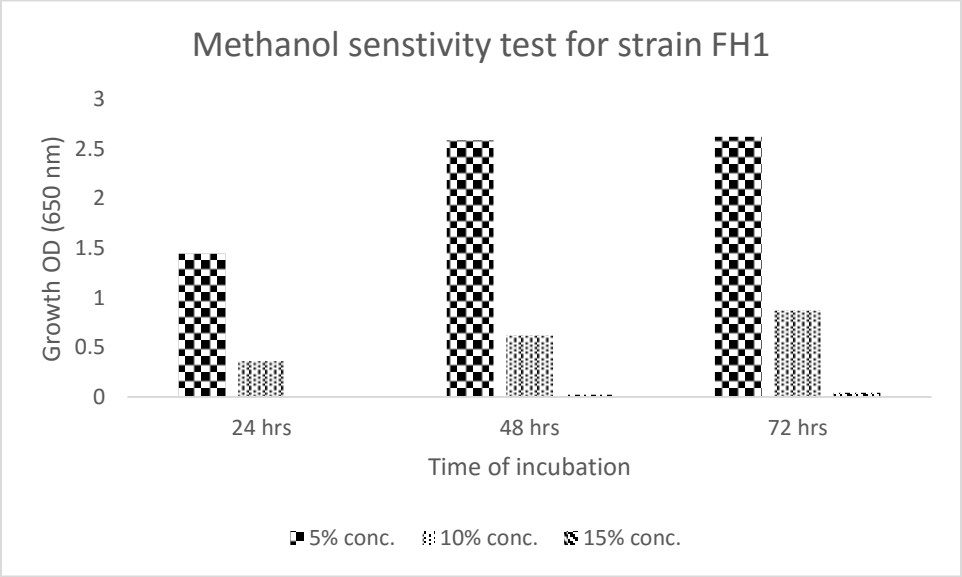
c)


Methanol senstivity test for strain Q6
Growth OD (650 nm)
2.5
2
1.5
1
0.5
0
24 hrs
48 hrs
72 hrs
Time of incubation
5% conc.
10% conc.
15% conc.

d)


Methanol senstivity test for strain Q8
Growth OD (650 nm)
2.5
2
1.5
1
0.5
0
24 hrs
48 hrs
72 hrs
Time of incubation
5% conc.
10% conc.
15% conc.

e)



f)

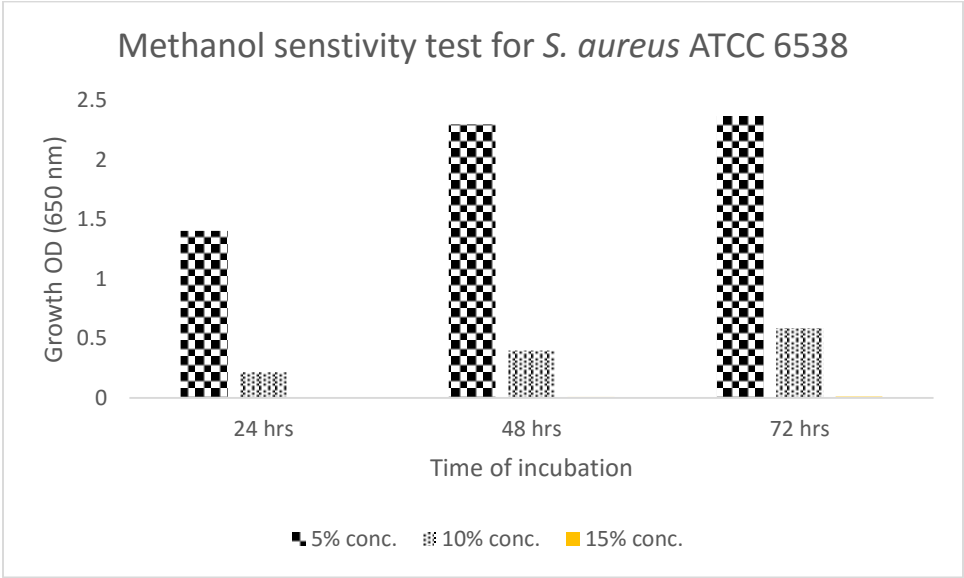
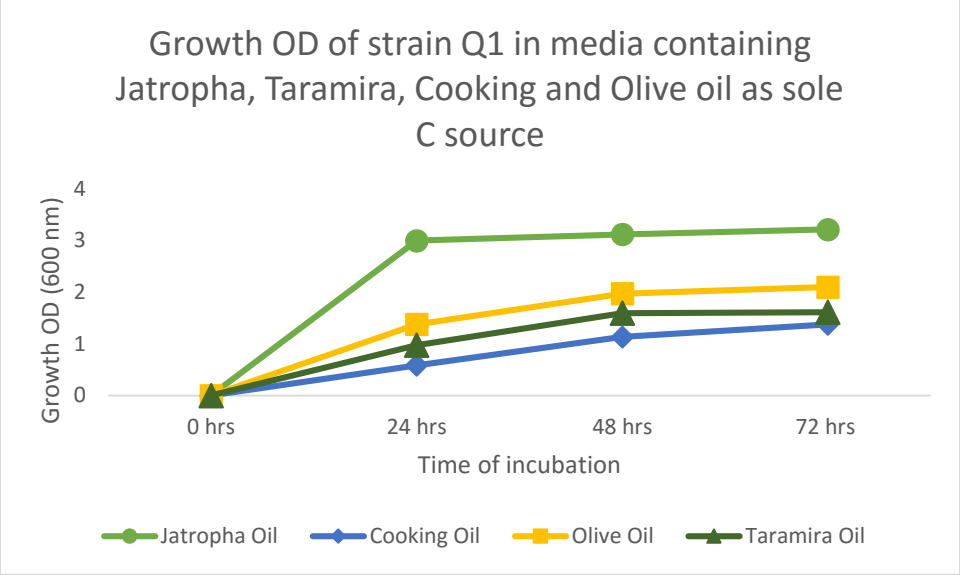


Figure 4.26 Growth ODs at 5%, 10% and 15% concentraion of methanol during incubation for 24, 48 and 72 hours for strain a) Q1, b) Q5, c) Q6, d) Q8, e) FH1 and f) *S. aureus* ATCC6538.

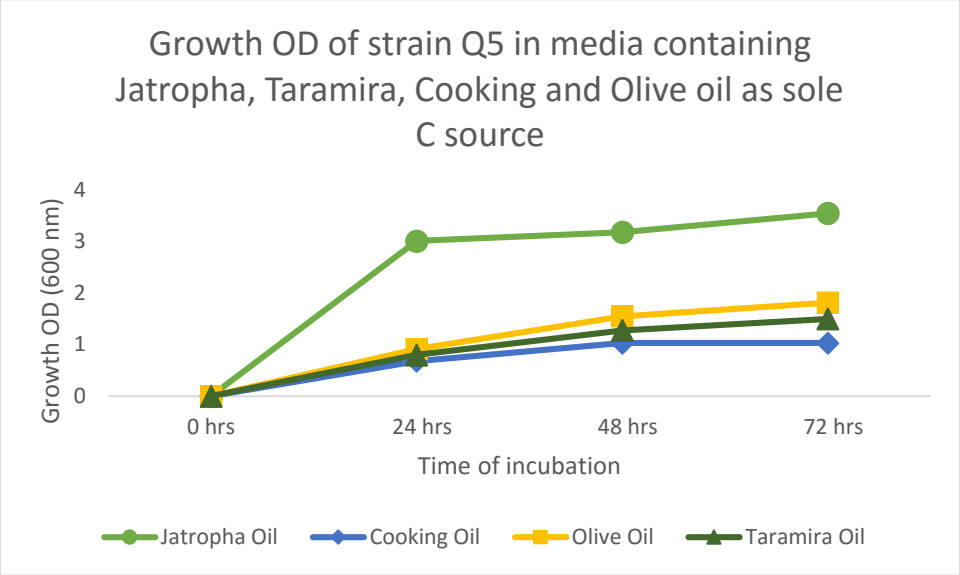
4.9 Oil Toxicity Test:

Jatropha oil and taramira oil used in this study were reported to have toxic effect on bacteria so oil toxicity test was conducted to check wether these oils inhibit the selected lipolytic bacterial strains for biodiesel production or not. Olive oil and Cooking oil were run as refrence in these experiments to relate the growth pattern of strains in these oils with growth pattern in jatropha oil and taramira oil. All the selected lipolytic strains showed good growth in both jatropha oil and taramira oil. All the strains achieved highest growth OD at 24 hours with jatrophs oil except for *S. aureus* ATCC 6538. No growth inhibition of strains was observed with the suspected oils. As oils were the carbon source in media, results showed that strains produced lipase in the pressence of all these oils.

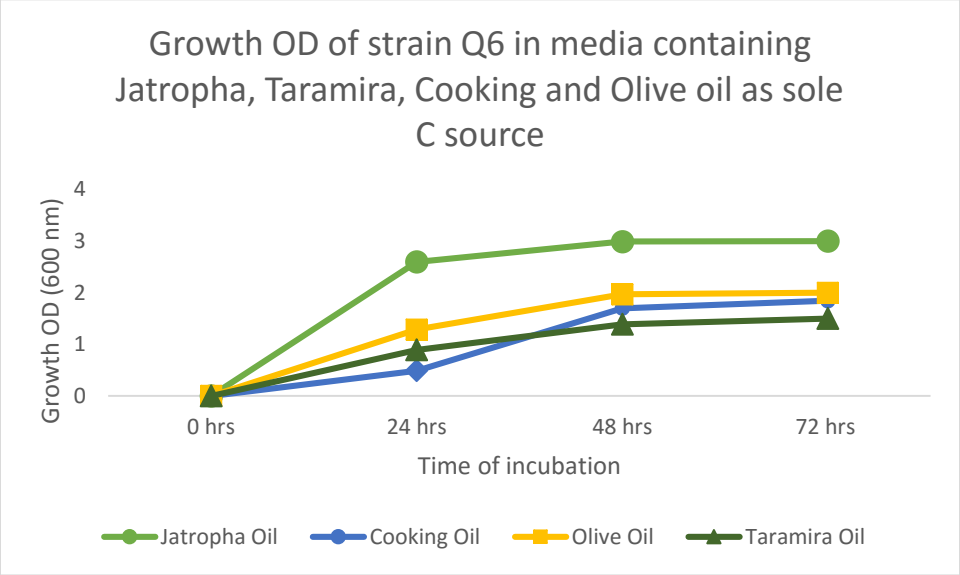
a)



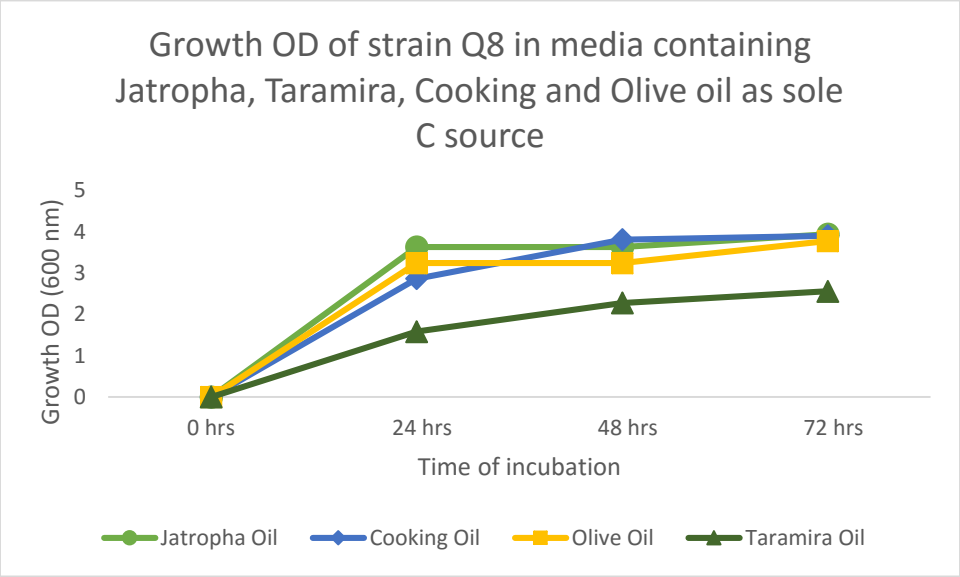
b)


Growth OD of strain Q5 in media containing Jatropha, Taramira, Cooking and Olive oil as sole C source
Growth OD (600 nm)
4
3
2
1
0
0 hrs
24 hrs
48 hrs
72 hrs
Time of incubation
Jatropha Oil
Cooking Oil
Olive Oil
Taramira Oil

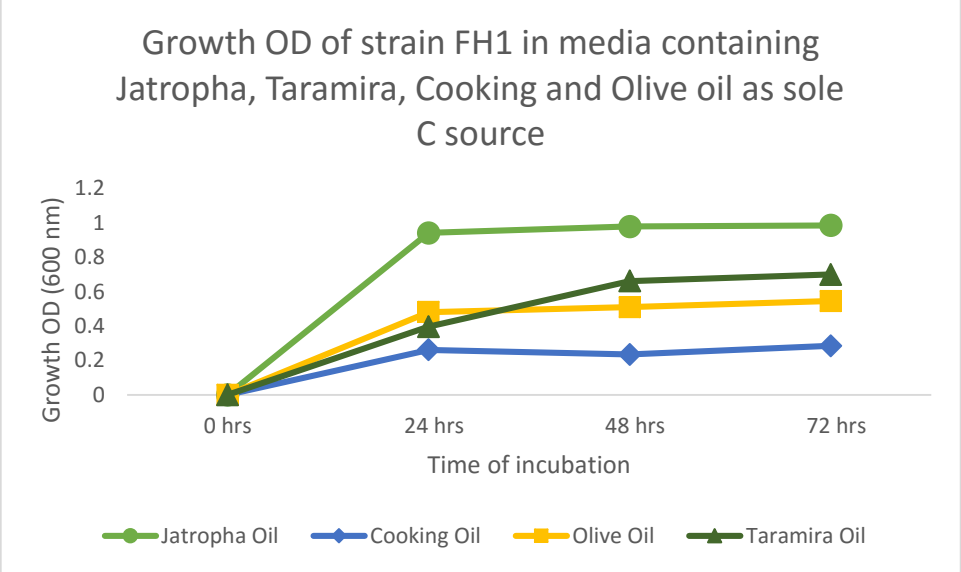
c)


Growth OD of strain Q6 in media containing Jatropha, Taramira, Cooking and Olive oil as sole C source
Growth OD (600 nm)
4
3
2
1
0
0 hrs
24 hrs
48 hrs
72 hrs
Time of incubation
Jatropha Oil
Cooking Oil
Olive Oil
Taramira Oil

d)


Growth OD of strain Q8 in media containing Jatropha, Taramira, Cooking and Olive oil as sole C source
Growth OD (600 nm)
5
4
3
2
1
0
0 hrs
24 hrs
48 hrs
72 hrs
Time of incubation
Jatropha Oil
Cooking Oil
Olive Oil
Taramira Oil

e)


Growth OD of strain FH1 in media containing Jatropha, Taramira, Cooking and Olive oil as sole C source
Growth OD (600 nm)
1.2
1
0.8
0.6
0.4
0.2
0
0 hrs
24 hrs
48 hrs
72 hrs
Time of incubation
Jatropha Oil
Cooking Oil
Olive Oil
Taramira Oil

f)

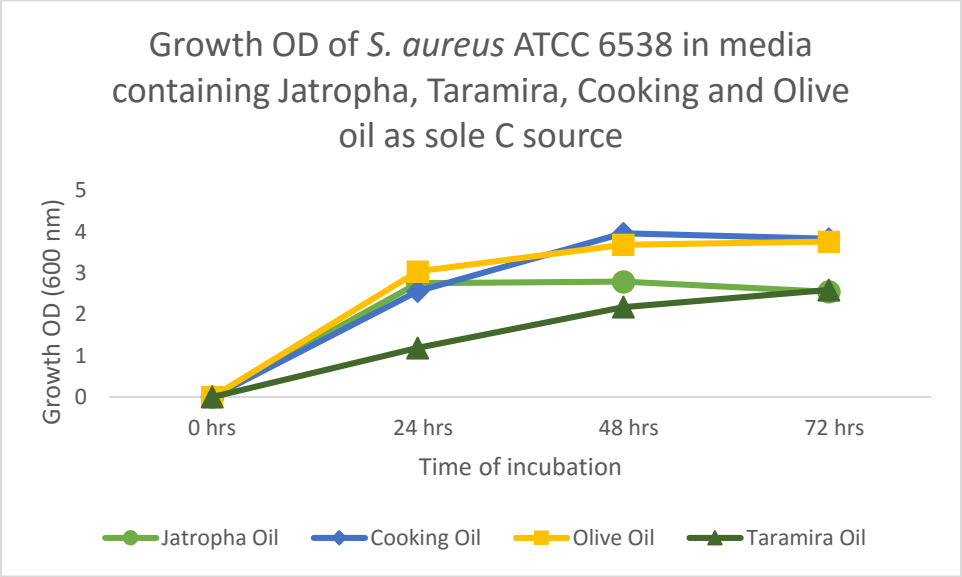


Figure 4.27 Growth OD, in media containing Jatropha, Taramira, Cooking and Olive oil as sole carbon source, of lipolytic bacterial strains a) Q1, b) Q5, c) Q6, d) Q8, e) FH1 and f) *S. aureus* ATCC6538.

4.10 Bioremediation study:

Bioremediation study for each strain and for two consortia C1 having strains Q1, Q6 and *S. aureus* ATCC 6538 and C2 having strains Q5, Q8 and *S. aureus* ATCC 6538 was conducted in batch system for 8 days. Initial COD after mixing MSM with Wastewater came out to be 2352 mg/L. Highest COD reduction was observed with both strain Q1 and consortia C2 with 90% reduction at eighth day. Strain Q5 gave the lowest percentage reduction of COD in eight days.

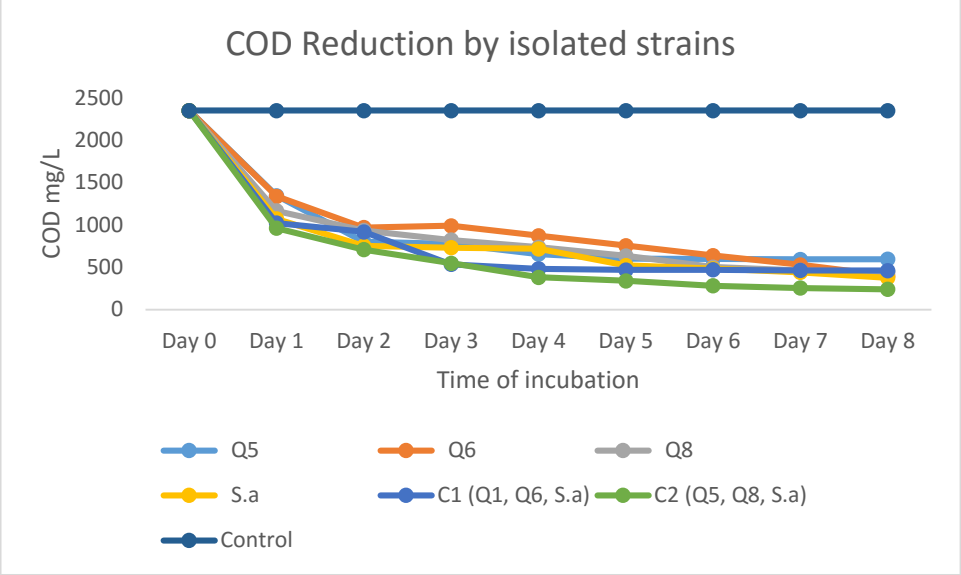


Figure 4.28 COD Reduction by bacterial strains in eight days.

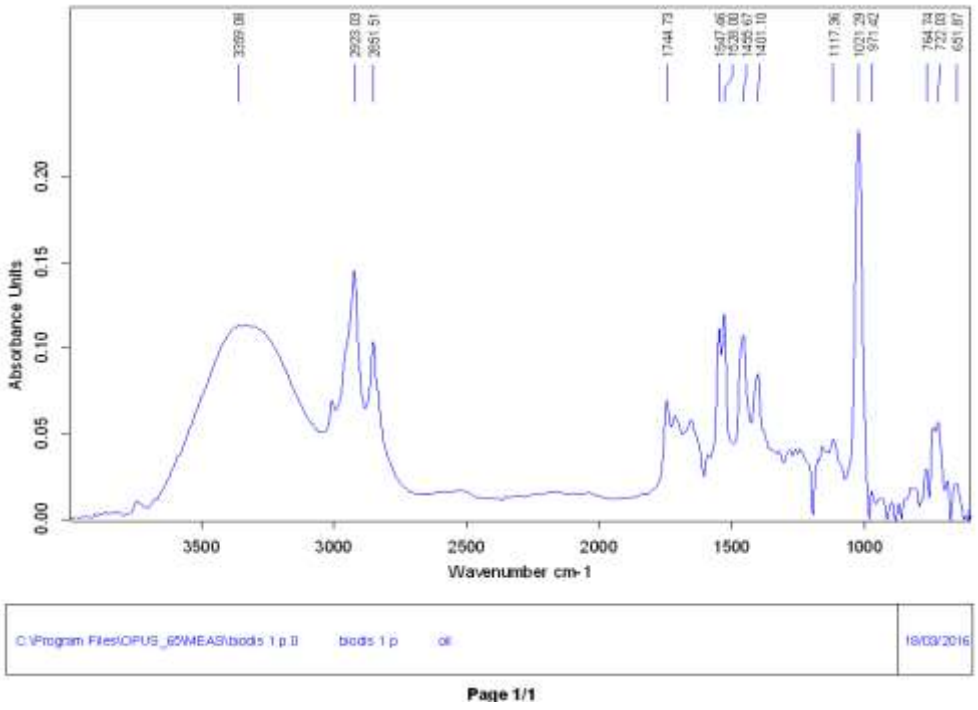
Table 4.4 Percentage Reduction of COD by Isolated Bacterial Strains.

Strains	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 8**
Q 1	46%	77%	85%	86%	88%	89%	89.2%	90%
Q 5	43%	66%	67%	72%	74%	74.5%	74.7%	74.7%
Q 6	43%	59%	60%	63%	68%	73%	77%	83%
Q 8	50%	60%	65%	69%	73%	79%	80%	81%
S. aur	54%	68%	69%	72%	78%	79%	81%	84%
C1	57%	60%	77%	79%	80%	80%	80%	80%
C2	59%	70%	77%	84%	86%	88%	89%	90%

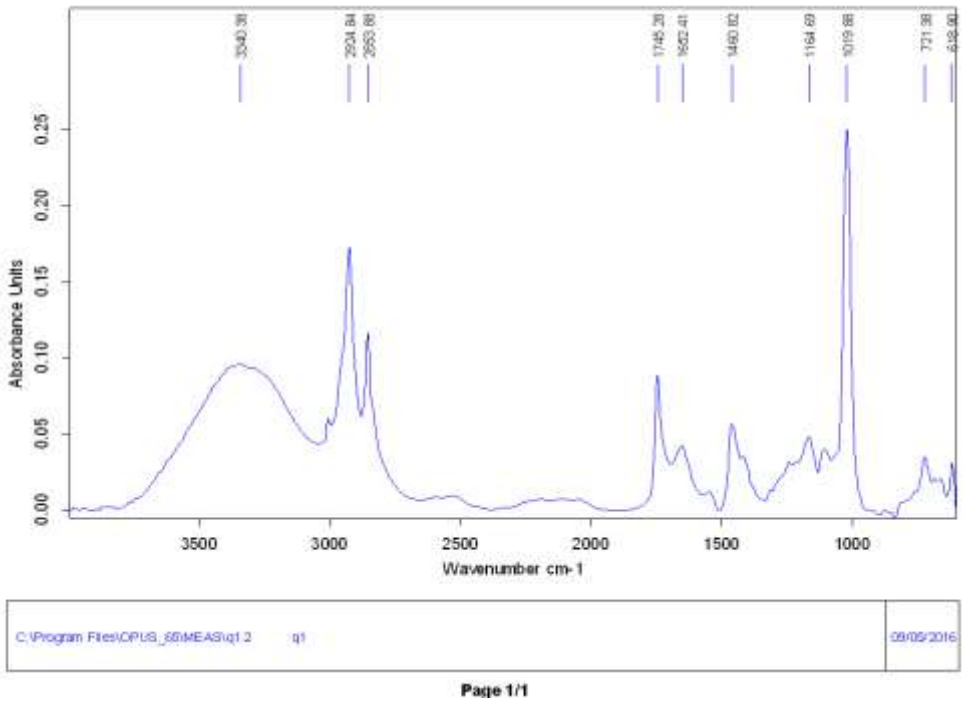
4.11 FTIR Analysis of Biodiesel produced via Microbial Synthesis:

Biodiesel production was carried out through all the six strains Q1, Q5, Q6, Q8, FH1 and *S. aureus* ATCC 6538 from four oils jatropha, taramira, soybean and mustard oil. Samples of produced biodiesel were then analyzed through FTIR. Biodiesel contains methyl or ethyl esters depending upon the type of alcohol used for transesterification reaction. If the sample contains ester, ester peak at frequency (cm^{-1}) range of 1735 to 1750 is observed in the infrared (IR) spectrum after infrared spectroscopy of sample. All the biodiesel samples had ester peaks in their IR spectra which confirmed biodiesel production.

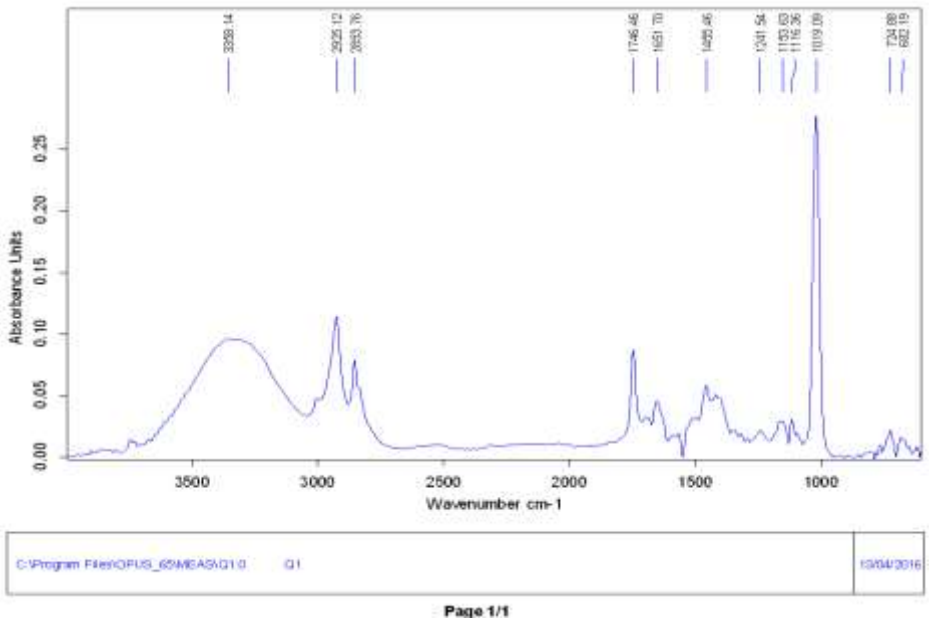
a)



b)


3340.38
2934.84
2853.88
1745.28
1652.41
1460.82
1164.69
1019.88
721.38
618.90
Absorbance Units
0.00
0.05
0.10
0.15
0.20
0.25
3500
3000
2500
2000
1500
1000
Wavenumber cm-1
C:\Program Files\OPUS_65\MEAS\q1 2 q1
09/05/2016
Page 1/1

c)


3359.14
2925.12
2853.76
1746.46
1651.70
1465.46
1241.54
1153.63
1116.36
1019.09
724.89
682.19
Absorbance Units
0.00
0.05
0.10
0.15
0.20
0.25
3500
3000
2500
2000
1500
1000
Wavenumber cm-1
C:\Program Files\OPUS_65\MEAS\Q1 0 Q1
10/04/2016
Page 1/1

d)

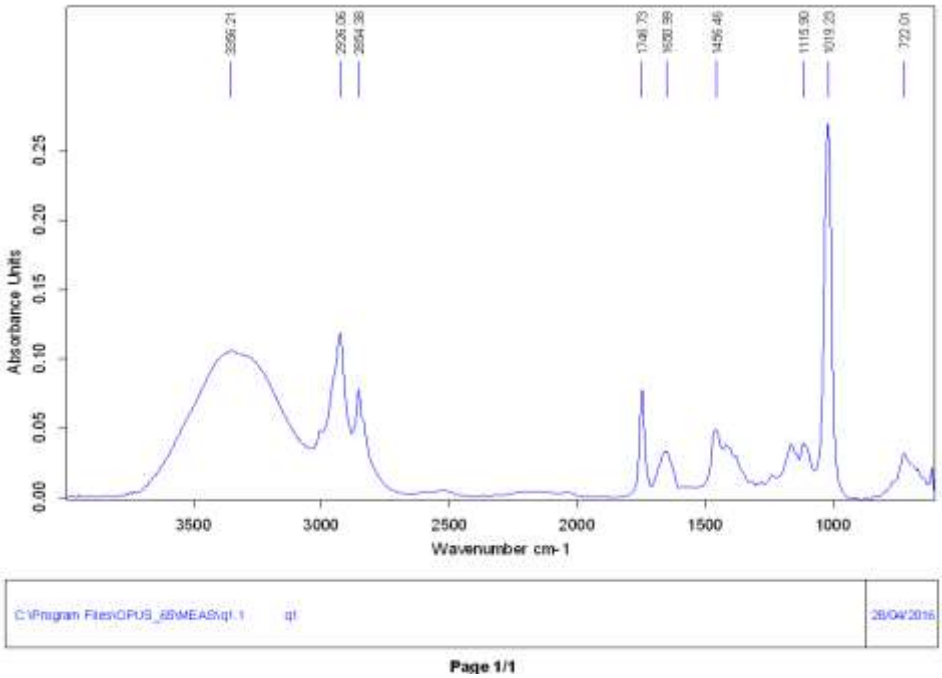
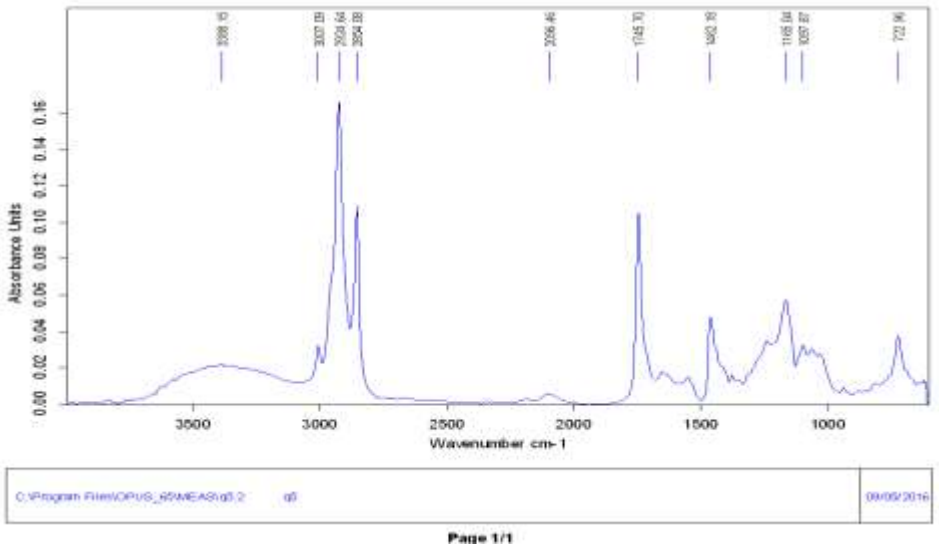
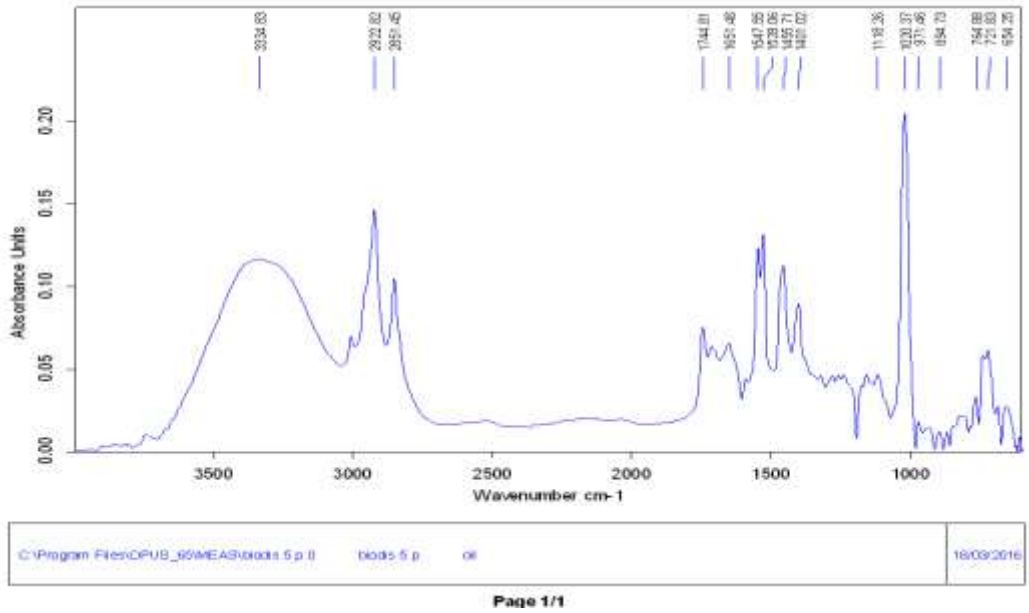


Figure 4.29 Infrared Spectra of Biodiesel produced by strain Q1 from a) Jatropha oil b) Taramira oil c) Mustard oil d) Soybean oil

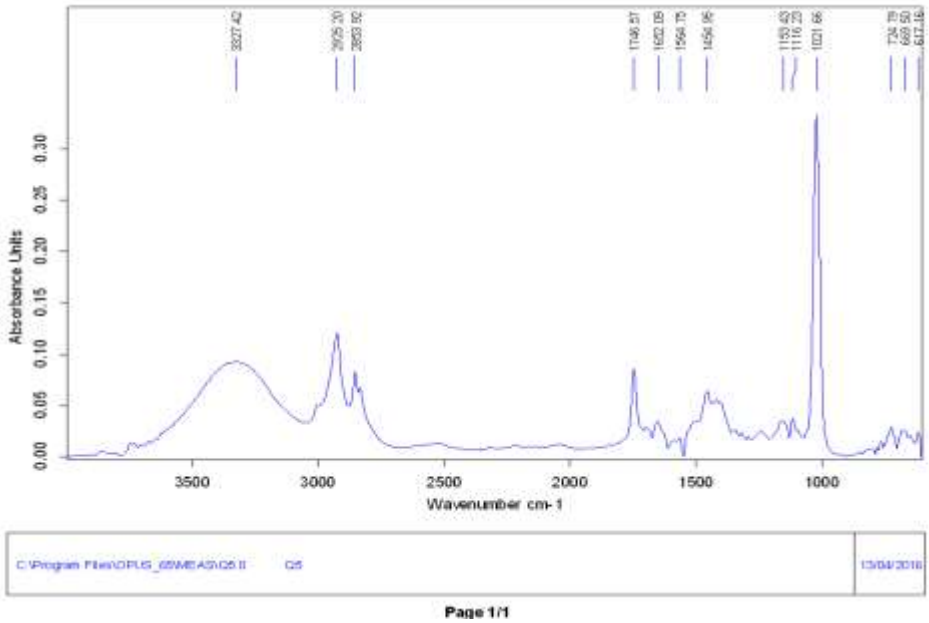
a)



b)


3334.83
2922.82
2851.45
1744.81
1651.48
1547.55
1538.06
1455.71
1401.02
1118.26
1030.37
971.96
894.73
794.88
721.83
654.35
Absorbance Units
0.00
0.05
0.10
0.15
0.20
3500
3000
2500
2000
1500
1000
Wavenumber cm-1
C:\Program Files\OPUS_65\MEAS\biodis 5 p.0
biodis 5 p
oil
18/09/2016
Page 1/1

c)


3327.42
2925.30
2853.92
1746.57
1652.08
1564.75
1456.95
1153.42
1116.23
1021.66
724.79
669.50
617.16
Absorbance Units
0.00
0.05
0.10
0.15
0.20
0.25
0.30
3500
3000
2500
2000
1500
1000
Wavenumber cm-1
C:\Program Files\OPUS_65\MEAS\05.0
05
13/04/2016
Page 1/1

d)

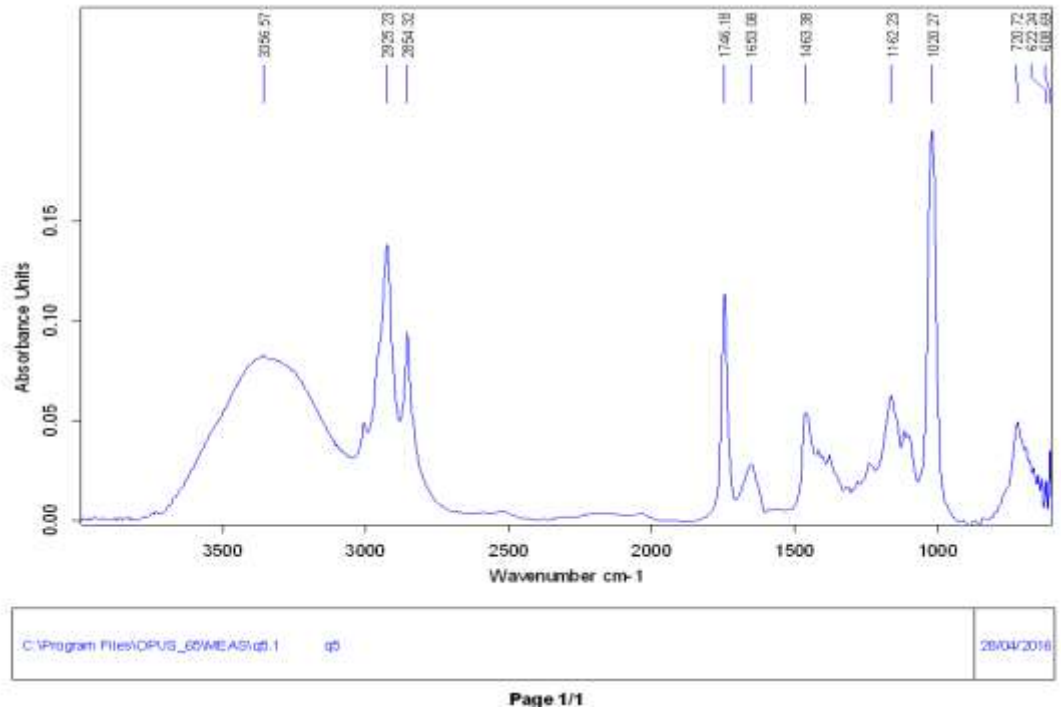
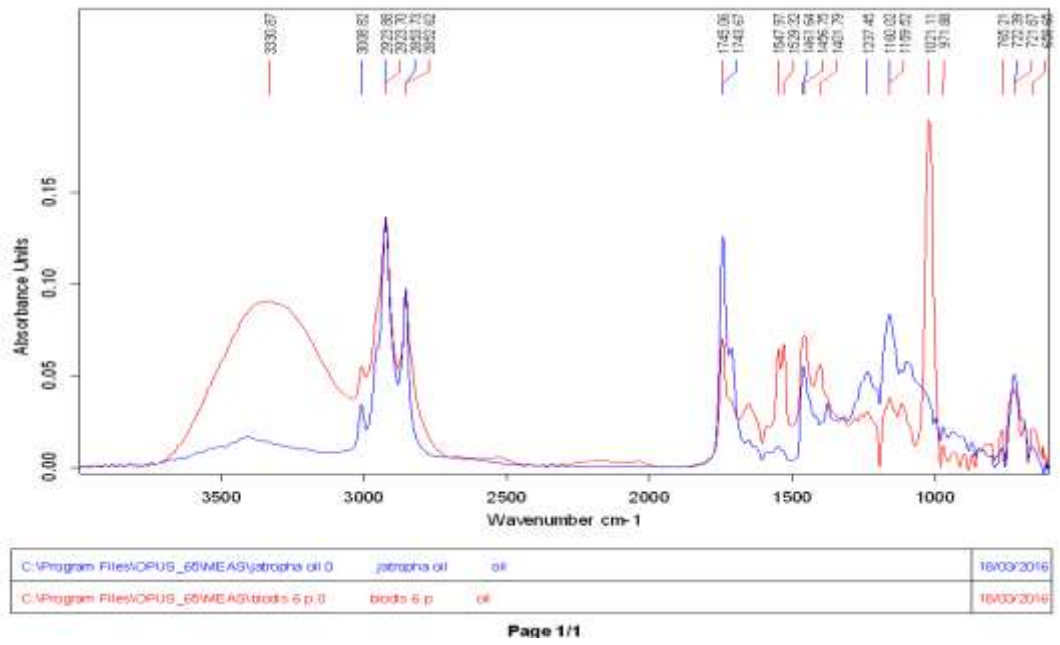
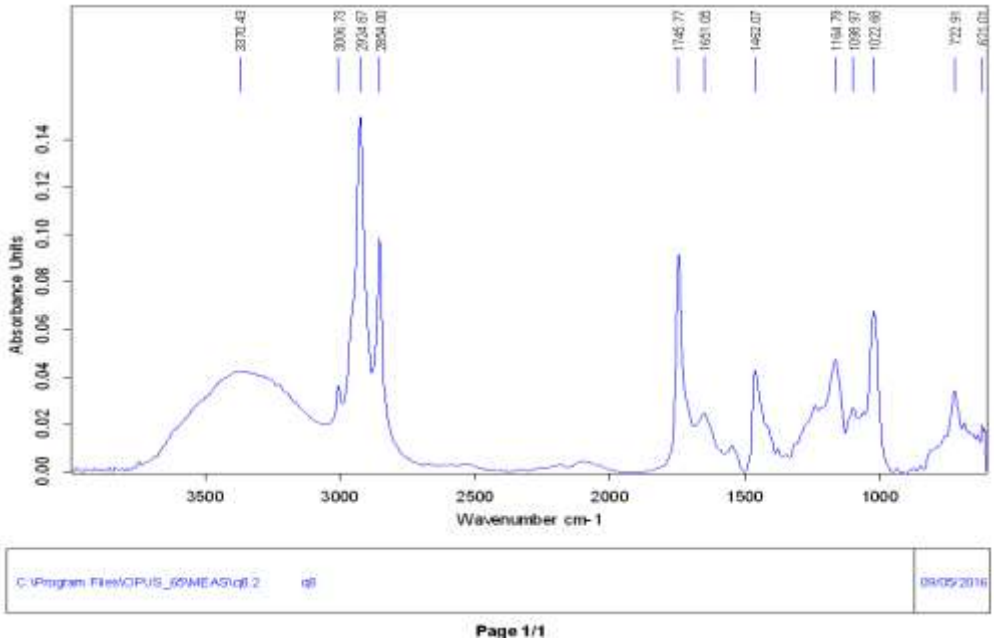


Figure 4.30 Infrared Spectra of Biodiesel produced by strain Q5 from a) Jatropha oil b) Taramira oil c) Mustard oil d) Soybean oil

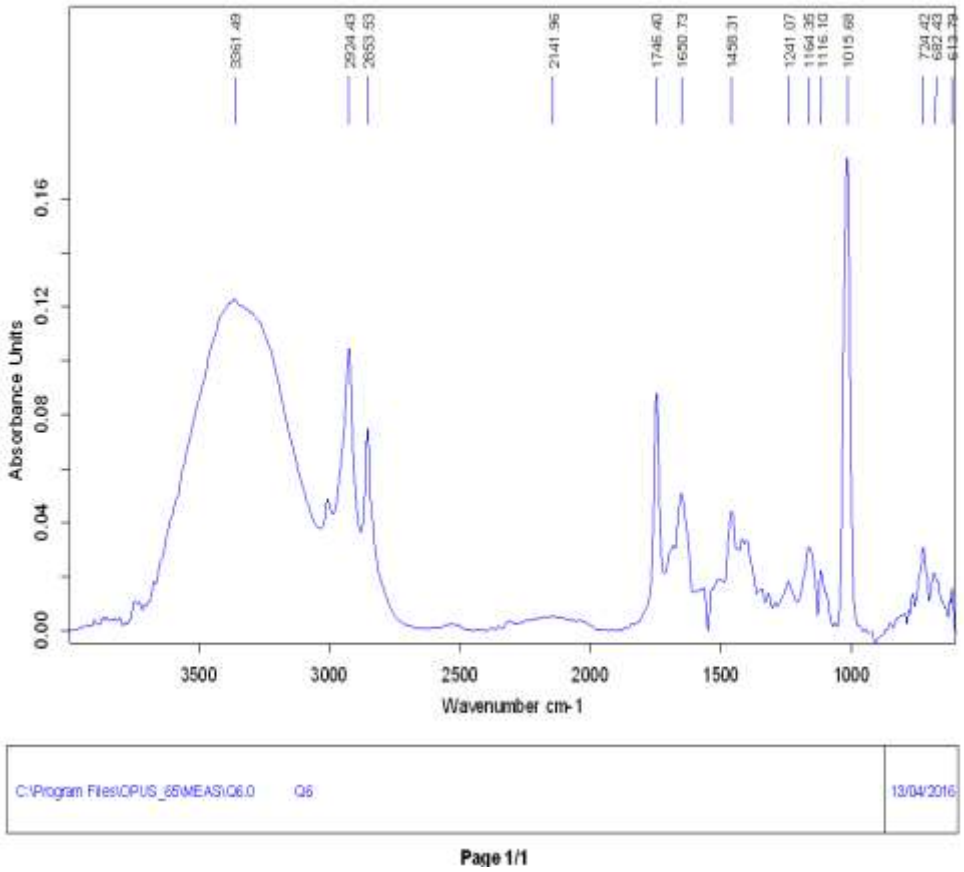
a)


3330.87
3008.83
2923.86
2923.70
2853.73
2852.62
1745.06
1743.67
1547.97
1529.33
1461.64
1456.75
1401.79
1237.45
1160.02
1099.62
1021.11
971.88
766.21
722.39
721.67
Absorbance Units
0.00
0.05
0.10
0.15
3500
3000
2500
2000
1500
1000
Wavenumber cm-1
C:\Program Files\OPUS_65\MEAS\jatropha oil 0 jatropha oil oil 16/03/2016
C:\Program Files\OPUS_65\MEAS\biodis 6 p 0 biodis 6 p oil 16/03/2016
Page 1/1

b)


3370.43
3006.73
2924.67
2854.00
1745.77
1651.05
1462.07
1164.79
1096.97
1022.69
722.91
Absorbance Units
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
3500
3000
2500
2000
1500
1000
Wavenumber cm-1
C:\Program Files\OPUS_65\MEAS\opt 2 opt 09/05/2016
Page 1/1

c)


3361.49
2924.43
2853.53
2141.96
1745.40
1650.73
1458.31
1241.07
1164.35
1116.10
1015.68
734.42
682.43
613.79
Absorbance Units
0.00
0.04
0.08
0.12
0.16
3500
3000
2500
2000
1500
1000
Wavenumber cm-1
C:\Program Files\OPUS_65\MEAS\Q6.0
Q6
13/04/2016
Page 1/1

d)

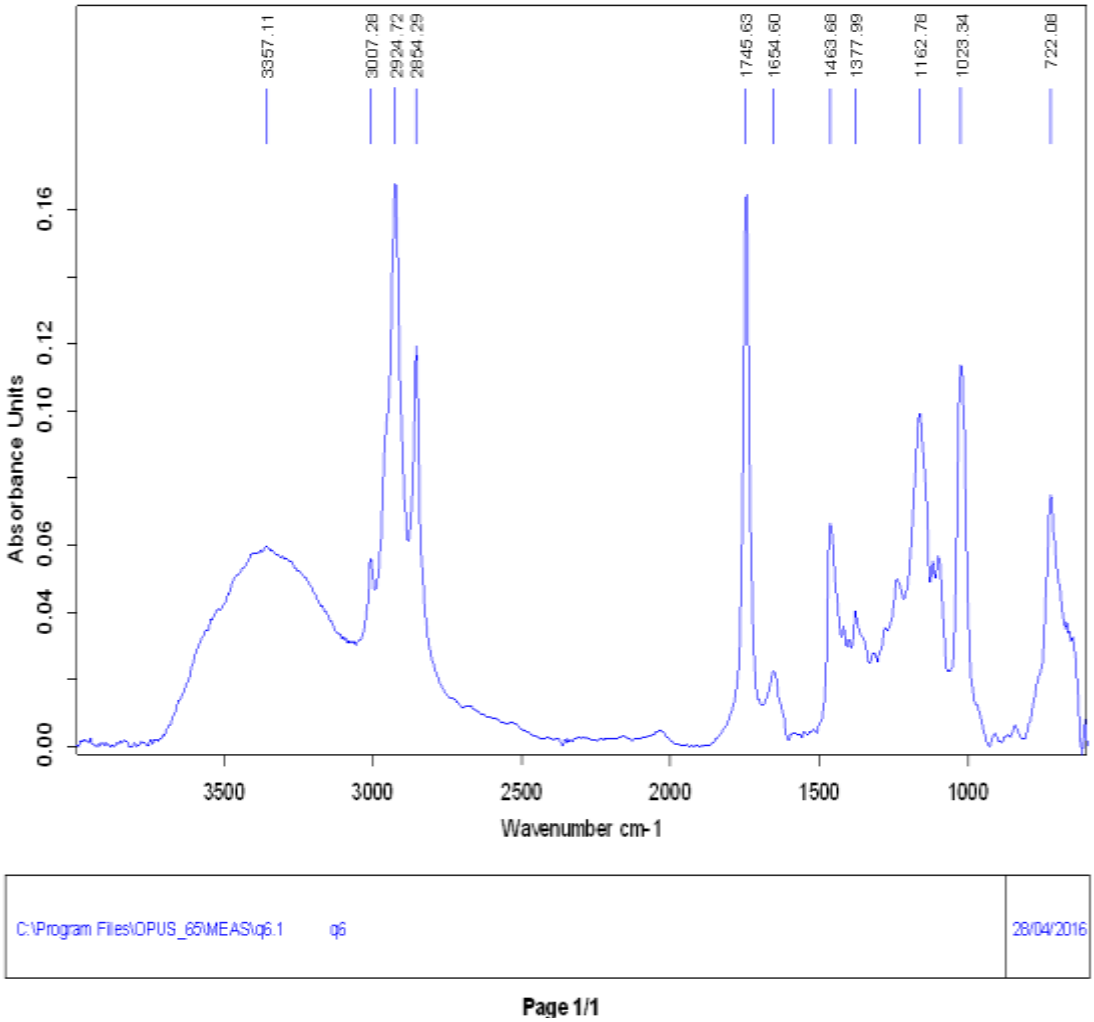
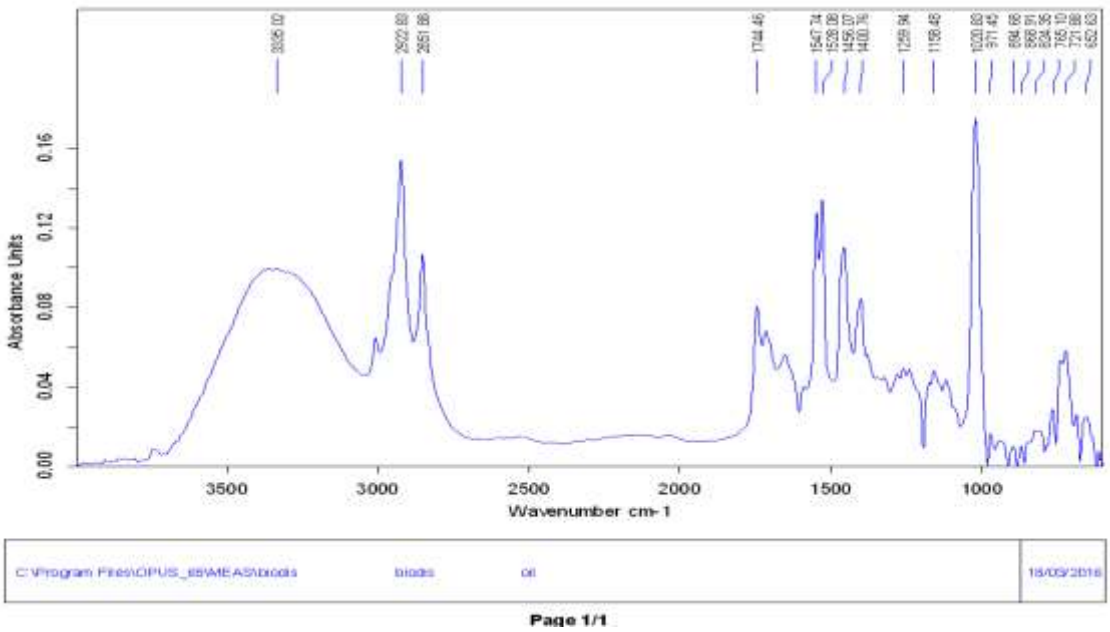
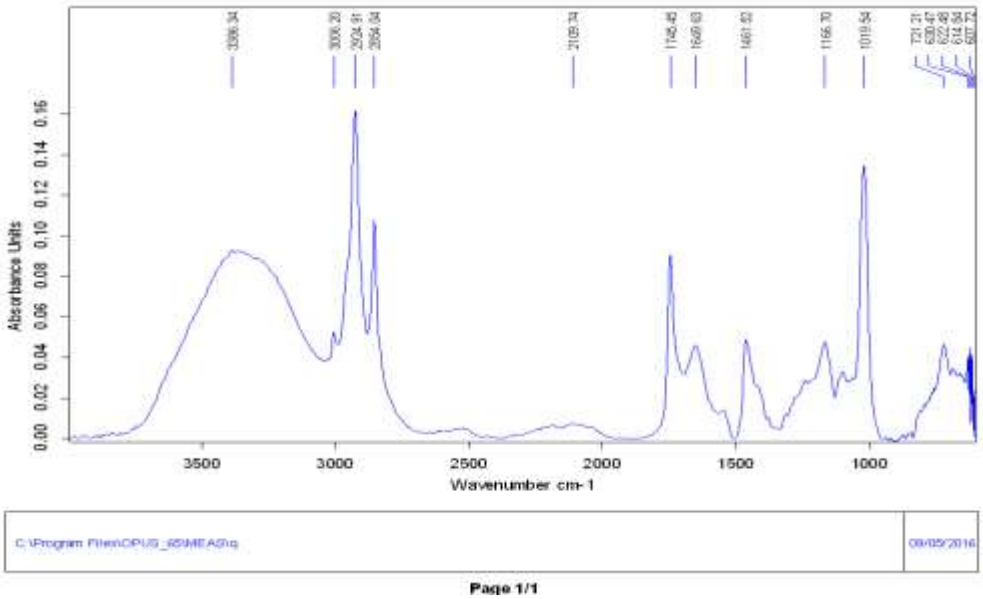


Figure 4.31 Infrared Spectra of Biodiesel produced by strain Q6 from a) Jatropha oil b) Taramira oil c) Mustard oil d) Soybean oil

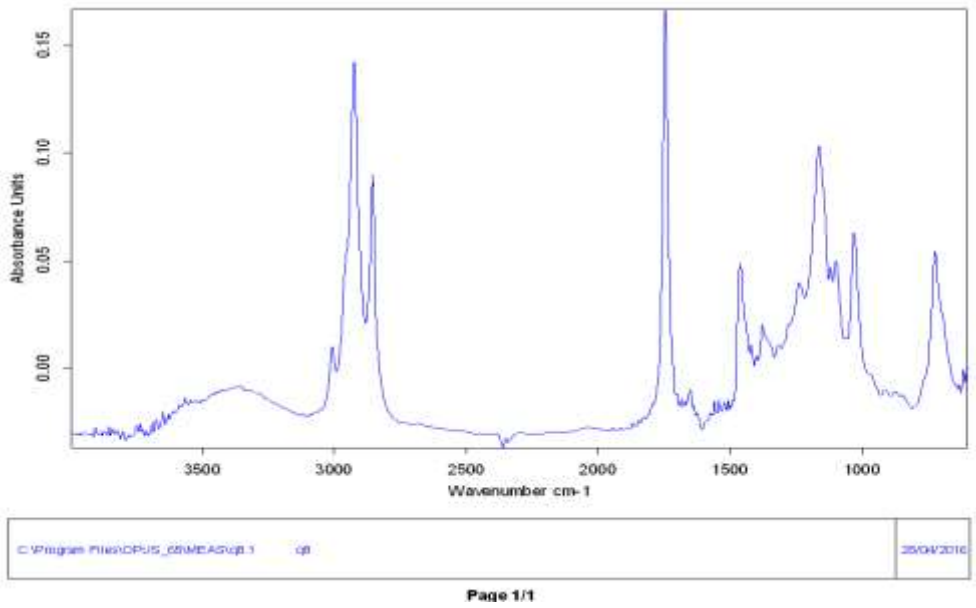
a)


Absorbance Units
0.00
0.04
0.08
0.12
0.16
3500
3000
2500
2000
1500
1000
Wavenumber cm-1
Page 1/1

b)


Absorbance Units
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
3500
3000
2500
2000
1500
1000
Wavenumber cm-1
Page 1/1

c)



d)

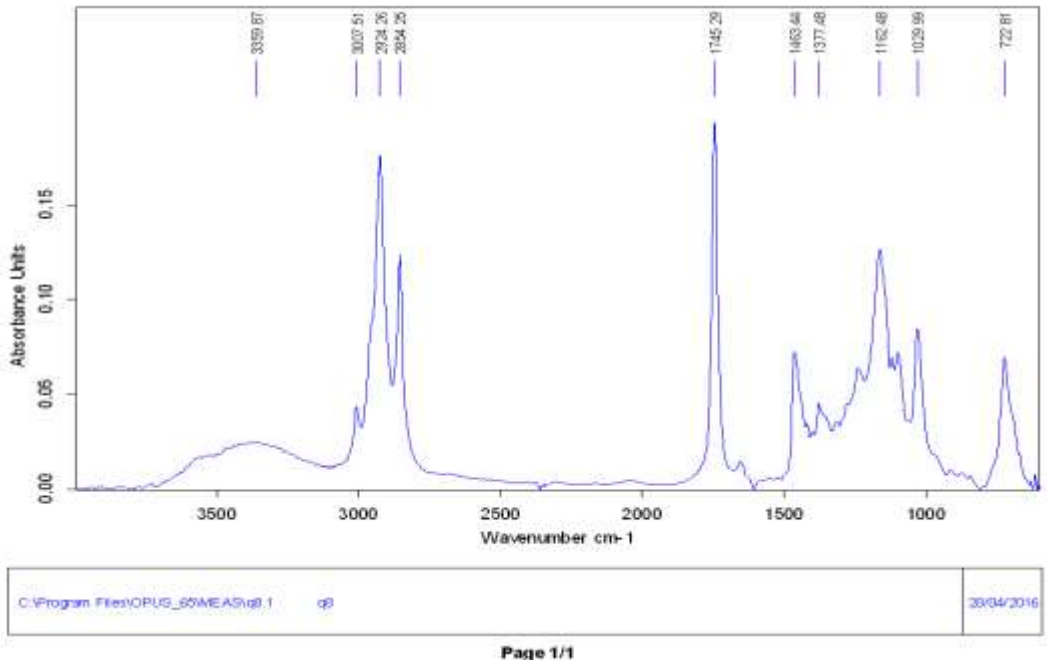
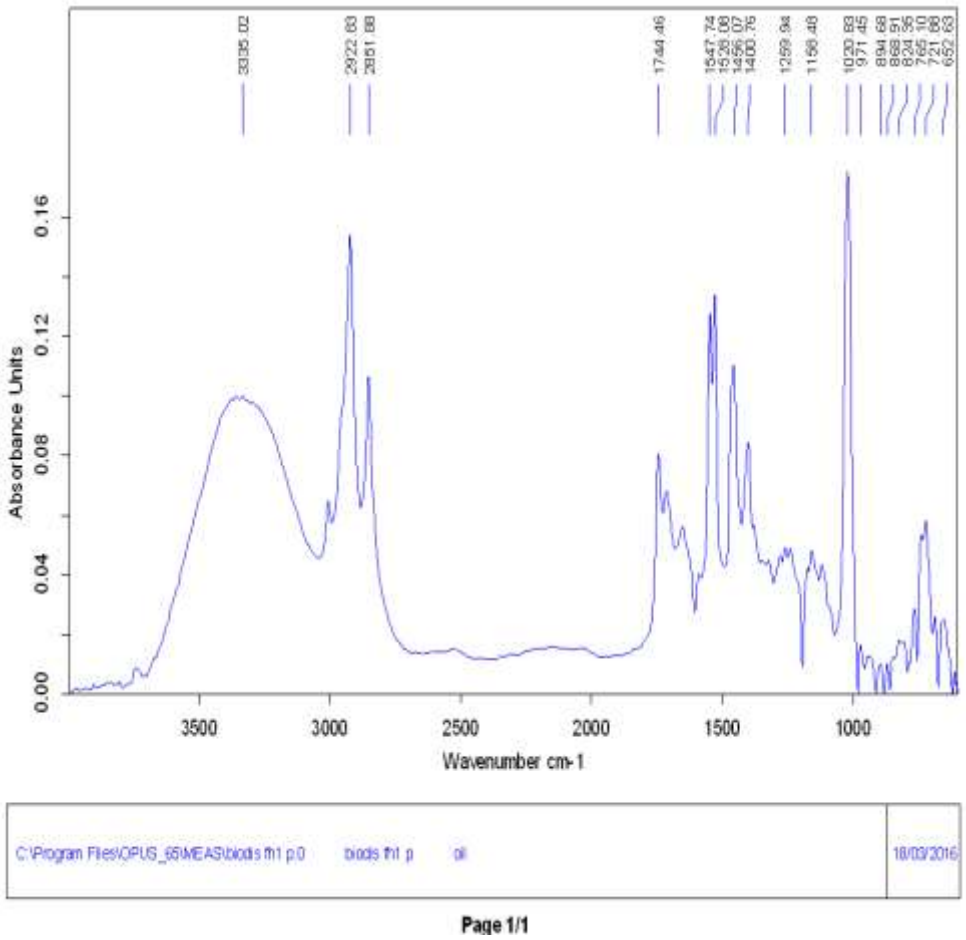


Figure 4.32 Infrared Spectra of Biodiesel produced by strain Q8 from a) Jatropha oil b) Taramira oil c) Mustard oil d) Soybean oil

a)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2922.63
2851.88
1744.46
1547.74
1538.08
1456.07
1400.76
1259.94
1156.48
1030.83
971.45
894.68
868.91
824.35
765.10
721.88
652.63
Absorbance Units
0.00
0.04
0.08
0.12
0.16
3500
3000
2500
2000
1500
1000
Wavenumber cm-1
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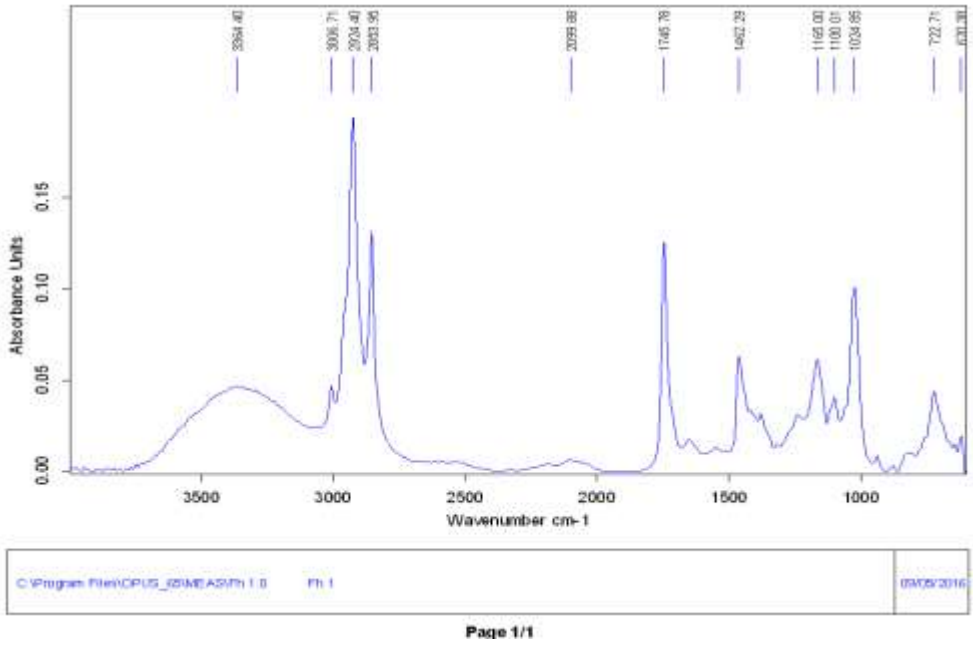
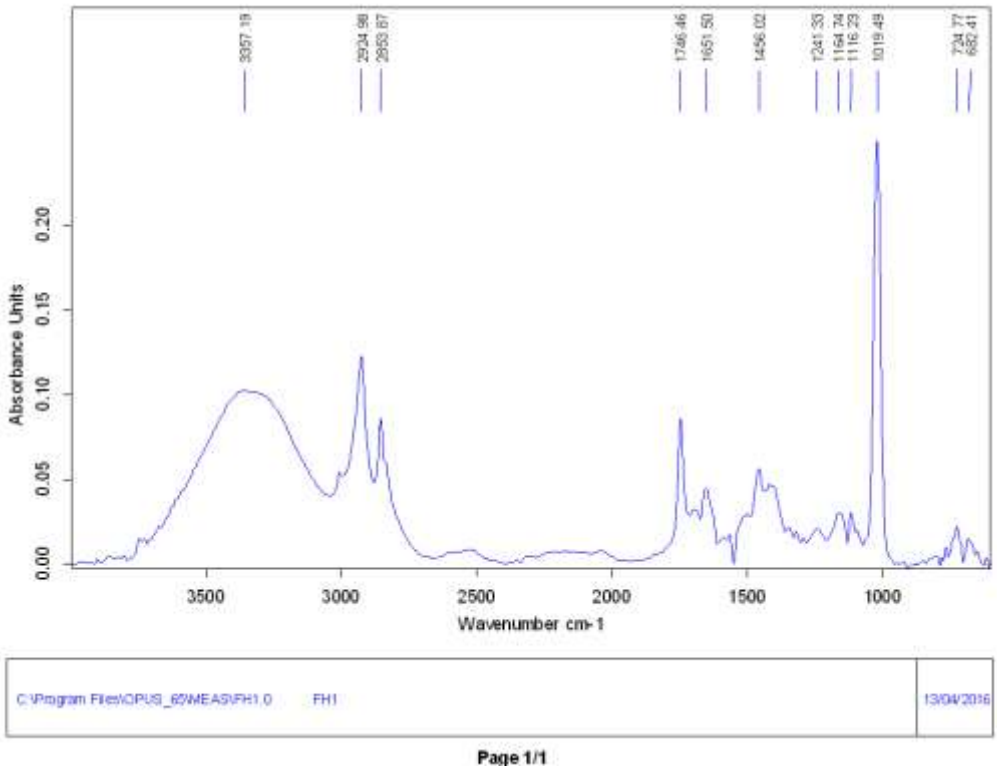
biodis fh1 p
oil
18/03/2016
Page 1/1

b)


3564.40
3006.71
2924.40
2853.95
2099.88
1745.78
1462.29
1165.00
1100.01
1034.85
722.71
631.39
Absorbance Units
0.00
0.05
0.10
0.15
3500
3000
2500
2000
1500
1000
Wavenumber cm-1
C:\Program Files\OPUS_65\MEAS\Fh 1.0
Fh 1
09/05/2016
Page 1/1

c)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2924.98
2853.67
1746.46
1651.50
1456.02
1241.33
1164.74
1116.23
1019.48
724.77
682.41
Absorbance Units
0.00
0.05
0.10
0.15
0.20
3500
3000
2500
2000
1500
1000
Wavenumber cm-1
C:\Program Files\OPUS_65\MEAS\FH1.0
FH1
13/04/2016
Page 1/1

d)

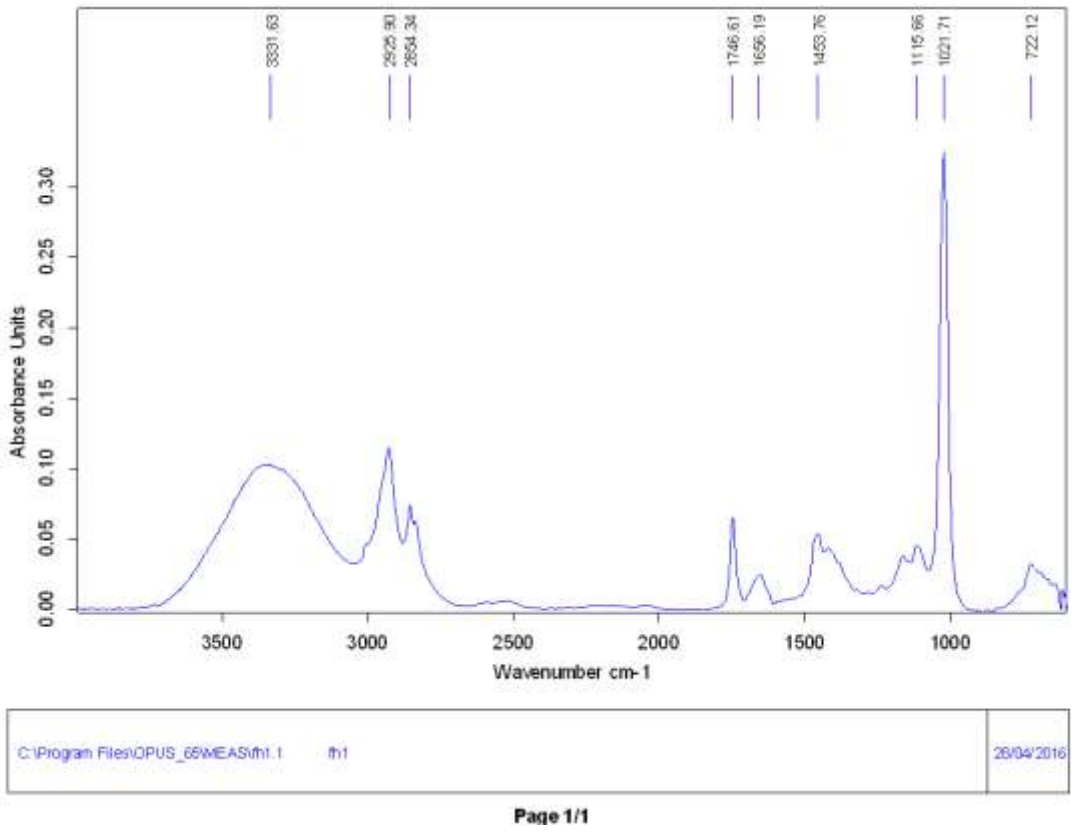
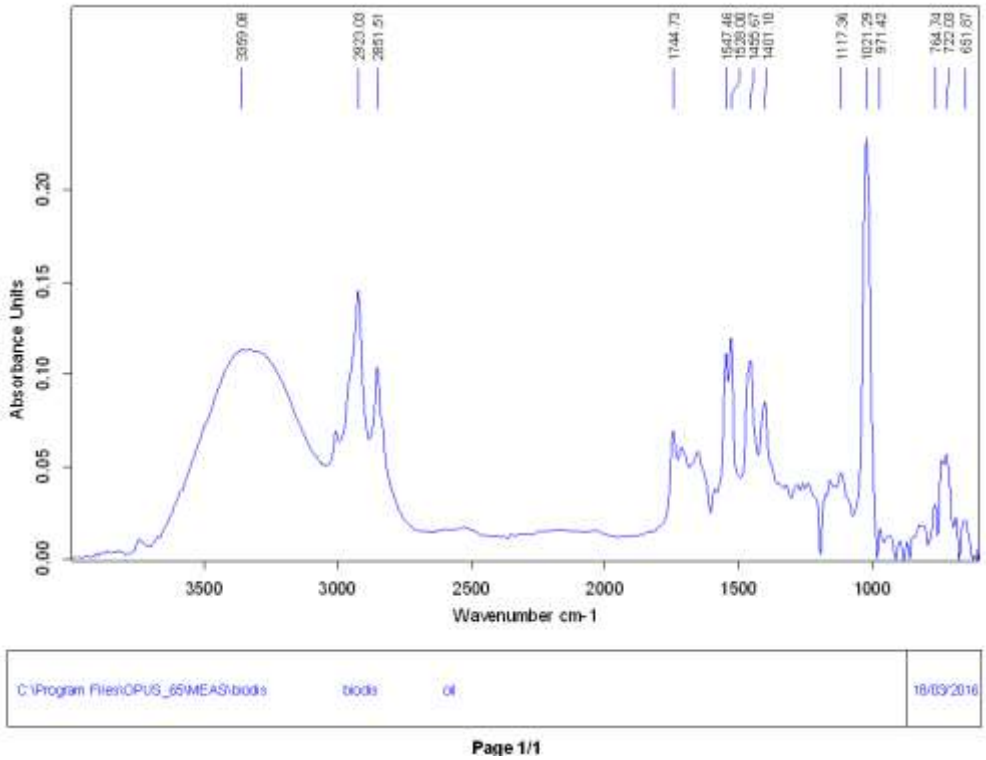


Figure 4.33 Infrared Spectra of Biodiesel produced by strain FH1 from a) Jatropha oil b) Taramira oil c) Mustard oil d) Soybean oil

a)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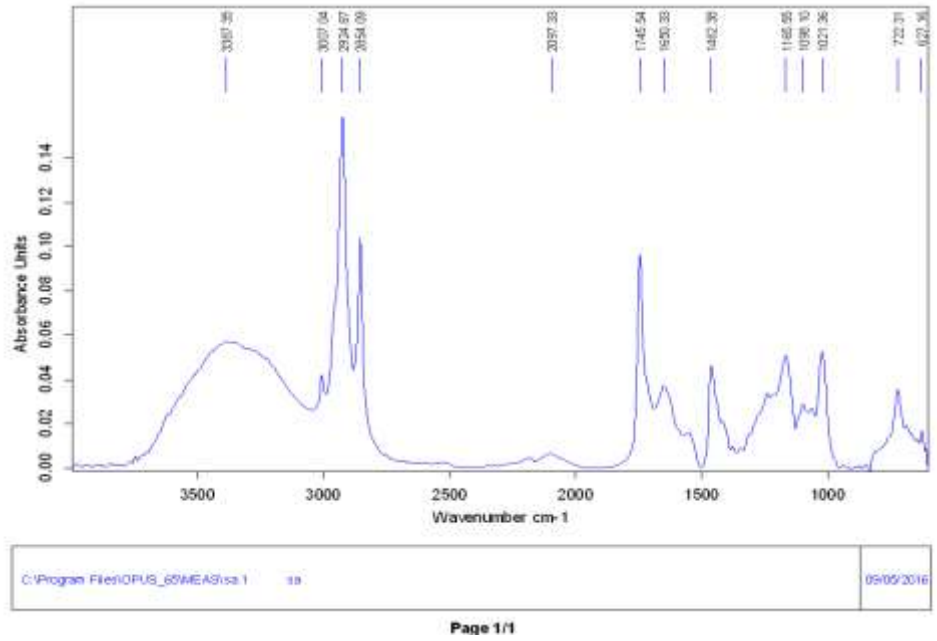
2923.09
2851.51
1744.73
1547.46
1539.00
1455.67
1401.10
1117.36
1021.29
971.42
764.74
722.09
651.87
Absorbance Units
0.00
0.05
0.10
0.15
0.20
3500
3000
2500
2000
1500
1000
Wavenumber cm-1
C:\Program Files\OPUS_65\MEAS\biodis
biodis
oil
18/03/2016
Page 1/1

b)


3357.36
3007.04
2924.87
2854.09
2357.33
1745.54
1650.33
1462.36
1165.55
1098.10
1021.36
722.31
Absorbance Units
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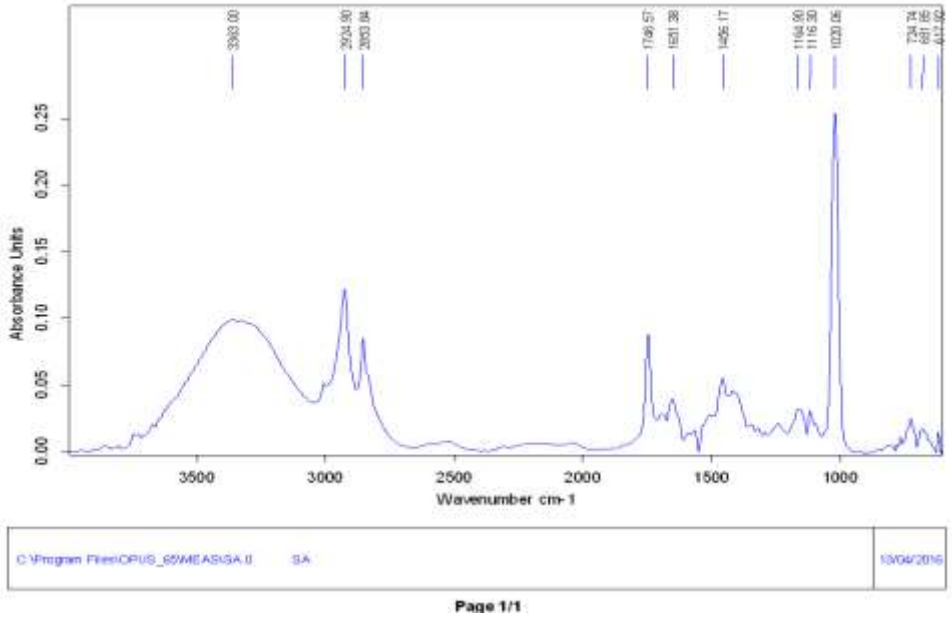
c)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Absorbance Units
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d)

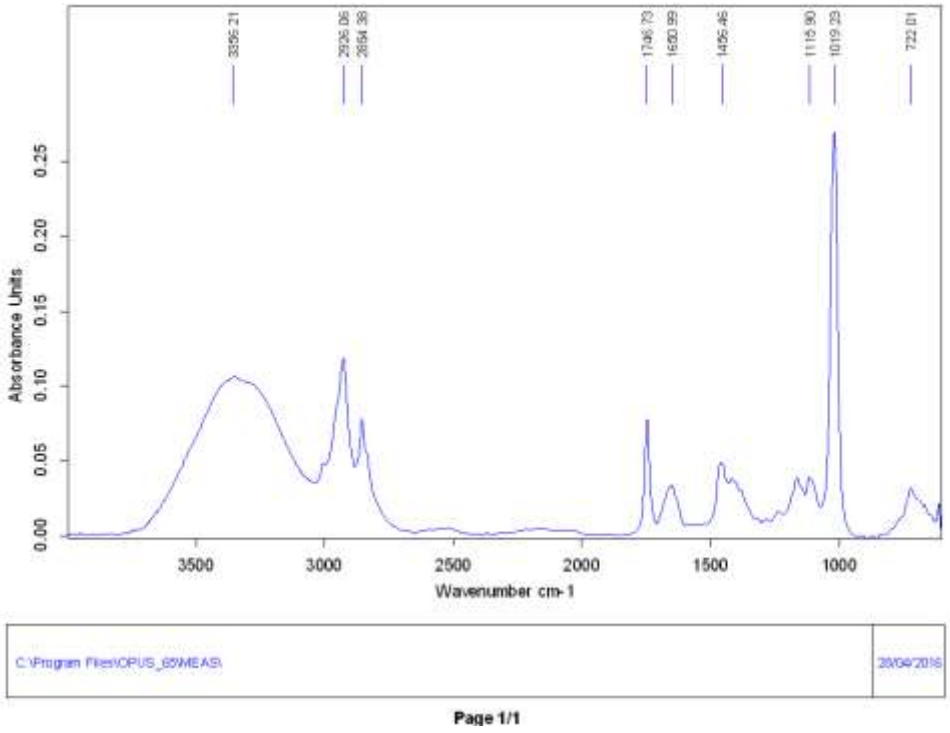

Figure 4.34 Infrared Spectra of Biodiesel produced by strain *S. aureus* ATCC 6538 from a) Jatropha oil b) Taramira oil c) Mustard oil d) Soybean oil

4.12 Optimization of Parameters for Biodiesel Production using Plackett-Burman Design.

Strain Q1and taramira oil were selected for optimized biodiesel production. Five factors namely Temperature, Agitation (RPM), Inoculum size, oil:methanol ratio and n-hexane percentage with respect to oil were optimized. Experiments were performed according to conditions given in 15 runs by Plackett-Burman design and response (percentage volumetric yield of biodiesel) was recorded.

Table 4.5 Percentage Volumetric Yield of Biodiesel in response to conditions specified by Plackett-Burman Design for each run.

Runs	Factor 1 A: Molar ratio	Factor2 B: Agitation RPM	Factor 3 C: Inoculum size ml	Factor 4 D: Temperature Celsius	Factor 5 E: n-hexane %	%age Volumetric Yield of Biodiesel
1	9.00	150.00	10%	37.00	10.00	**100%**
2	9.00	300.00	10%	37.00	6.00	**100%**
3	9.00	150.00	30%	55.00	6.00	**84%**
4	3.00	150.00	10%	37.00	6.00	**60%**
5	6.00	225.00	20%	46.00	8.00	**98%**
6	3.00	150.00	30%	37.00	10.00	**98%**
7	9.00	150.00	30%	55.00	10.00	**94%**
8	3.00	150.00	10%	55.00	6.00	**0%**
9	6.00	225.00	20%	46.00	8.00	**100%**
10	3.00	300.00	30%	37.00	10.00	**60%**
11	9.00	300.00	10%	55.00	10.00	**98%**
12	6.00	225.00	20%	46.00	8.00	**98%**
13	3.00	300.00	30%	55.00	6.00	**0%**
14	9.00	300.00	30%	37.00	6.00	**102%**
15	3.00	300.00	10%	55.00	10.00	**0%**

Highest volumetric yield of biodiesel 102% was obtained with Run 14 with oil:methanol ratio of 1:9, agitation speed of 300 RPM, inoculum size 30%, temperature 37°C and n-hexane 6%.

ANOVA analysis of the Plackett-Burman designed experimental model was done by the Stat-Ease Design Expert Software which gave The Model F-value of 20.92 which implies that the model is significant and there is only a 0.02% chance that a "Model F-Value" this large could occur due to noise.

F-test on each factor was also applied by the software to identify significant factors. Values of "Prob > F" less than 0.0500 indicated that model terms (factors) are significant. In this case A and D are significant model terms with Prob > F value of 0.002 and 0.04 respectively, where A is Molar ratio and D is temperature. Molar ratio affected the response i.e., %age volumetric yield of biodiesel positively which means increasing the molar ratio increases the yield whereas temperature affects the yield negatively which means that increasing the temperature decreases the yield.

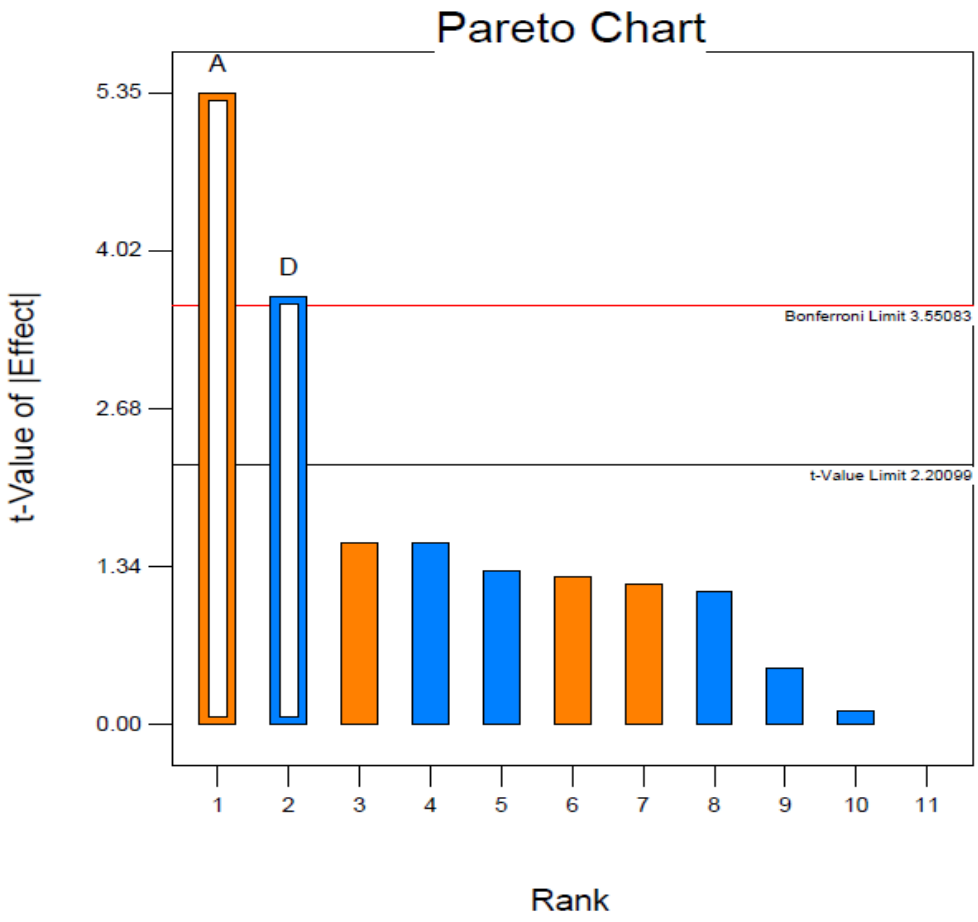

Figure 4.35 Illustration of significant factors via Pareto Chart, where A corresponds to Molar Ratio and D corresponds to Temperature.

Orange color shows positive effect and blue color depicts negative effect.

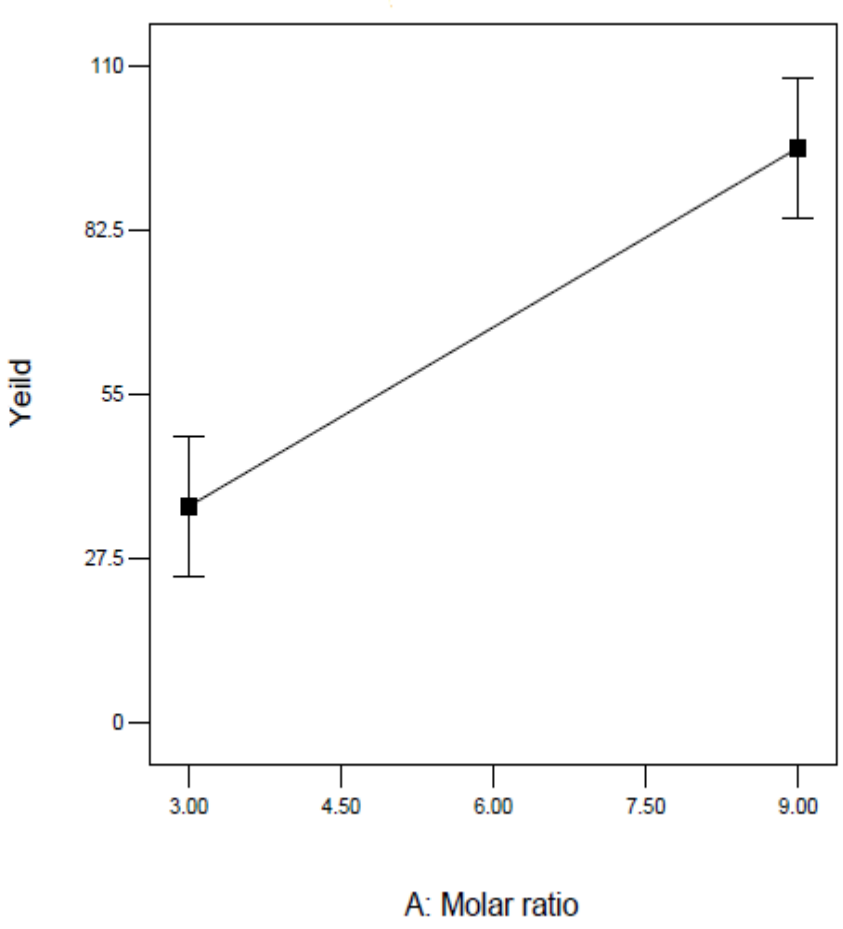

Figure 4.36 Plot of volumetric yield (response) versus molar ratio (significant factor).

The above plot illustrates the positive effect of molar ratio on volumetric yield of biodiesel. Increase in molar ratio increased yield.

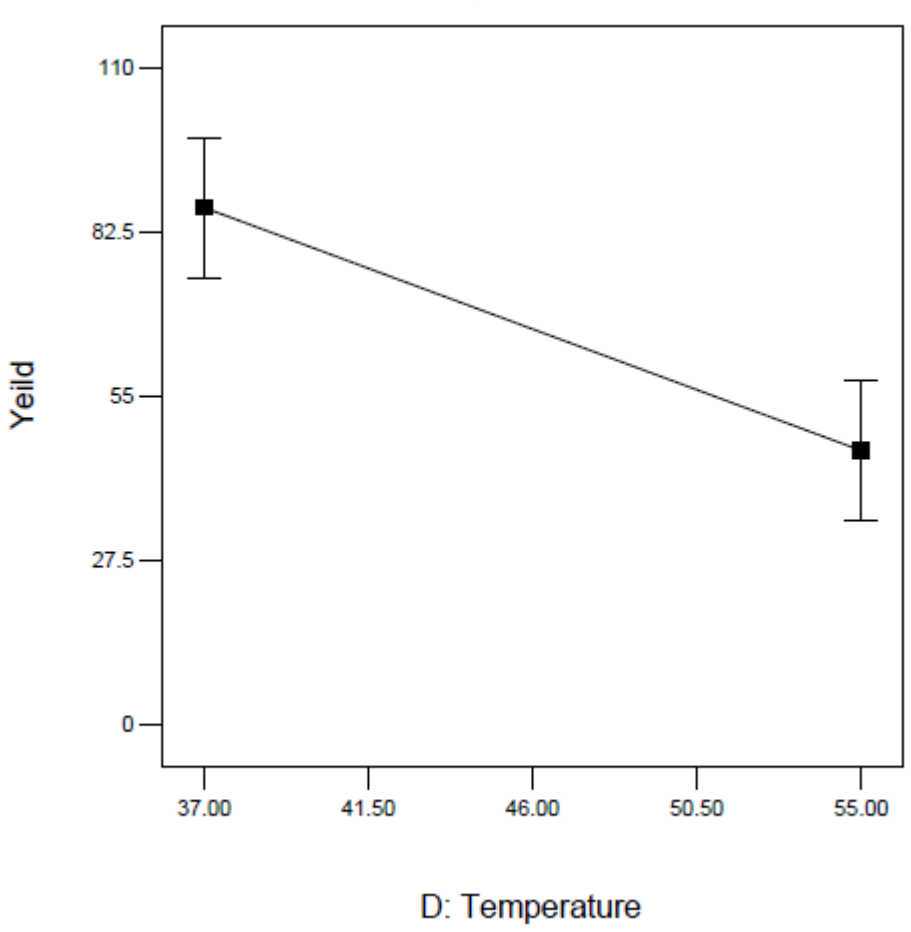

Figure 4.37 Plot of volumetric yield (response) versus temperature (significant factor).

The above plot illustrates the negative effect of temperature on volumetric yield of biodiesel. Decrease in molar ratio decreased yield.

Final Equation in Terms of Coded (Significant) Factors for %age volumetric yield of biodiesel as given by software is:

Yields = + 66.33 +30.00 * A -20.33 * D

Where, A is Molar Ratio

D is Temperature

Final Equation in Terms of Actual (Significant) Factors for %age volumetric yield of biodiesel as given by software is:

Yields = +110.25926 +10.00000 * Molar ratio -2.25926 * Temperature

4.13 Simultaneous Bioremediation of Oil Contaminated Wastewater and Biodiesel Production from Non-Edible Taramira oil using strain Q1:

Bioremediation experiment (Continues system):

For continuous bioremediation reaction volume of reactor was kept 100 ml, retention time 3 days and flow rate was calculated to be 33.3 ml. The initial COD of Wastewater and MSM mixture came out to be 2249 mg/L. A complete reduction in COD was attained in 3 days (72 hours).

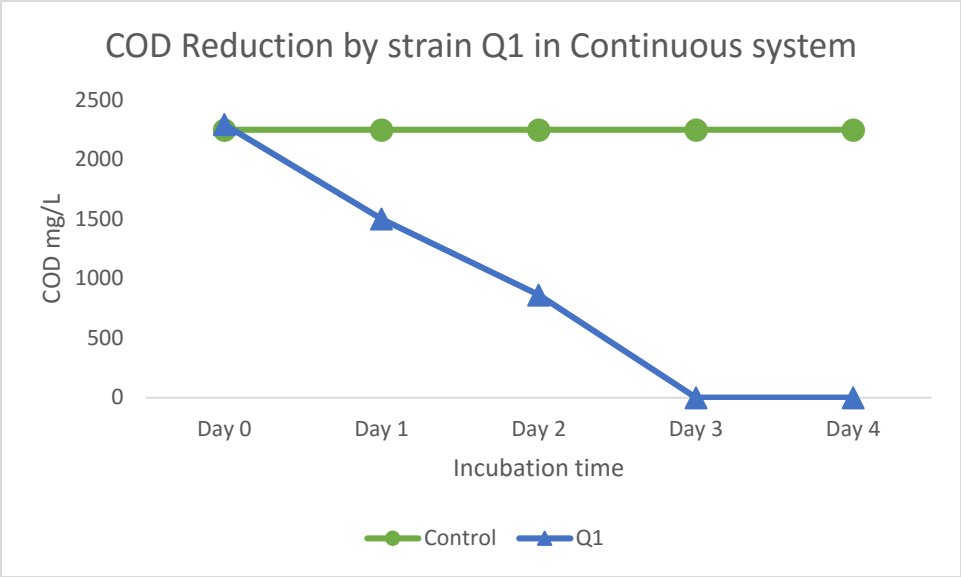

Figure 4.38 COD Reduction by Strain Q1 in Continuous System

Table 4.6 Percentage COD Reduction by strain Q1 in continuous system.

Days	**Percentage COD Reduction**
01	33%
02	62%
03	100%
04	100%

Biodiesel Production from taramira oil by cells of Q1 harvested from continuous bioremediation reaction:

For biodiesel production with strain Q1 from taramira oil the conditions of all factors were kept in accordance with the 14th run of Plackett-Burman design which gave the highest volumetric yield of biodiesel. Reaction was performed at 37°C temperature, 300 RPM agitation speed, 6% n-hexane, molar ratio of oil:methanol 1:9 and 30% inoculum size. Incubation time was 48 hours. The %age volumetric yield of biodiesel came out to be 100% which was less than 102% attained by cells grown in LB broth. The infrared spectrum of produced biodiesel gave showed ester peak at 1745.28 cm^{-1}.

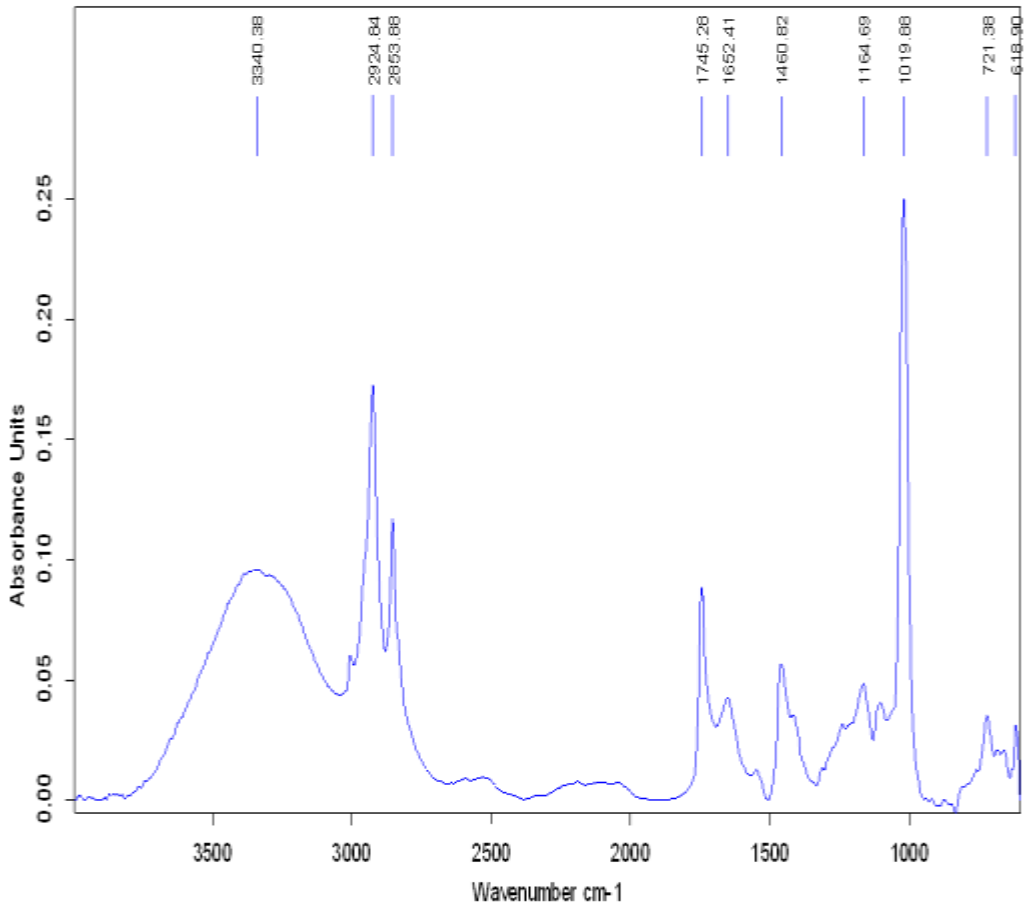

Figure 4.39 Infrared Spectrum of Biodiesel produced by strain Q1 cells obtained from continuous bioremediation reactor.

Vegetable oil industry generates a large amount of wastewater which contains oil. This water needs to be treated before discharging it into natural waterbodies. Bioremediation is the biological treatment of this wastewater to degrade oil present in it. The wastewater obtained from vegetable oil industry has only went through filtration as a treatment for the removal of FOGs present in it. As filtration does not completely remove all the FOGs present in wastewater effectively so the amount of FOGs in wastewater through APHA standard method performed in laboratory came out to be quite high along with its COD. The pH of wastewater was however neutral. COD of the wastewater was further enhanced with the addition of oil from same origin to determine the ability of the isolated strains to cope with high organic load. The COD and FOGs values obtained are typical for the wastewater collected from vegetable oil industries (Table 4.1).

As wastewater contained oil as contaminant, this made it an excellent substrate for isolation of lipase producing oil degrading bacteria. For the isolation and screening of lipolytic bacteria Tween-20 agar medium was first used. Bacteria with the ability to produce lipases degraded Tween-20 present in the media into short fatty acids. These fatty acids then reacted with $CaCl_2$ and formed white precipitate. Thus, lipase producing bacteria formed white hallows around their colonies. The colonies with largest white hallows around them were selected and further screening was done using Phenol-Red and Rhodamine B Olive oil agar plates. Phenol-Red is pH indicator. At 7.5 pH of media the color of Phenol-Red was red but as lipase producing bacteria inoculated on media degraded olive oil into shorter fatty acids the pH of media shifted towards acidic side and the color of Phenol-Red Olive oil agar plates changed from red to yellow. In the case of Rhodamine B agar as lipolytic bacteria inoculated on Rhodamine B Olive oil agar plates degraded olive oil into shorter fatty acids, these fatty acids reacted with Rhodamine B and resulted in fluorescent orange illumination of colonies under UV light. On the basis of these tests, five isolated strains were selected.

For identification of isolated bacterial strains biochemical testing was done first which showed that out of five isolated selected strains four were specie of *Bacillus* and one was *Pseudomonas* sp. (Table 4.3). A known lipase producing *S. aureus* ATCC 6538 was used as reference strain for all the experiments. Out of five isolated strains two strains were selected for sequencing. After extraction of DNA through standard protocols 16S rRNA sequencing was commercially done by

MACROGEN Korea. BLAST search program showed the nearest phylogenetic neighbor of these strains Q1 and Q5 were strains of *Bacillus subtilis* so both the isolates were designated as *Bacillus subtilis* strain Q1 and *Bacillus subtilis* strain Q5.

All the strains showed appreciable lipase activity both crude and specific even without optimization and at conditions specified for enzyme assay (Figure 4.25 and 4.26). FH1 gave the highest crude enzyme activity at both 24 and 48 hours. Highest specific activity at 24 hours was given by Q8 and at 48 hours by Q5. The results of enzyme assay depicted that these strains can be further used for the purpose of both bioremediation and biodiesel production.

Biodiesel is monoalkyl esters of long chain fatty acids derived from oils. The transesterification reaction for biodiesel production is also known as alcoholysis in which an alcohol is reacted with long chain fatty acids to convert them into mono alkyl esters of long chain fatty acids which are known as biodiesel (Ma and Hanna, 1999). The alcohol used in this study was methanol. Methanol is an organic solvent which inhibits bacteria by dissolving in their cell membrane and then disturbing its permeability and integrity. Certain bacteria have developed mechanisms to resist the toxicity of these organic solvents by changing the cis/trans structure of their fatty acids still most of the microorganisms are sensitive to methanol (Heipieper et al., 1994). Methanol sensitivity test at low concentration of methanol was carried out on the bacterial isolates to check their methanol tolerance level (Figure 4.26). All the strains gave good growth with 5% concentration of methanol at 24, 48 and 72 hours. At 10% concentration of methanol all strains showed reasonable growth after 72 hours of incubation. With 15% methanol concentration an accountable growth OD was observed with strains Q1 and Q5 after 72 hours, strain Q6 showed very little growth whereas FH1 and Q8 did not show any growth even after 72 hours of incubation.

Two of the oils used in this study are reported to be toxic. Jatropha oil contains phorbol esters which are responsible for its toxicity. This toxicity is not only restricted to microorganisms but also affects animals and humans. Its biological toxic effects include alteration of cell morphology by affecting its biochemistry, promotion of tumor growth, mutagenesis of lymphocytes and induction of platelet aggregation (Ahmed and Salimon, 2009). Considering the inhibitory effect of jatropha oil on microorganisms the bacterial isolates was tested by carrying out jatropha oil toxicity test (Figure 4.27). Results showed that all *Bacillus* strains including *Bacillus subtilis*

strain Q1 and Q5 did not show any inhibition in growth in fact highest Growth OD with jatropha oil was achieved by these strains as compared to taramira and reference oils used in this study. *S. aureus* ATCC 6358 and strain Q8 (*Pseudomonas* sp.) also did not show any inhibition with jatropha oil. These results were supported by the results reported by Devappa et al., (2012) which showed *B. subtilis* to be least susceptible to concentrated phorbol esters extracted from jatropha oil whereas *Pseudomonas putida* and *S. aureus* depicted moderate susceptibility.

Taramira oil contains phytochemicals like tannins, phenolics, alkaloids, flavonoids, cardiac glycosides and saponins which are all known for their antimicrobial activity (Gulfaraz et al., 2011). All bacterial strains showed a general growth pattern in media containing taramira oil (Figure 4.27). Growth OD was low in first 24 hours as compared to jatropha and reference oils used in the experiment but it kept on increasing as the incubation time increased from 24 to 72 hours which showed adaptation of microorganisms towards taramira oil as a carbon source in media.

The bioremediation study using batch culture with single bacterial culture and mixed culture showed that *Bacillus subtilis* strain Q1 and consortia C2 (strain Q5. Q8 and *S. aureus* ATCC 6358) showed highest COD reduction of 90% in 8 days of incubation period (Table 4.4). *Bacillus subtilis* strain Q5 showed least reduction (75%) out of all the strains. Almost all bacterial strains and consortia showed their maximum COD reduction potential in first four days of incubation after which COD reduction was stabilized to 1 or 2% reduction every day. Consortia C1 (strain Q1, Q6 and *S. aureus* ATCC 6358) showed only 80% reduction in COD whereas *Bacillus subtilis* strain Q1 alone showed highest COD reduction of 90%. It was may be due to the antimicrobial activity of both *Bacillus* strains Q1 and Q6 against *S. aureus* ATCC 6358 as *Bacillus* sp. are reported to produce antimicrobials against *S. aureus* when grown in media together. COD reduction percentages of *Bacillus subtilis* strain Q1 was in consistence with the results reported by Kanmani et al., (2016) for *Bacillus subtilis* COM6B which gave 92.7% removal efficiency with 15% oil and grease content in wastewater.

Four oils jatropha, taramira, soybean and mustard oil were selected for the production of biodiesel using lipase producing bacterial strains. Production of biodiesel was then confirmed by performing its FTIR analysis (Figure 4.29 to 4.34). For FTIR analysis infrared radiation is passed through the sample. Depending upon the functional groups infrared radiation is absorbed and

emitted by the sample and an IR spectrum of the sample is generated with each peak representing a functional group. As biodiesel is mono alkyl ester of long chain fatty acids thus, its IR spectrum should contain peaks of ester groups which are observed at the frequency (cm^{-1}) range of 1735 to 1750. The infrared spectra of all 24 samples of biodiesel produce by all six bacterial strains from all the four oil gave peaks in the range of 1735 – 1750 cm^{-1} which showed the presence of esters in sample thus confirming the production of biodiesel.

Bacillus subtilis strain Q1 and taramira oil were further selected for the optimization of parameters for biodiesel production. Plackett-Burman Design was used for optimization to achieve statistically significant sets for experiment as well as statistically significant results (Table 4.5). Temperature, agitation speed, molar ratio, inoculum size and n-hexane percentage were the factors optimized, incubation time was kept constant at 48 hours and response was measured as percentage volumetric yield of biodiesel. ANOVA analysis of the design gave F-value 20.92 which depicted that the experimental model generated by design was significant. F-test on each factor was also applied by the software to identify significant factors. Values of "Prob > F" less than 0.0500 indicated that factors are significant. In this case Molar ratio and Temperature are significant factors with Prob > F value of 0.002 and 0.04 respectively (Figure 4.35). Molar ratio affected the response i.e., %age volumetric yield of biodiesel positively which means increasing the molar ratio increases the yield (Figure 4.36). However, in most of the reported biological synthesis of biodiesel (Devanesan et al., 2007 and Aranisola et al., 2014) increase in oil: methanol molar ratio decreases the yield of biodiesel as it inhibits lipases. In this study the non-occurrence of inhibition with increase of molar ratio is may be due the utilization of high inoculum size from 10-30% with respect to amount of oil so that some of the cells remained protected from methanol till end or it may be due to direct use of cell pellet instead of immobilizing it and then mobilizing the cell pellet in oil layer to protect it from sudden direct contact with methanol as it is added. Moreover, the isolated strains also shoed tolerance to methanol at lower concentrations as depicted by the results of methanol sensitivity test. Temperature affects the yield negatively which means that increasing the temperature decreases the yield (Figure 4.37). This gives biological synthesis of biodiesel an advantage over chemical synthesis as chemical synthesis requires high temperature which means more energy to increase temperature which leads to higher cost. However, highest yield of biodiesel obtained in this

study was at 37°C (optimum growth temperature od strain Q1) which renders this process energy and cost efficient.

For simultaneous bioremediation and biodiesel production bioremediation study was conducted using continuous culture so that alive cells can be obtained at the end of the reaction. *Bacillus subtilis* strain Q1 which gave highest COD reduction was selected for simultaneous reaction. Complete COD reduction (100%) was achieved after 3 days (Table 4.6). Cells were then harvested from the continuous reaction and utilized for biodiesel production with taramira oil at optimized conditions. The percentage volumetric yield of biodiesel attained was this time was 2% less then yield obtained with cells grown in LB broth at same conditions. This may be due to inoculum used in simultaneous reaction may have included a higher percentage of dead biomass with cells which have achieved their death phase along with cells in log and stationary phase as continuous system contains cells present in all the phases at the same time. Moreover, the cell pellet may also have contained higher percentage of cell debris and suspended impurities present in wastewater along with viable lipase producing cells and hence, a lower percentage of viable cells may have led to decrease in percentage yield but still this decrease is very small and can be neglected. The IR spectrum of biodiesel produced in simultaneous reaction include ester peaks which confirmed it to be biodiesel (Figure 4.39).

Conclusions:

The study concludes that isolated lipolytic bacteria from wastewater of vegetable oil industry were efficient in reduction of COD at specified parameters. These strains were further found to be methanol tolerant and resistant to taramira and jatropha oils which are otherwise reported to be toxic. This made them an attractive candidate for biodiesel production from these non-edible oils. Biodiesel production with these strains was positive for mustard, soybean, taramira and jatropha oils which implies that they can effectively synthesize biodiesel from all of these oils. *Bascillus subtilis* strain Q1 with highest COD reduction potential when optimized for biodiesel production from taramira oil gave highest percentage volumetric yield of biodiesel at incubation time of 48 hours, 1:9 oil/methanol ratio, 300 RPM Agitation speed, 30% inoculum size with respect to oil, 37°C Temperature and 6% concentration of n-hexane with respect to oil. Biodiesel production at low temperature gave biological synthesis advantage over chemical synthesis as biodiesel production at low temperature makes the process both cost and energy efficient. Simultaneous bioremediation and biodiesel production using *Bacillus subtilis* strain Q1 and taramira oil gave 100% COD reduction in 3 days and 100% volumetric yield of biodiesel.

Future Prospects

Future prospects include:

- Optimization of isolated strains for bioremediation to achieve maximum COD reduction in minimum time period.
- Optimization of all the isolated strains for biodiesel production from jatropha, mustard, soybean and taramira oils to achieve maximum yield of biodiesel.
- Optimization of biodiesel production from mustard oil obtained from *Brassica campestris*.
- Development of an efficient industrial scale process for onsite effective bioremediation of wastewater contaminated with oil.
- Development of an industrial scale process for efficient microbiological synthesis of biodiesel from non-edible oils.
- Pilot scale investigation of simultaneous bioremediation and biodiesel production study to develop an efficient and applicable industrial scale process for simultaneous bioremediation of oil contaminated wastewater and microbial synthesis of biodiesel from non-edible oils simultaneously in vegetable and cooking oil industries.

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