Evaluating Indigenous Microalgae as Potential Source for Bioethanol production

By

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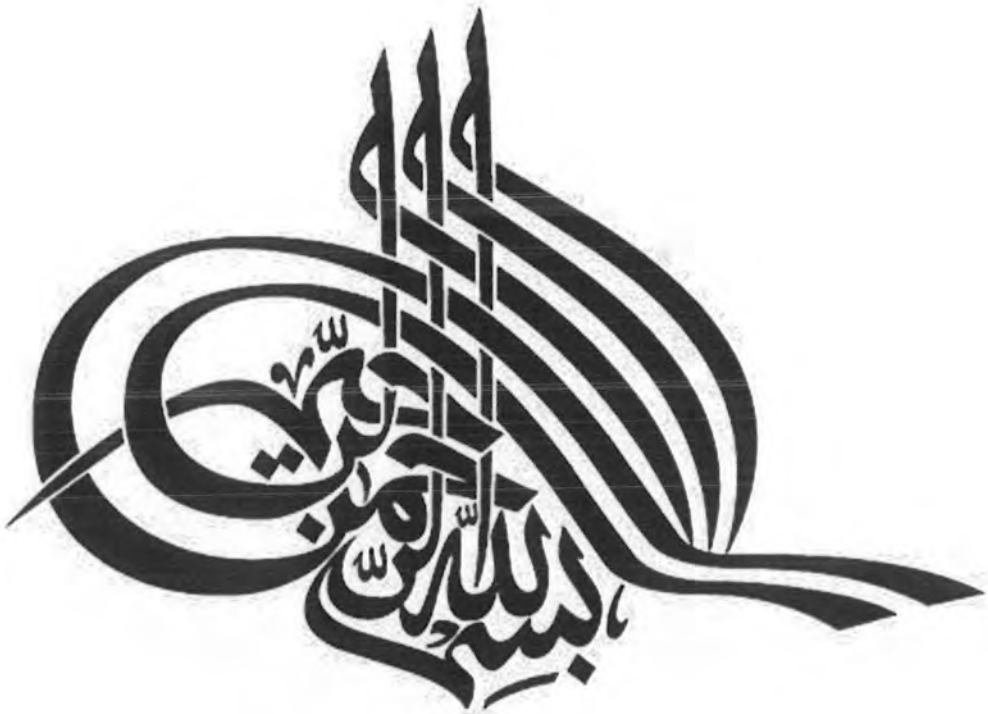
بسم الله الرحمن الرحيم

DEDICATED

To

the sweetest mother on earth my Ammi G and the greatest father of the world my Abu G with deepest affection of my heart

And

All those who made my life more beautiful and precious for me

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.

Saira Bano

Certificate

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

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List of Contents

List of Abbreviations

°C	Degree Celsius
AD	Anaerobic digestion
ASTM	American Society for Testing and Materials
BBM	Basal Bold Medium
$CaCl_2$	Calcium chloride
CFCs	Chlorofluorocarbons
CH_4	Methane
Cm	Centimeter
CO	Carbon Monoxide
CO_2	Carbon Dioxide
$CO(NO_3)_2$	Cobalt Nitrate
COD	Chemical Oxygen demand
CTAB	Cetyl trimethylammonium bromide
$CuSO_4$	Copper Sulphate
DNA	Deoxyribonucleic acid
DNSA	Di-nitrosalicyclic acid
EDTA	Ethylenediaminetetraacetic acid
et al.	et alii/alia, and others
FAME	Fatty Acid Methyl Ester
$FeSO_4$	Iron Sulphate
FFA	Free Fatty Acids
FTIR	Fourier Transform Infrared Spectrometer
g	Gram
GC	Gas Chromatography
GHG	Greenhouse Gases
GW	Gega Watt
H_2	Hydrogen
H_2SO_4	Sulfuric Acid
H_3BO_3	Boric Acid
ha^{-1}	Per hector
HPLC	High Performance Liquid Chromatography
K	Kelvin
K_2HPO_4	Potassium phosphate
kg	Kilogram
KH_2PO_4	Potassium bisphosphate
km^2	Kilometer square
KNO_3	Potassium nitrate
KOH	Potassium hydroxide
L	Liter
LB	Luria-Bertani

M	Molar
m^2	Meter square
mg	Milligram
$MgSO_4$	Magnesium Sulphate
ml	Milliliter
$MnCl_2$	Manganese Chloride
MoO_3	Molybdenum trioxide
MPa	Mega pascal
N	Nitrogen
N_2O	Nitrous Oxide
Na_2CO_3	Sodium carbonate
Na_2SO_3	Sodium sulfite
NaCl	Sodium chloride
Na-K tartarate	Sodium potassium tartarate
$NaNO_3$	Sodium nitrate
NaOH	Sodium Hydroxide
NOx	Nitrogen oxides
NH_4Cl	Ammonium chloride
nm	Nanometer
OD	Optical density
OECD	Organization for Economic Cooperation and Development
pH	Power of Hydrogen
Rpm	Round per minute
rRNA	Ribosomal ribonucleic acid
SO_2	Sulfur Dioxide
TAE	Tris base, acetic acid and EDTA
TDS	Total dissolved solids
TE	Tris base and EDTA
TG	Tri glyceride
TS	Total solids
v/v	Volume by volume
WEO	World Economic Outlook
YPD	Yeast peptone dextrose
ZnSe	Zinc selenide
$ZnSO_4$	Zinc Sulphate
µ	Micron
e.g.	Exempli gratia, for example
i.e.	id est, that is

List of Tables

List of Figures

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Abstract

Increasing energy demand and consumption leading to early fossil fuels depletion and severe energy crises along with environmental hazards. Biofuels could be the only possible sustainable alternative energy approach. Microalgae being third generation feedstock seems to be an attractive source for biofuels production as it can accumulate high amount of lipids and carbohydrates content in its biomass. The current study was primarily focused on evaluation of indigenous microalgae for bioethanol and biodiesel production. 5 microalgal strains were isolated from local fresh water samples and MSB strain with highest growth rate, biomass productivity, total carbohydrates and sugars content of 0.175μ/day, 0.187gL^{-1} $day^{-}1$, 2.23% and 3.14% respectively, was selected for further its optimization and bioethanol production. Effect of four different parameters was observed on MSB strain, and highest growth rate and biomass productivity was achieved at pH 7 when urea used as nitrogen source with proper aeration and 24 h continuous light supply. Highest biomass production of 1.75g/L was noticed on average temperature of 29°C in the month of August. In optimized conditions strain was observed with increase in total carbohydrates, sugars and lipids content up to 8% ,7.5% and 19.6% respectively and after 11 days nitrogen starvation these concentrations further increased up to 10%, 9% and 22.1% accordingly. Dried biomass was pre-treated with dilute acid hydrolysis and maximum sugars extraction was achieved when treated 5g/L biomass with 1% concentrated Sulfuric acid. Bioethanol was produced from extracted sugars hydrolysate in 72 h yeast based fermentation and its production was indicated by yeast culture growth along with gas collection in sample reactor. GC-MS was performed for bioethanol yield and quality evaluation (results are awaiting). FTIR analysis was performed for biodiesel production confirmation and C=O of ester stretch was observed at 1734.20 and 1740.93 in microalgae oil while this stretch was located at 1742.14 in biodiesel. C-O of ester stretch in microalgal oil spectra was observed at 1160.90 while it was existed at 1107.96, 1157.20, 1193.71 in biodiesel spectra and presence of these ester groups indicated the biodiesel production from algal oil. With respect to this study it can be concluded that indigenous microalgae can be effectively optimized for enhanced biofuels production.

INTRODUCTION

Primary energy consumption and emerging energy demands are directly proportional to the alarming increase in the world population, urbanization and industrialization. This extended commercialization resulting in excessive energy consumption leading to early fossil fuels depletion and severe energy crises as well as drastically increased prices of petroleum-based fuels. Another key disadvantage linked to extensive fossil fuels consumption is the environmental hazards concerns including global warming by high carbon dioxide (CO_2) emission to the atmosphere together with other factors like air pollution, acid precipitation, forest destruction, ozone depletion and radioactive substances emission. Keeping in view all the above discussed energy production and consumption scenario, production of alternative, efficient, sustainable, eco-friendly and cost-effective fuel is needed as a suitable alternate energy source termed as biofuels. (Gupta and Tuohy., 2013; Nigam and Singh., 2010).

Pakistan being an over populated and underdeveloped country, inhabited with above 190 million population by 2014 (Shakeel *et al.,* 2016). By the end of 2050, three-fold increase in energy demand is predicted whereas, our oil and gas reserves are exhausting within duration of 10 and 19 years respectively (Rafique and Rehman., 2017). On the other hand, fossil fuels import accounts for about 60% of Pakistan's overall foreign exchange (Khan and Pervaiz., 2013). Based on environmental concerns, alarming increase in CO_2, NOx and SO_2 emissions is currently the most highlighted issue in Pakistan. With renewable energy applications, these emissions are predicted to be reduced by 50% (CO_2), 61% (NOx) and 60% (SO_2) accordingly (Shakeel *et al.,* 2016). To fill the gap between national energy demand and supply, attentions are needed to be triggered towards the exploration, development and establishment of sustainable energy source (Rafique and Rehman., 2017).

Renewable energy can be a solution for all energy crisis in Pakistan, as it is self-sufficient in crops and agricultural residues, as well as holds approximately 800,000 km^2 area with plenty of water, wind and sunshine that could be utilized effectively for energy production (Shakeel *et al.*, 2016). Strategically important emerging alternative sustainable energy sources includes biofuels, hydrogen, natural gas and syngas. However, among all these alternative fuel sources, presently biofuels are gaining much attention because of their eco-friendly nature with respect to global warming, their biodegradability, renewability and acceptable emission of exhaust

gases. Biomass derived solid, liquid, or gas fuels are referred as biofuels and broadly classified into two categories primary and secondary biofuels. Unprocessed biomass with their native chemical structures used in their natural form are considered as Primary biofuels. It includes firewood, wood chips and pellets and generally used as heating or cooking fuel and electricity generation at small and large-scale. But when primary fuels are modified into solids like charcoal, liquids e.g. Biodiesel, bioethanol and bio-oil and gaseous forms like synthesis gas, biogas and hydrogen, these fuels are referred as Secondary fuels. They are having wide range of applications, including power generation, transportation and use in large-scale industrial processes (Nigam and Singh., 2011).

Biofuels are classified as first, second and third-generation biofuels based upon raw material and their production technologies being used. Fuels derived from food and oil crops like sugarcane, sugar beet, vegetables oils and animal fats are considered as first-generation biofuels. While second-generation biofuels are generally derived from non-edible and non-food crops like jatropha and miscanthus, agricultural residue and wood processing residues (Milano *et al.*, 2016). They are having reduced environment impact as compared to first generation biofuels, as they do not directly compete with arable land. However, the conversion processes are not yet economically feasible due to their low conversion rates (Adenle *et al.*, 2013). Microalgae derived fuel are recognised as third generation biofuels. They are having large-scale production capabilities and supposed to be the only possible feedstock that can replace the fossil fuel completely (Ziolkowska and Simon., 2014).

Algae being third generation biomass seems to be an attractive substitute renewable source for biofuels production (Menetrez., 2012), as it can overcome the sustainability issue mainly linked to first generation feedstocks including food crops security (Dębowski *et al.*, 2013; Rajkumar *et al.*, 2013). Being more photosynthetically active, algal biomass is having5–10 times higher productivity rate than second-generation feedstocks e.g. land-based plants, (Chen *et al.*, 2013). Along with this microalgal pre-treatments and enzymatic hydrolysis is much easier as compared to lignocellulosic materials as it lacks lignin thus making the process beneficial for ethanol production. It can be intensively cultivated in photobioreactors or open pond with nutrients and CO_2 availability and capable of surviving in harsh conditions, thus demonstrating

itself to be considered as the cheapest feedstock for biofuel production (Milano *et al.*, 2016). The biomass processing of algae is relevant to other lipid-based feed stocks and its carbohydrates content could be proficiently fermented into bioethanol (Bibi *et al.*, 2017).

Indigenous microalgal strains can be preferred for fuel production because of their better compliance to native environmental conditions. These strains can be collected from ocean, lakes, pond, river, soil, rocks and extreme conditions like ice-lakes, hot spring lakes, brine environment and nuclear reactors (Ghosh *et al.*, 2016). Photo-bioreactors (PBR), open ponds system (OPR)and hybrid systems are normally used in microalgae cultivation in laboratory. Factors effecting microalgal growth includes water availability, nutrients concentration, light intensity, CO_2 supply, temperature and pH (Jankowska *et al.*, 2017). Eco-friendly attitude, greater lipids and carbohydrates storage, maximum biomass yield and cheaper substrates requirements e.g. wastewater are main features making microalgae an attractive feedstock for biofuel production (Gill *et al.*, 2016).

Key challenge occurs in microalgal downstream-processing is its biomass harvesting from growth medium and accounts for almost 20–30% of total production cost. Holding a cell size of about 2–20μm, microalgae gets colloidal stability and remain in suspension form, thus making the separation process more critical. To expand overall microalgae biomass production, an efficacious harvesting approach with minimal energy and cost consumption is needed (Vandamme *et al.*, 2013; Wan *et al.*, 2015). Generally applicable microalgal biomass harvesting techniques incorporates as centrifugation, gravity sedimentation, flocculation, filtration and screening, flotation and electrophoresis techniques. Appropriate technique selection is based upon cellular properties like density, size and preferred product's worth (Pragya *et al.*, 2013).

Bioethanol is ecologically sustainable and mainly a renewable biofuel. In altered spark-ignition engines either ethanol can be used as blend with petrol or burned in its pure form. As compared to petrol, ethanol contains just traces of Sulphur therefore, ethanol-petrol blends can reduce the Sulphur oxide emissions which is carcinogenic and responsible for acid rain (Nigam and Singh., 2011). Carbohydrates are stored in microalgal cells in the form of cellulose, starch and glycogen. Cell wall embedded cellulose and cellular starch content makes algae a suitable feedstock for bioethanol

production (de Farias Silva and Bertucco., 2016). During hydrolysis, in optimized conditions cell wall embedded carbohydrates are released into hydrolysate and then broken down into fermentable sugars in the step of saccharification (Harun *et al.*, 2014). Hydrolysis or pre-treatment is generally classified into physical, chemical and enzymatic approaches (Daroch, *et al.*, 2013). Most commonly used method for polysaccharides hydrolysis is chemical method while, enzymatic hydrolysis is a latest efficient approach (Jambo *et al.*, 2016).

By the action of certain microorganisms like yeast, bacteria and fungi, fermentable sugars extracted through saccharification are converted into bioethanol and some other products (Wei *et al.*, 2013). Bioethanol productivity mainly depends upon pre-treatment conditions of biomass (Daroch *et al.*, 2013). For bioethanol fermentation, commonly used microorganisms embrace the specie of bacterial genus *Zymomonas and* yeasts like *Saccharomyces* (de Farias Silva and Bertucco., 2016). Two renowned latest fermentation techniques are known as simultaneous saccharification and fermentation (SSF) and Separated hydrolysis and fermentation (SHF). These techniques are responsible for economically suitable and better production rate of bioethanol as compared to simple fermentation process (Jambo *et al.*, 2016).

Biodiesel is a blend of mono-alkyl esters of long chain unsaturated fats (FAME) and comprised of 90–98% by weight triglycerides, insignificant contribution of mono- and di-glycerides,1–5% free unsaturated fats and minor portion of carotenes, phospholipids, sulphur mixes, phosphatides with tiny water quantity (Bozbas., 2008). Microalgae can accumulate significant quantities of lipids by optimizing factors, like CO_2 concentration, stressed nitrogen level, temperature, intensity of light, salinity and harvesting method. These lipids can be easily converted into biodiesel by the process of transesterification (Brennan and Owende., 2010).

For biodiesel production, lyophilized biomass undergoes through solvent treatment resulting in lipids and fatty acids extraction prior to transesterification (Mata *et al.*, 2010). Transesterification process involves the conversion of triacylglycerol into diacylglycerol and fatty acid ester in initial step leading towards the production of fatty acid and monoacylglycerol from diacylglycerol. In terminating step monoacylglycerol are converted into fatty acid esters and glycerol as an ending product. Conversion of one mole of triglycerides requires three moles of alcohol

however, maximum biodiesel yield can be achieved by higher molar ratios of oil and alcohol. Alcohols normally used in transesterification process involves ethanol, methanol, and propanol (Rathore and Madras., 2007), while various catalysts used in the process encompasses biological catalysts, acid catalyst, alkaline catalyst and used alcohol in supercritical state. (Mythili *et al.,* 2014). Based on catalyst there are three types of transesterification incorporating as alkaline catalysed transesterification (Rathore and Madras., 2007), acid catalysed transesterification (Meher *et al.*, 2006) and enzymatic transesterification (Antczak *et al*., 2009).

Aim and objectives

Aim of the current study is to evaluate the bioethanol production potential of indigenous microalgae strain.

Objectives

- To isolate the microalgae strains from freshwater bodies and their identification and molecular characterization.
- Selection and optimization of the strain for bioethanol production and to evaluate its biochemical composition.
- To evaluate the bioethanol production via yeast based fermentation and its yield estimation.
- To investigate the strain for biodiesel production potential by alkaline catalyzed transesterification and its confirmation analysis through FTIR.

LITERATURE REVIEW

The world population, urbanization and industrialization is increasing in an alarming rate which is directly proportional to the increase in primary energy consumption and emerging energy demands. Till 2010 energy consumption rate had reached to maximum growth of 5.6% in almost 40 years (Jones and Mayfield., 2012; Alam *et al.*, 2012). Extensive consumption of fossil fuels in past decades resulting in rapid fossil fuels depletion and energy crises leading to elevated prices of petroleum-based fuels. fossil fuels used for power generation and as transportation fuels are responsible for global warming by high carbon dioxide (CO_2) emission to the atmosphere and an eager approach is needed to decrease GHG emission to avoid its disastrous effects. Increasing energy demands in future together with the environmental hazards concern, national security has directed the attention towards production of efficient, alternative, renewable, sustainable, cost-effective and clean liquid fuel, termed as biofuels, as a suitable alternative source of energy (Gupta and Tuohy., 2013; Nigam and Singh., 2010).

Biofuel was first prepared by Rudolph Diesel, who invent compression ignition engine in 1900. History reveals that corn derived ethanol was firstly used as a fuel for early cars like Henry Ford's Model-T. The concept of using microalgae as a biofuel production source was proposed for the first time in early 1950s. The idea was comprised of methane gas production from algae (Amberg and Meints.,1991). Much of the research conducted by "The Aquatic Species Program" (ASP) from 1978 to 1982 was focused on hydrogen production from using algae as biomass. While in early 1980s, program's intentions were shifted to other transportation fuels production, specifically biodiesel in the beginning and then bioethanol (Sheehan *et al.*, 1998).

2.1. Worldwide scenario of Fossil fuels unsustainability

2.1.1. Fossil fuels depletion and Energy security issue

The Energy Information Administration (EIA) has predicted the increase in energy consumption from an average rate of 1.1% per annum, from 500 quadrillion Btu in 2006 to 701.6 quadrillion Btu in 2030 (Shafiee and Topal., 2009). "global oil supplies will only meet demand until global oil production has peaked sometime between 2013 and 2020" (Salameh., 2003). According to Edigera and his colleagues turkey has

already reached to fossil fuel production peak and their reserves are depleting in 038 (Edigera *et al.*, 2006; Edigera and Akar., 2007).

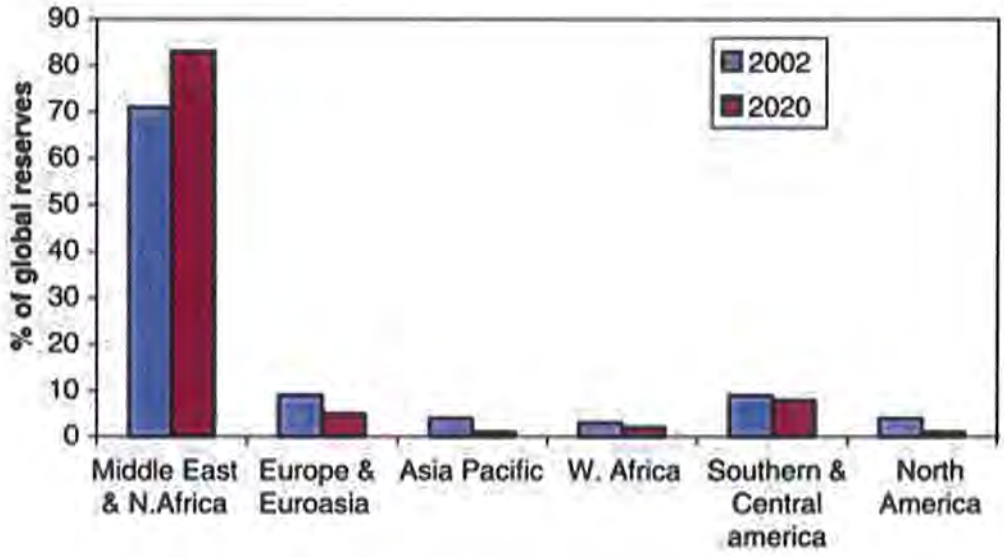

Figure 2.1: World reserves for 2002 and 2020 (Shafiee *and Topal., 2009)*

By the middle of 21st century, World's population is expected to be doubled, along with this industrialization and commercialization will continue to grow steadily resulting primary demands of energy are predicted to be raised by 1.5–3 times (Dincer., 2000). Coal, natural gas and petroleum are the sources of 90% energy coming from fossil fuels and these reserves are depleting rapidly therefore sustainable energy supplies are needed (Chen *et al.*, 2011; Demirbas., 2010). WEO 2006 estimated the average life time of various fossil fuels which shows the estimated ratio for oil between 39 and 43 years, 164 years for coal and 64 years for gas (Shafiee and Topal., 2009). World oil reserves may run out by 2050 with respect to current consumption rate, (Harun *et al.*, 2010; Ho *et al.*, 2011).

2.1.2. Environmental concerns

Problems are not only related to fossil fuels depletion but the biggest concern is related to global warming and a lot of other environmental protection issues like ozone depletion, air pollution, acid precipitation, radioactive substances emission and forest destruction.

2.1.2.1. Acid rain

Combustion of fossil fuels in smelters for nonferrous ores, transportation vehicles and industrial boilers, results in the pollutants formation that are responsible for acid rain. SO_2 and NOx emissions are the major contributors of acid rain (Dincer., 1998). Disasters caused by acid rain includes acidification of streams and lakes, plants toxification, aquatic life damage and more importantly deterioration of buildings (Dincer and Rosen., 1999). It is obvious that some energy-related activities are major sources of acid precipitation. 80% of SO_2 is emitting from electric power generation, industrial energy and while the use of coal individually accounts for 70% SO_2 emissions. In OECD countries road transport is major contributor and accounts for 48% of NOx emissions while China and countries from the former Soviet Union, and United States are the largest contributors of pollutants (Dincer., 2000).

2.1.2.2. Stratospheric Ozone depletion

Between altitudes of 12 and 25 km in stratosphere, there exist an ozone layer having natural, equilibrium-maintaining role for Earth and provide protection from harmful radiations by absorbing ultraviolet (UV) and infrared radiation of 240±320 nm (Dincer., 1998). Emissions of halons (chlorinated and brominated organic compounds), CFCs and NOx are the primary factors involved in ozone depletion. This stratospheric Ozone depletion results in bigger rate of eye damage, skin cancer and harm to other organisms through reaching destructive UV radiation to ground. CFCs mostly comes from air conditioners and refrigerators while NOx emissions are contributed by biomass combustion processes and fossil fuel (Dincer and Dost., 1996).

2.1.2.3. Greenhouse effect (Global warming)

Global warming is the biggest concern of fossil fuels unsustainability. The global climate change termed as greenhouse effect is associated with increase in earth's temperature with the contribution of CO_2 and some other gases including N_2O, CH_4, halons, peroxyacetyl nitrate, ozone and CFCs, emitted from domestic and industrial activities. However, the major contributor of anthropogenic greenhouse effect is CO_2 contributes about 50% effect. over the last century, sea level is estimated to have risen perhaps 20 cm due to increase of about 0.68°C in earth's surface temperature. It is

predicted that if the current trend in fossil fuel consumption continues till next century, there will be steady increase in atmospheric concentrations of GHG. Consequently, temperature of earth may increase by 28°C to up to 48°C and the rise in sea level perhaps reach between 30 and 60 cm prior to the end of the 21st century, this could endanger the survival of entire populations (Dincer and Rosen., 1999).

Keeping in mind all the above discussed factors, many countries have shown increased interest in developing alternative energy sources that would be renewable, economically competitive and environmentally friendly (Mussgnug *et al.*, 2010).

2.2. Current scenario of energy situation in Pakistan

Pakistan is inhabited with above 190 million of population in 2014 and underdeveloped and over populated country. The primary supplies and demands of energy elevated over 90% last decades, between 34 million tons of oil equivalent (MTOE) and 64.7 MTOE from 1992 to 2012, while the native production rate persistent around 45.2 MTOE, creating a shortage of 20.5 MTOE to be imported (Shakeel *et al.*, 2016). Around 60% of Pakistan's overall foreign exchange devotes on fossil fuels import (Khan and Pervaiz., 2013). There is prediction of three-fold increase in energy demand by the end of 2050, whereas the primary supply is not so inspiring. Oil and gas reserves are exhausting within 10 and 19 years interval respectively (Rafique and Rehman., 2017).

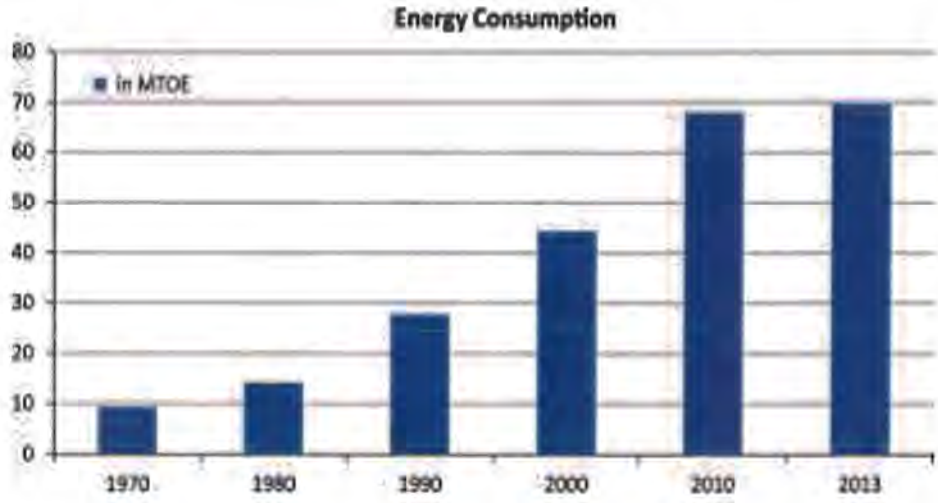

Figure 2.2: Pakistan energy consumption from 1970 to 2013 (Shakeel *et al.*, 2016)

In Pakistan CO_2 emissions is a big problem with respect to environmental concern. Above 80% of CO_2 contribution is from energy sector. In 2010, its emission was 64 million metric tons (MMT) and reached to over 166 MMT by the end of 2013. Similarly, NOx and SO_2 emissions share climbed up to four and seven folds, respectively. By renewable energy applications these emissions predicted to reduce by 61% in NO, 60% of SO_2 and 50% in CO_2 (Shakeel *et al.*, 2016).

2.3. Alternative sustainable energy solutions

Finding out an alternate source for fossil fuel reserves is currently the focus of global community. There are so many emerging sustainable energy alternatives, among these strategically important fuel sources are biofuels, hydrogen, natural gas and syngas (Nigam and Singh., 2011). Palm oil can be used as a source of renewable energy as it can be converted to hydrogen in supercritical water through gasification reaction (Kelly-Yong *et al.*, 2007). With respect to another suggestion that countries like turkey as one of the major agricultural nations can have high share of biomass energy (Demirbas., 2008). Nuclear power can also act as another alternative for energy but there is a big threat of the GHG effect, because of producing a carbon similar to coal (Walters *et al.*, 2002). Goldemberg suggested to increase the contribution of renewable energy to extend the life of fossil fuel reserves (Goldemberg., 2006). Currently increased attention is towards exploring biofuels to replace fossil fuels as they can provide some relief from the reliance on imported oil and are most eco-friendly energy source with respect to global warming and can be a favourable choice of fuel based upon their renewability, biodegradability and generating acceptable quality exhaust gases (Nigam and Singh., 2011).

2.4. Renewable energy potential of Pakistan

Total renewable energy potential of Pakistan is about 167.7 GW, 8 times higher than country total 21 GW demand of electricity (Farooq and Shakoor., 2013). Renewable energy can be the solution for all energy crisis in Pakistan as it holds approximately 800,000 km^2 with abundance of water, wind and sunshine. Also, self-sufficient in crops, agricultural residues and waste that could be utilized efficiently for energy production (Shakeel *et al.*, 2016). Pakistan has significant biofuels production potential like bioethanol and biodiesel, more than 400,000 tons $year^{-1}$ of ethanol can

be produced with respect to current sugarcane cultivation rate though, about 120,000 tons is being produced which is a third portion of total potential (Raheem *et al.*, 2016). Castor bean is another potential source of biodiesel, a self-grown crop in different parts of the country, capable of producing 1180 kg oil ha^{-1} drastically higher than other oil producers like soybean 375 kg oil ha^{-1}, corn 140 kg oil ha^{-1} and sunflower 800 kg oil ha^{-1} (Asif., 2009).

2.5. Biofuels and their importance

Liquid, gas and solid fuels chiefly produced from biomass are referred to as biofuels. Fuels variety which can be derived from biomass includes methanol, ethanol, methane, hydrogen, biodiesel and Fischer-Tropsch diesel (Demirbas., 2008). Biofuels are important replacement of petroleum fuels and key to dropping reliance on foreign oil, decreasing GHG emissions also accomplishing rural development goals (Nigam and Singh., 2011). Biofuels are broadly classified as primary and secondary biofuels.

2.5.1. Primary Biofuels

Primary biofuels are unprocessed biomass, utilized in their natural and non-modified chemical form it includes wood chips, firewood, and pellets. These fuels usually supply cooking fuel, heating or electricity generation for small and large-scale industrial applications (Nigam and Singh., 2011).

2.5.2. Secondary Biofuels

These fuels are the modified form of primary fuels, processed and modified in solids (e.g. charcoal), liquids (e.g. ethanol, biodiesel and bio-oil), or gaseous forms (e.g. biogas, synthesis gas and hydrogen). These secondary fuels have huge ranges of applications, including transportation and in thermophilic industrial processes (Nigam and Singh., 2011). Based on raw material and used technology for their production, secondary biofuels are categorized into first, second and third-generation biofuels.

2.5.2.1. First Generation Biofuels

Food and oil crops based fuels are considered as the first-generation biofuels, they are mainly derived from sugar beet, sugarcane, animal fats and vegetables oils. They have already reached to commercial level in USA, Brazil, and the European Union

while still limited in transportation sector due to food security issue (Milano *et al.*, 2016). However, food shortage issue is created by using food crops for biofuels production. Along with this, their cultivation cycle longer and also needs large area of arable land. Use of first-generation feedstocks for biofuels production is not a better solution as resolving energy crises could create food crises (Naik *et al.*, 2010).

2.5.2.2. Second Generation Biofuels

Second-generation biofuels like biodiesel and bio-synthetic gas etc. are derived mainly from non-edible components from food crops, agricultural residue, wood processing residues and non-food crops cultivation like tobacco seed, jatropha and Miscanthus (Milano *et al.*, 2016). In comparison with first generation, they have reduced environment impact as they are not directly competing with arable land. Despite of this, the conversion processes for second generation biofuels are currently not economically because of having low conversion rates (Adenle *et al.*, 2013).

2.5.2.3. Third Generation Biofuels

Microalgae derived biofuel are known as third generation biofuels and they are potentially capable for large-scale production. Microalgae appears to be the only feasible feedstock having the ability to replace fossil fuel completely (Ziolkowska and Simon., 2014). Microalgae shows high growth rate and having the capability to survive in harsh conditions, therefore it could be considered as the cheapest biomass for biofuel production. Usually photo-bioreactors or (PBR) large open ponds are used for microalgae cultivation follow up by harvesting for further conversion to biofuels (Milano *et al.*, 2016).

2.6. Microalgae

Algae are the primitive plants lacking stems, leaves and roots having primary photosynthetic pigment chlorophyll, considered among the oldest forms of life and capable of living in wide range of environmental conditions (Mata *et al*, 2010).

2.6.1. Biofuels production from microalgae

The impression of using algae as a raw material for energy generation dates to the late 1950s, but now it is taken critically (Bibi *et al.*, 2017). Microalgae can be

photoautotrophic, mixotrophic or heterotrophic. Phototrophic microalgae under natural growth conditions absorb sunlight, and sequester CO_2 into lipids, proteins and carbohydrates within short periods of time. These products can be further processed into both biofuels and valuable co-products (Brennan and Owende., 2010). Microalgae can fix three different sources of CO_2 including soluble carbonates, atmosphere and discharge gases (Wang *et al.*, 2008). It also requires some inorganic nutrients for its growth like nitrogen, phosphorus and Sulphur (Hu *et al.*, 2008). They are photo-synthetically more efficient than terrestrial plants because of their efficient CO_2 fixation and also can be used to reduce greenhouse gas (GHG) emission (Patil *et al.*, 2008). Recently two novel microalgae strains *Desmodesmus* sp. FG and yet unidentified strain SP2-3 has been screened out, having the carbohydrates generating potential of 57% and 70% respectively on dry biomass basis, under controlled growth conditions and nitrogen deficiency (Rizza *et al.*, 2017).

2.6.2. Advantages associated to microalgae-derived biofuels:

- Capable of producing oil throughout the year, therefore exceeding in yield from all known oil seed crops (Schenk *et al.*, 2008).
- Although they require aqueous growth medium, but their water necessity is much less than terrestrial crops so dropping the burden on freshwater sources (Dismukes *et al.*, 2008).
- Can be cultured on non-arable land and in brackish water thus reducing associated environmental impacts (Searchinger *et al.*, 2008).
- Having a rapid growth rate with a doubling time of3.5 h during exponential phase and many species with high lipid content about 20–50% of dry weight (Chisti., 2007).
- Capable of improving air quality maintenance through bio-fixation of waste CO_2, about 1.83 kg of CO_2 is utilised by 1 kg of dry algal biomass (Chisti., 2007).
- Capable of obtaining nutrients for their growth like nitrogen and phosphorus from wastewater thus having potential for organic effluent treatment from the agri-food industry (Cantrell *et al*, 2008).

- Their cultivation does not require any pesticides or herbicides application (Rodolfi *et al.*, 2009).
- Having potential for valuable co-products like oil extracted proteins enrich residual biomass may be used as feed, fertilizer or can be fermented into ethanol or methane (Spolaore *et al.*, 2006).
- Oil yield can be significantly enhanced by optimizing different growth conditions (Brennan and Owende., 2010).
- They are capable of photobiological production of Biohydrogen (Ghirardi *et al.*, 2000).

Biofuels from algae are considered as the biggest alternative of petroleum energy, yet there is need to achieve the commercial grade fuels quality by genetic modifications of algal cells to make them more efficient in their oil storing abilities (Nikolić *et al.*, 2009).

2.6.3. Microalgae culturing techniques

2.6.3.1. Sample collection

Indigenous microalgal strains are having greater adaptability to their native environmental conditions, consequently preferred for fuel production purpose. Most of microalgae species are being photoautotrophic and can be collected from ocean, lakes, pond, river, soil and rocks, while some strains can be found on extreme conditions like hot spring lakes, ice-lakes, nuclear reactors and brine environment like *spirulina*, are halo-thermotolerant and *Zygnemopsis* sp. is extremely high sunlight intensity tolerant (Ghosh *et al.*, 2016).

2.6.3.2. Isolation

Isolation of axenic microalgae cultures is usually achieved by either spread plate, streak plate, pour plate and serial dilution methods. Capillary method is another technique in which desired species' s inoculum obtained by operating a capillary pipette handled under a microscope followed by dilution method. Fluorescence Activated Cell Sorting (FACS) is also an effective isolation method follow-up by

subsequent isolation (Ghosh *et al.*, 2016). Paper-based device is an additional latest and cheaper technique for single cell separation of microalgae and the main driving force behind this technique is forthright capillarity lacking complex pump connection that is part of most devices for micro fluidics (Chen *et al.*, 2015).

2.6.3.3. Bioreactor types and cultivation parameters

There are three different types of systems involved in microalgae cultivation consisting of open ponds reactors (OPR), photo-bioreactors (PBR) and hybrid systems (Jankowska *et al.*, 2017). OPR are exposed to the environment and having comparatively cheaper construction, installation and maintenance cost and easy operation. Drawbacks associated to these reactors includes high contamination chances, vaporization (Kröger and Müller-Langer., 2012) and relatively low biomass of around 10–25 g per day per m^2 (Borowitzka., 1992). Raceway ponds and High Rate Algal Pond (HRAP) are examples of OPRs. On the other hand, photo bioreactors are closed systems with optimized algal growth conditions leading to high biomass production rate of 20-100 g per day per m^2 and controlled conditions against other algal contaminations but it requires higher operating and maintenance costs (Jankowska *et al.*, 2017). Its examples include flat-tank (Samson and LeDuy., 1983), tubular (Ras *et al.*, 2011), serpentine and bubble column reactors (Zamalloa *et al.*, 2012).

Various factors are involved in efficient cultivation of microalgae like nutrients concentration, water availability, CO_2 supply, light intensity of 1000–10000 lx, optimum temperature of 16–27 °C, pH between 4-11, culture density, turbulence, traces metals and salinity of 12–40 g/L (Jankowska *et al.*, 2017).

2.6.3.4. Strain selection

Higher lipids and carbohydrates contents, increased biomass yield, eco-friendly attitude and capability of growing on cheaper substrates like wastewater are main features of microalgae for being efficient feedstock for biofuel production. Potent strains selection can be based upon higher growth rates and enhanced carbohydrates and lipid accumulations (Gill *et al.*, 2016) like *Botryococcus braunii* is having lipid content up to 75% of its total biomass (Lee *et al.*, 2010), while *Chlorella vulgaris* can accumulate carbohydrates up to 37–55% of its dry biomass (Chen *et al.*, 2013).

2.6.3.5. Optimization

Selection of fast growing microalgae strains and its optimization for producing higher energy content is important to improve the economic value of microalgae derived biofuels. Microalgal cells growth can be enhanced by optimizing various factor like nutrient-rich medium, appropriate pH, temperature, light intensity and proper aeration/mixing. According to recent study carbohydrate or lipid content can increased when microalgae cultivated under nitrogen-deficient conditions. This could be achieved by supplying microalgal cell with nutrient-rich medium firstly, later on culture is shifted to specific nutrient-deficient medium when over 90% nitrates are consumed, it will trigger the quick accumulation of either lipids or carbohydrates (Ho *et al.*, 2013b).

2.6.3.6. Harvesting

Harvesting of microalgae from their growth medium is a key challenge in microalgae downstream-processing (Vandamme *et al.*, 2013). As cell density of microalgae is below 1.0 g L^{-1} in open pond cultivation systems and requires a huge volume of water removal. Furthermore, microalgal cells size of 2–20μm remain in suspension form due to their colloidal stability, making the separation process more complicated and overall harvesting step accounts for 20–30% of total production cost. An effective harvesting technology with minimal investment of cost and energy is needed to improve overall microalgae biomass production process (Wan *et al.*, 2015). Currently, applied techniques for microalgal biomass harvesting includes gravity sedimentation, centrifugation, filtration and screening, flocculation, electrophoresis and flotation. Appropriate harvesting technique selection depends upon cellular properties like size, density and worth of preferred products (Pragya *et al.*, 2013).

2.7. Algal biofuels conversion technologies

Technologies involved in microalgae biomass energy derivation can be divided into two major categories that includes biochemical and thermochemical conversion. Factors responsible for choosing appropriate conversion method are comprised of the biomass type and quantity, desired energy form, economic consideration and nature of desired final product.

2.7.1. Thermochemical conversion technologies

2.7.1.1. Gasification

Term gasification is stand for the process responsible for partially oxidizing biomass into the form of a gas mixture. Gasification is performed at higher temperature, when at 800-1000°C temperature conditions water and oxygen react with biomass resulting in the production of syngas. Syngas is the mixture of CH_4, CO_2, CO, N and H_2. Its calorific value is very low so it can be taken as fuel in gas turbines and engines (McKendry., 2002).

2.7.1.2. Thermochemical liquefaction

Thermochemical liquefaction is a higher pressure and lower temperature process. In this technique at temperature of 300–350.8°C under high pressure of about 5–20 MPa, wet algal biomass is converted into liquid fuel. This process is performed by a catalyst in the presence of hydrogen to yield bio-oil (Patil *et al.*, 2008; Goyal *et al.*, 2008).

2.7.1.3. Pyrolysis

Pyrolysis involves the conversion of biomass at 350–700.8°C temperatures in the absence of oxygen into bio-oil, charcoal and syngas. Change in condition leads to products formation in different proportions like pyrolyzing the biomass at slow heating rates of 5–7 K/min will lead to higher char production and less liquid and gaseous product development (Goyal *et al.*, 2008).

2.7.2. Biochemical conversion

Different biological processes are used to generate green energy. These technologies embrace anaerobic degradation, enzymatic conversion and alcoholic fermentation resulting in variety of energy products like photobiological hydrogen, biodiesel, biogas and bio-alcohols e.g. bioethanol, bio-methanol and biobutanol.

2.7.2.1. Biohydrogen

Microalgae is equipped with essential hereditary enzymatic and metabolic characteristics to generate H_2 gas. In anaerobic conditions microalgae produce

hydrogen which is used as an electron donor during CO_2 fixation (Ghirardi *et al.*, 2000). During photosynthesis, microalgae convert water molecules into ions by tic process. Resulting hydrogen ions are further converted into H_2 in anaerobic conditions by the activity of hydrogenase enzyme (Cantrell *et al.*, 2008). Reaction reversibility consequences in either production or consumption of hydrogen through simple proton transformation into hydrogen (Clark and Macquarrie., 2008). Hydrogen production could be failed by oxygen production during photosynthesis which causes hindrance to enzymatic activity responsible for hydrogen generation (Cantrell *et al.*, 2008).

2.7.2.2. Anaerobic Digestion

Technology involves in the conversion of complex organic matter into CH_4 and CO_2 is named as anaerobic digestion (AD). The process of AD is based on four subsequent stages categorised as hydrolysis, acidogenesis, acetogenesis and methanogenesis. In the step of hydrolyses, microbial secreted extracellular enzymes hydrolysed the complex organic compounds into sugars, fatty acids amino acids and alcohols. Succeeding conversion of these molecules into H_2, CO_2 and volatile fatty acids takes place during acidogenesis and acetogenesis. Lastly, methanogenesis comes which is comprised of methanogenic archaea that utilises formed H_2, CO_2, fomites and butyrate as substrate and producing CH_4 as main product along with CO_2 (Gonzalez-Fernandez *et al.*, 2015). Methanogenesis is normally carried out by either catabolic acetoclastic pathway generating almost 70% methane, or by hydrogenotropic pathway activated during stress induced conditions (Carballa *et al.*, 2015). Advantages associated with AD contains renewable energy production, reduced greenhouse gas emissions and resulting produced liquid digestate with fertilizer properties (Gonzalez-Fernandez *et al.*, 2015).

2.7.2.3. Bioethanol

Bioethanol is being produced by the fermentation first generation feedstocks like cornstarch, sugarcane, potato, wheat, rye, beet, cassava derived sugars and second-generation lignocellulosic biomass (Ajit *et al.*, 2017). Typically, sugars are fermented into bioethanol through yeasts (Sulaiman *et al.*, 2011), whereas bacterial based fermentation is also gaining much attention (Ajit *et al.*, 2017).

According to US Energy Policy Act 2005, the oil industry is obligatory to blend 7.5 billion gal of renewable fuels into gasoline by 2012 and ethanol is considered as the most common renewable fuel (Gray *et al.*, 2006). Bioethanol is environmentally sustainable and primarily a renewable biofuel. In altered spark-ignition engines ethanol can be burned in its pure form or can be used as blend with petrol. Though ethanol comprised of only 66% energy per litre in comparison with energy per litre of petrol but due to having higher octane level, it improves the latter performance when blended with petrol as transportation fuel. It also reduces the lethal vehicles emissions like carbon monoxide, carcinogens and unburned hydrocarbons by improving the fuel combustion process. As ethanol contains just traces of Sulphur, in comparison with petrol thus ethanol-petrol blends can also reduce the Sulphur oxide emissions which is main constituent of acid rain, and a carcinogen (Nigam and Singh., 2011).

2.7.2.4. Biodiesel

A blend of mono-alkyl esters of long chain unsaturated fats (FAME) is known as biodiesel which is derived from sustainable lipid feedstock like oil-crops, second-generation feed stock e.g. jatropha and algae extracted oils. These lipid feedstocks are comprised of 90–98% by weight triglycerides, 1–5% free unsaturated fats and minute contribution of mono and diglycerides along with slight portion of carotenes, phospholipids, sulfur mixes, phosphatides and minute quantity of water (Bozbas., 2008). Biomass extracted oil can be subsequently converted into biodiesel through the process of transesterification. Transesterification can be considered as synthetic response among triglycerides and liquor in the presence of an incentive to generate mono-esters named as biodiesel (Sharma and Singh., 2009).

2.8. Bioethanol production from microalgae

As compared to first and second-generation feedstocks algae being third generation biomass seems to be very attractive alternative renewable source for bioethanol production (Menetrez., 2012). It can also resolve the sustainability problems mostly associated with first generation feedstocks including food crops security (Dębowski *et al.*, 2013; Rajkumar *et al.*, 2013). Being more photosynthetically efficient algae biomass production rate is 5–10-times higher than land-based plants, categorized as second-generation feedstocks (Chen *et al.*, 2013), along with this algae pre-treatment

and enzymatic hydrolysis is much easier than lignocellulosic materials as it lacks lignin therefore making the process beneficial for ethanol production. The algal biomass is processed similarly for biofuel production as other lipid-based feed stocks and its carbohydrates content could be fermented into bioethanol (Bibi *et al.*, 2017).

2.8.1. Microalgae-based carbohydrates composition

Microalgal cell wall primarily consist of an inner layer and outer layer and the composition of outer layer differs in each specie but normally comprised of polysaccharides like pectin, alginate and agar while the inner cell wall layer is made-up of cellulose, hemicellulose and glycoprotein (Yamada and Sakaguchi., 1982). Due to having carbohydrates source like cellulose-based cell walls and accumulated starch, microalgae supposed to be a promising feedstock for bioethanol production. These both carbohydrate types can be reduced into fermentable sugars leading to microbial fermentation for subsequent bioethanol production (Chen *et al.*, 2013). Study shown that *Chlorella vulgaris* can accumulate carbohydrates up to 37–55% of its dry biomass likewise 45–60% of dry biomass content of *Chlamydomonas reinhardtii* and *Scenedesmus obliquus* is also comprised of carbohydrate (Kim *et al.*, 2006). Therefore, species with higher carbohydrates accumulating ability are potential candidates for carbohydrate-based bioethanol production (Chen *et al.*, 2013).

2.8.2. Carbohydrates extraction or hydrolysis of algal biomass

Cell wall-bound carbohydrates in microalgal cells released into hydrolysate under optimized conditions and converted into fermentable sugars by saccharification. Bioethanol production efficiency of fermentation mainly depends upon pre-treatment or hydrolysis conditions of algal biomass. Therefore, an effective pre-treatment is necessary for better carbohydrates availability and enhanced bioethanol production (Harun *et al.*, 2014). Pre-treatment or hydrolysis is usually categorized into physical, chemical and enzymatic approaches (Daroch, *et al.*, 2013). chemical approach is the most commonly used method for polysaccharides hydrolysis while, enzymatic hydrolysis is a latest efficient approach (Jambo *et al.*, 2016).

2.8.2.1. Chemical hydrolysis/pre-treatment

Commonly studied technique is Extremely low acid (ELA) pre-treatment in which inter and intra-molecular hydrogen bonds broken down resulting in release of carbohydrates into the hydrolysate (Harun *et al.*, 2014). Acid being a catalyser will break polysaccharide into monosaccharide by hydrolysing the glycosidic bonds (Jambo *et al.*, 2016). Maximum sugar recovery can be achieved by optimizing the chemical pre-treatments parameters (Daroch *et al.*, 2013). A study showed the acid treatment of *C. vulgaris* FSP-E with 0.036–1.8 N sulfuric acid, used 10–80 g L^{-1} of biomass concentration resulted in up to 95% saccharification of the glucose content leading to 90% of the theoretical fermentation yield with *Zymomonas mobilis* ATCC 29191 and proved it to be better than enzymatic treatment (de Farias Silva and Bertucco., 2016).

Alkaline pre-treatment of algal biomass was used by Harun *et al.*, (2014) for the very first time and found 0.75% NaOH exposure at 120°C for 30 min, as the optimized conditions for maximum glucose yield (Harun *et al.*, 2014).

2.8.2.2. Mechanical pre-treatment

It is also known as Physical pre-treatments comprised of cell wall disruption by using technologies like milling, supercritical CO_2 exposure and ultrasonication. Ultrasonic assisted extraction (UAE) is an efficient method for carbohydrate extraction from algal biomass as compared to fluidized bed extraction (FBE) and conventional solvent extraction (CSE) method (Zhao *et al.*, 2013). Gamma radiation can be also use of for weakening the algal cell wall for carbohydrates extraction (Yoon *et al.*, 2012). Hydrothermal pre-treatment is another method responsible for splitting algal biomass into sugar and lipid fractions for further bioethanol and biodiesel production processing (Kim *et al.*, 2012). Another alternative pre-treatment technique is supercritical fluid exposure. As compared to intact algal biomass, 60% more bioethanol has been produced by applying Supercritical CO_2 treatment to lipid-extracted biomass of *Chlorococum* sp (Harun *et al.*, 2014).

2.8.2.3. Enzymatic hydrolysis/ pre-treatment

Enzymatic hydrolysis is a simple way to convert complex sugars into simple form. Having the conversion efficiency of more than 80% seems to be an attractive approach for bioethanol production. Enzymatic hydrolysis typically employed *cellulases* enzymes for polysaccharides degradation it includes *exo-glucanases*, *endo-glucanases* and *β-glucosidases* (Jambo *et al.*, 2016). Enzymatic hydrolysis of *Chlamydomonas reinhardtii* by using amylases for liquefaction and glucoamylase for saccharification in separated hydrolysis and fermentation (SHF) achieved 94% carbohydrates hydrolysis and further fermentation achieved a yield of 60% through *Saccharomyces* cerevisiae S288C (de Farias Silva and Bertucco., 2016).

2.8.3. Drawbacks linked to acid and enzymatic hydrolysis

Drawback associated to acid hydrolysis is the fermentation inhibition due to formation of salts after neutralization of the liquor, if high acid concentration is being used (de Farias Silva and Bertucco., 2016). While, the biggest hindrances in the way of enzymatic hydrolysis are increased enzymes prices, time taking and intensive labour processes (Daroch *et al.*, 2013).

2.8.4. Two steps Saccharification

Introduction of two-step process in saccharification is capable for enhanced biomass treatment. Process involve the initial acidic treatment of cells carries towards the subsequent enzymatic treatment. brown algae *Laminaria sp.* was effectively saccharified by acid pre-treatment followed by the combination of many enzymatic cocktails (Daroch *et al.*, 2013).

2.8.5. Fermentation or bioethanol production

Sugars extracted from hydrolysis step converted into bioethanol and some by-products by the activity of few microorganisms like bacteria, yeast and fungi (Wei *et al.*, 2013). Bacterial genus *Zymomonas* and yeasts like *Saccharomyces* are commonly used microorganisms for bioethanol fermentation (de Farias Silva and Bertucco., 2016). Embden-Meyerhof pathway of glycolysis is involved in glucose conversion into ethanol. This is two-step process, initially sugar is converted into glucose-6-

phosphate followed by the subsequent conversion of this intermediate into pyruvate (van Maris *et al.*, 2006). Depending upon microorganism end product varies, in case of yeast pyruvate is reduced into ethanol and CO_2 during alcoholic fermentation (Jambo *et al.*, 2016).

For economically feasible and the better production rate of bioethanol, simultaneous saccharification and fermentation (SSF) and Separated hydrolysis and fermentation (SHF) are well known latest techniques instead of a simple fermentation process (Jambo *et al.*, 2016).

2.8.5.1. Separated hydrolysis and fermentation (SHF)

Mechanism involved in SHF is the initial separate degradation of feedstock into monomeric sugars, followed by the subsequent fermentation reaction. Key problem associated to SHF is the inhibition of fermentation end-product by sugars formed in the process of hydrolysis (Alfani *et al.*, 2000)

2.8.5.2. Simultaneous saccharification and fermentation (SSF)

In SSF, single reactor and a single step is involved in simultaneous hydrolysis and fermentation. Reaction comprised of the collective addition of feedstock, yeast and enzyme in an orderly manner for the rapid conversion of released sugars into bioethanol. End-product inhibition can be limited in SSF by removing the residual sugar (Dahnum *et al.*, 2015) and under the appropriate conditions, higher bioethanol yield can be achieved.

2.8.6. Drawbacks Linked to SHF and SSF

Each process has their own advantages and disadvantages like SHF can be conducted at optimum temperature independently, though high contamination and end-product inhibition incidence cannot be avoided in this process. As for as SSF is concerned, the process requires only a small amount of enzyme, decreased inhibitory effects and less contamination chances while, the only problem associated to SSF is the elevated temperature requirement for enzymatic hydrolysis making the process difficult to control. Keeping in view the above all discussion SSF is typically preferred over the SHF process due to its reduced costs and high production rate (Jambo *et al.*, 2016).

2.8.7. Purification

Techniques involve in bioethanol purification step includes distillation, rectification and dehydration that can highly influence the end products (Demirbas., 2011). Among these most widely used technique is distillation although it is highly energy consuming process. Basic principle of distillation is the component volatilities based separation of mixtures and resulting bioethanol must accomplish the international standards such as ANP and ASTM for its commercialization (Jambo *et al.*, 2016).

2.9. Biodiesel production from microalgae

2.9.1. Lipids accumulation in microalgae

Various microalgae strains are known for accumulating considerable lipids quantities and responsible for high oil yields. concentration can be increased by optimizing various growth influential factors like CO_2 concentration, stressed nitrogen level, temperature, intensity of light, salinity and harvesting method (Brennan and Owende., 2010). Their lipid storage properties fluctuate between the average of 1-70% while some species can hold up to 90% of their dry weight under certain conditions. Fatty acid composition may be affected by various environmental and nutritional factors, growth phases and cultivation conditions (Mata *et al.*, 2010). Normally microalgae stores carbohydrates proteins and lipids inside the cell but under stress conditions its cell division is terminated and CO_2 is shifted to energy storage in the form of lipids resulting in increased lipid content of algal cell (Xin *et al.*, 2010).

2.9.2. Lipid Extraction

Before biodiesel production, lyophilized biomass is exposed to solvent treatment for lipids and fatty acids extraction from the algal cells. Some most widely used solvents are comprised of 96% hexane, 96% ethanol, or a 96% ethanol-hexane mixture. These solvent extraction methods can obtain up to 98% quantity of purified fatty acids. Though ethanol can be used as proficient solvent but incase when only lipids extraction is required it could not be desirable as it can also extract other cellular impurities like sugars, salts, amino acids, proteins and pigments (Mata *et al.*, 2010).

2.9.3. Transesterification

Process by which reaction between acyl-acceptors and triglycerides takes place, following up the conversion of triglycerides into lower molecular weight alkyl esters is referred to as transesterification (Helwani *et al.*, 2009). During the process of transesterification glycerol is formed when alcohol acts as acyl acceptor and as a result when esters are being used triacylglycerol is produced (Robles-Medina *et al.*, 2009). It is a three steps process; diacylglycerol and one fatty acid ester is formed from triacylglycerol in first step carrying towards the production of monoacylglycerol and fatty acid from diacylglycerol. In final step conversion of monoacylglycerol into terminal product fatty acid esters and glycerol takes place. Three moles of alcohol are needed for the conversion of one mole of triglycerides however, higher molar ratios of oil and alcohol are required to achieve maximum biodiesel yield. Based upon type of catalysts, feedstock, reaction time and temperature these molar ratios are varied. Ethanol, methanol, and propanol are mainly used alcohols in the process (Rathore *et al.*, 2007). In this reaction ester bond is firstly broken down leading to the subsequent breakage of hydroxyl group, resulting in formation of glycerol as a by-product (Al-Zuhair *et al.*, 2007). Different types of catalysts used in the process of transesterification encompasses acid catalyst, alkaline catalyst, used alcohol in supercritical state or biological catalysts. (Mythili *et al.*, 2014).

2.9.3.1. Alkaline catalytic transesterification

In this process reaction between triacylglycerol and alcohol is catalyzed by an alkali at pH above 7. frequently used catalysts include potassium hydroxide, Sodium hydroxide, and sodium methoxide. Alkaline catalysts are preferred due to their easy usage. Base catalyzed transesterification is usually carried out at lower temperature and pressure and resulting in highest yield of 98% achievement within short period of time (Rathore and Madras., 2007). Whereas, the key drawback linked to alkaline catalyst is its sensitivity to water content and free fatty acids and cannot work in condition with FFA and water content more than 0.5wt% (Sivasamy *et al.*, 2009). High FFA accumulation in reaction utilizes the catalyst carries towards the excessive soap formation, which can affect the yield and creates separation and purification problems in downstream processing. To avoid these hurdles pre-treatment is needed (Al-Zuhair *et al.*, 2007).

2.9.3.2. Acid catalyzed transesterification

Acid catalyzed transesterification involves the reaction between TGs and alcohol in the presence of acid. Commonly used acids as catalyst includes phosphoric and hydrochloric acid (Meher *et al.*, 2006). Acid catalyzed reaction is rarely used method and it is 4000 times slower than alkaline catalyzed reaction and enlarged convergence of catalyst is needed with higher alcohol concentration. Studies shown that molar methanol and oil ratio of 30:1 at 55– 80°C along with 0.5 to 1mol% catalysts convergence required for accomplishing 99% yield in 50 hours (Akoh *et al.*, 2007).

2.9.3.3. Enzymatic Transesterification

The process is comprised of using biological catalyst like lipases enzymes for the conversion of triglycerides into biodiesel. Lipases are lipid degrading enzymes showing the catalytic properties in the presence of methanol. Lipase enzymes are included in the class of hydrolases enzymes produced extracellularly or intracellularly by both bacteria and fungi. They broke down triglycerides into single fatty acids and glycerol leading to subsequent esters molecule formation by lipase catalysis in transesterification process (Robles-Medina., 2009; Ranganathan *et al.*, 2008). There is no byproduct formation occurs in enzyme mediated catalysis and having easy operational conditions. In enzymatic transesterification biodiesel production could be accomplish at lesser temperature of 35-45°C (Antczak *et al.*, 2009). During reaction, free fatty acids and triglycerides are converted into biodiesel and unlike chemical catalysis, soap production does not take place so no washing is required (Noureddini *et al.*, 2005).

MATERIAL AND METHODS

3.1. Sample collection and storage

Fresh water samples were collected from Mirpur, Shahdara stream and Rawal Lake Islamabad Pakistan for microalgae isolation. Samples were collected in 50ml sterile falcon tubes against the flow rate and temperature and pH of collection site were noted. Samples were brought to laboratory and stored at 4°C to cease any type of microbial activity prior to further processing.

3.2. Bold's Basal Medium (BBM) media preparation

Table No. 3.1: BBM media recipe

	Components	Stock solution (g/200ml)	Working volume (ml/L)
Stock 1	$NaNO_3$	5	10
	$CaCl_2$	0.5	10
	$MgSO_4$	1.5	10
	K_2HPO_4	1.5	10
	KH_2PO_4	3.5	10
	NaCl	0.5	10
	Components	**Stock solution (g/200ml)**	**Working volume (ml/L)**
Stock 2	EDTA	1.0	1
	KOH	6.2	1
	$FeSO_4.7H_2O$	0.98	1
	H_3BO_3	2.28	1
	Components	**Stock solution (g/50ml)**	**Working volume (ml/L)**
Stock 3	$ZnSO_4$	0.441	0.2
	$MnCl_2.4H_2O$	0.072	0.2
	MoO_3,	0.035	0.2
	$CuSO_4.5H_2O$	0.078	0.2
	$CO(NO_3)_2.6H_2O$	0.024	0.2

3.3. Microalgae isolation

Microalgae culture was firstly enriched in QAU wetland effluent water and BBM medium. The flasks were incubated at room temperature for 15 days under continuous illumination and aeration provided by fluorescent lamps and aerator pumps respectively. BBM agar medium was prepared by adding 7.5g of technical agar in 500ml working volume of BBM broth along with 1g/L Amoxil and 5 drops/ L of Nystatin as antibacterial and antifungal agents respectively to avoid any fungal or bacterial contamination. 1 ml of sample from each enrichment culture poured onto agar medium and dispersed equally by spreader. Plates were putted under illumination at room temperature for growth development. Pure cultures were obtained by streaking and further re-streaking methods.

3.4. Broth cultures development

Five different strains were isolated based upon their morphology, colour change, shape and appearance. Small inoculums were picked from re-streaked plates with sterile wire loop and inoculated into the flasks. Flaks were incubated at room temperature and sunlight was provided as source of illumination while aeration and mixing was supplied by stone bubblers associated with electric aerator pumps. Algal growth was observed by measuring optical density at 682nm with UV-visible spectrophotometer after the interval of every two days.

3.5. Molecular identification

For molecular level identification of strains, DNA was extracted and sent to MACROGEN, Korea for sequencing.

3.5.1. DNA Extraction

Modified CTAB method was used for microalgae DNA extraction. 2 ml of each algal sample was taken in Eppendorf tubes and centrifuged at 10,000rpm for 10 minutes. After discarding the supernatant, about 1-1.5ml of 1X TE buffer was added to Eppendorf and vortexed to dissolve the pellet. Centrifuged again at 10000rpm for 10 minutes and TE buffer was discarded. The pellet was re-dissolved via vortexing into 0.6ml of TE buffer and then added 3-5µl proteinase k along with 5-10µl of lysosome

and incubated in water bath for 1 hour. After this incubation 100µL of CTAB buffer along with 80µl of 5M NaCl was added and again incubated in water bath at 65°C for10 minutes. Then 500µl of phenol: chloroform: isoamyl alcohol was added in 25:24:1 ratio and centrifuged at 10000rpm for 20 minutes. After the centrifugation, upper layer was picked up carefully and transferred it to new Eppendorf tube which was supplemented with 500µl P:C:I. tube was centrifuged again at 10000rpm for 10 min . Now upper layer was taken, added with 500µl isopropanol and 300µl 3M sodium acetate and left for overnight incubation. Centrifuged again at 10,000rpm for 6 minutes and discarded the supernatant. Then 200µl 70% ethanol was used to wash the pellet and again centrifuged at same temp and rpm for 3 min. After discarding the supernatant pellet was dissolved in 100µl TE buffer and stored at freezing temp.

3.5.2. Gel Electrophoresis

To confirm the presence of DNA, gel electrophoresis technique was performed. To make 1X TAE buffer, 3ml of 10X TAE buffer was added to 27ml of deionized water. Then gel was prepared by dissolving 0.3g agarose into 30 ml 1X TAE buffer and heated for two minutes in microwave. After cooling to 40°C, 3µl ethidium bromide was added to gel and it was poured into the gel-tray with already embedded comb in it to make the wells. Comb removed after solidification of gel and wells were formed. Then total volume of 5µl (made after mixing of sample with 2 µl of DNA loading dye) was loaded into the wells and process was run for 30 minutes at 110 volts and 500mA in gel-tank. After removing gel from tank, it was observed in UV-illuminator to detect the DNA bands.

3.5.3. 18S Ribosomal RNA sequencing

Extracted DNA samples were sent to MACROGEN standard custom DNA sequencing services (Macrogen Inc, Seoul, Korea) for microalgal identification by18S Ribosomal RNA sequencing.

3.6. Growth kinetics and Biomass productivity

3.6.1. Growth kinetics

Growth kinetics of each microalgae strain was calculated by measuring the light absorbance intensity within their cell suspensions. For this purpose, optical density of

cultures was observed at 682nm in UV–visible spectrophotometer (Santos-Ballardo *et al.*, 2015).

3.6.2. Cell growth efficiency

Both the specific growth rate and duplication time for microalgae strains were calculated by using following growth kinetics (Godoy-Hernández and Vázquez-Flota, 2006).

Specific growth rate:

$$\mu=(lnX-lnX°)/t$$

Whereas: μ is the specific growth rate

X°=initial biomass concentration

X =the final biomass concentration

t= time (days).

3.6.3. Biomass productivity

During the exponential growth phase, grams of dry biomass produced per liter per day is known as biomass productivity. Samples were collected during their exponential phase and centrifuged at 5000rpm for 10 min at 10°C. supernatant discarded and pellets were washed with distilled water, dried overnight at 60°C in oven and weighed in weighing balance (Ranga-Rao., 2007).

Biomass productivity=DW/SV/Day*1000

DW= Dry weight

SV= Sample volume

3.7. Carbohydrates and sugars content determination

Carbohydrates content of all the 5 isolated microalgal strains was determined for the selection of single strain with higher carbohydrates content. As carbohydrates are

embedded in cell wall of microalgae, therefore proper extraction methods were used for their release into the hydrolysate. Two types of extraction methods were used for carbohydrates extraction as discussed below:

3.7.1. Acidic extraction

All the five microalgal strains were collected during their exponential phase and harvested by centrifugation at 5000rpm for 10 min at 10°C. After discarding the supernatant, pellets were washed by using distilled water, dried overnight at 60°C in oven. 0.1 g of dried biomass of each sample was treated separately with 3 ml of 72% concentrated H_2SO_4 (v/v) in a flask and stirred for 30 min at 30 °C on a magnetic stirrer. After stirring solution was diluted to final acid concentration of 2.5 with addition of distilled water. These flasks were then autoclaved at 121 °C for 30 min for sample hydrolysis. Each hydrolyzed sample was individually distributed into two flasks. NaOH was used to neutralize the sample from first flask for reducing sugar determination of by DNSA method whereas the other flask was neutralized with Na_2CO_3 for total carbohydrates determination by Phenol–Sulfuric acid method. Both the solutions were kept at 4°C and centrifuged at 8000rpm before use.

3.7.2. Alcoholic extraction

10 ml of 70% ethanol was used to treat 0.1 g of dried algal biomass for 2 hours at 80°C in a water bath. Sample was then filtered after cooling. Residual sample was again treated with 70% ethanol in water bath at 80°C for complete soluble sugars extraction. Both the filtrates thoroughly mixed and diluted to the volume of up to 100 ml with distilled water. This filtrate solution was centrifuged at 80000rpm for 10 min and used for total soluble carbohydrates estimation.

3.7.3. Carbohydrates determination

3.7.3.1. Phenol-sulfuric acid method

Total carbohydrates present inside microalgal cells was estimated by phenol-sulfuric acid method and its standard protocol is discussed below.

Standard curve preparation

Stock solution:100g of glucose dissolved in 100 ml of water.

Working volume:10 ml is taken from stock solution and diluted up to volume of 100 ml with distilled water, making the final sugars concentration of 0.1 mg/ml. A range of 0µl-1000 µl from this solution is taken into 6 tubes as discussed below in table No. 3.2.

Table No. 3.2: Working volume of standard sugar solution

Test tube No.	Glucose solution quantity (µl)	Distilled water quantity (µl)
1	0	1000
2	200	800
3	400	600
4	600	400
5	800	200
6	1000	0

Protocol: Total carbohydrate in algal samples were estimated by phenol–sulfuric acid method (Dubois *et al.*, 1956). 0.2 ml of sample was pipetted out and made up to volume of 1 ml with distilled water in test tube and distilled water was used as blank in another tube. 5 ml of 96% H_2SO_4 (v/v) along with 1ml of 80% phenol was added to each tube (including tubes containing standard glucose concentrations for standard curve preparation). All tubes were then vortexed for 1 min until solution in tubes become transparent and these tubes were placed in water bath at 30 °C for 20 min and orange-yellow colour was developed in tubes. Tubes were then cooled at room temperature and optical density (OD) was recorded at 490 nm in UV-visible spectrophotometer. Standard curve was drawn and OD values of samples were putted in trendline equation and by solving the equation total carbohydrates present in samples were estimated.

Total soluble carbohydrates, total insoluble carbohydrates and total carbohydrates were calculated as following:

Total soluble carbohydrate = Carbohydrate determined in alcoholic extracts

Total carbohydrate = Carbohydrate determined in acidic extracts

Total insoluble carbohydrate = Total carbohydrate – Total soluble carbohydrate

3.7.4. Reducing sugars estimation

3.7.4.1. Dinitrosalicylic acid method (DNSA)

For reducing sugars estimation dinitrosalicylic acid (DNSA) method was used (Miller, 1959). Method based on the principal that dinitrosalicyclic acid is reduced by reducing sugars resulting in reddish-orange colored complex formation and its optical density can be observed at 540nm (Ghose, 1987).

DNSA reagent preparation

Table No. 3.3: Reagents required for1 Liter stock

Reagents	Quantity per Liter
3, 5-Dinitrosalicylic acid	7.5g
NaOH	13.99g
Na-K tartarate	216.10g
Phenol	5.4ml (melted at 50°C)
Na_2SO_3	5.87g

Standard curve preparation

Stock solution: 250 mg dissolved in 100 ml distilled water.

Working volume: 10 ml taken from stock solution and diluted up to 100 ml containing the final sugars concentration of 0.25 mg/ml. 6 test tubes were taken and 0-1000µl of final sugar solution was transferred in these tubes and volume was made up to 1 ml for each tube in the same order as shown in the table No. 3.2.

Protocol:1 ml of acid treated algal samples which were neutralized with NaOH, taken in test tube and added with 1 ml of DNSA reagent. Tubes were them incubated in water bath for 10 min at 100°C and then diluted up to volume of 10 ml with distilled

water after cooling at room temp. OD was measured at 540 nm and sugars content was determined by putting the OD values of the samples in trendline equation of standard curve.

3.8. Strain selection and optimization

For subsequent bioethanol production potential, MSB strain was selected due to its higher growth rate, biomass productivity and carbohydrates accumulation. Four parameters were optimized to enhance growth rate and biomass productivity of the strain that includes susceptible nitrogen source, pH, dark/light durations and air bubble mixing/without mixing.

3.8.1. Nitrogen source optimization

Four different nitrogen sources (NH_4Cl, Urea, $NaNO_3$, KNO_3) in quantity of 0.125g/500ml, were taken in four flasks containing BBM media (without $NaNO_3$) with working volume of 500ml and 5ml of inoculum (MSB strain) was added to each flask. The experiment was carried out for 15 days under sunlight with continuous aeration and constant pH of 7.1 at room temperature. Optical density of each flask was observed at 684nm in UV-visible spectrophotometer with every 2 days interval. At 15^{th} day, 50 ml of sample from each flask passed through pre-weighted Whatman's filter paper, dried in oven and reweighted to calculate the dry biomass. Based on these observation nitrogen source with higher growth rate and biomass productivity was selected for further microalgae cultivation.

3.8.2. pH optimization

Five flasks with working volume of 500ml BBM culture medium were inoculated with 5ml of sample into each flask and adjusted with five different pH ranges (5, 6, 7, 8, 9) for pH optimization. Experiment settled for 15 days and optical density of each flask was recorded at 684nm in UV-visible spectrophotometer with interval of 2 days. After 15 days, 50 ml sample from each flask filtered through dried, pre-weighted Whatman's filter paper and reweighted after drying in oven to calculate the dry biomass. pH of medium with higher growth rate and biomass productivity further selected for microalgal cultivation media.

3.8.3. Effect of Dark-Light cycle on growth

Two flasks with working volume of 500ml, added with BBM medium and inoculated with 5ml of sample into each flask. One flask was incubated under sun light 12:12 hours dark and light intervals while, other was incubated under 24 hours continuous illumination provided by fluorescent lamps. Experiment was carried out for 15 days in constant conditions and growth OD recorded at 684nm with 2 days interval. Thus, illumination dependent growth rate of microalgae (MSB) was determined.

3.8.4. Effect of mixing (Air bubbles) on growth

To check the effect of mixing through air bubbles on microalgal growth, two flasks were taken with 500ml BBM medium in each and inoculated with 5ml MSB strain culture in each. Proper air bubble mixing was provided in one flask through pump aerator while other was incubated without aeration source. Experiment was conducted for 15 days at room temp under continuous sunlight. Growth OD was measured after every two days interval at 684nm and effect of aeration/mixing was calibrated accordingly.

3.9. Microscopy of selected strain

Morphology of MSB strain was observed with compound microscope. Slides were prepared by placing small drop of broth culture onto glass slide with addition of few emulsifying oil droplets and covered with coverslip. Slides were placed under lens of microscope and observed at 10X and 40X.

3.10. Scaling up the cultivation of optimized MSB strain

For scaling up the biomass production, MSB strain was shifted into two 5000ml flasks with working volume of 4000ml BBM medium. The flasks were incubated under sunlight and optimized conditions were provided. Optical density was recorded regularly at 684nm through spectrophotometer with 2 days interval and culture was grown till exponential phase.

3.11. Total carbohydrates, Reducing sugars and Lipids content determination before starvation

Optimized MSB strain sample was collected during exponential phase. Sample was centrifuged and dried overnight in an oven at 60°C. Dried biomass was grinded and processed by acidic treatment for carbohydrates extraction. Total carbohydrates from acidic treated sample were estimated by Phenol-sulfuric acid method while reducing sugars were estimated by DNSA method. On the other hand, lipid content was estimated through below discussed protocol.

Protocol: 0.5g of dried sample was dissolved in chloroform: methanol solution in 10:20 ratio and left for 4 minutes. Solution was further added with 10ml of chloroform and filtered through Whatman filter paper No. 1 into graduated cylinder. Filter paper was then washed with water and that water was blended with 10ml of chloroform and re-filtered into cylinder. In cylinder solution allowed to settle down for 10 minutes and separated into two layers comprising lower layer of oil and chloroform mixture while upper layer of water and methanol. Total volume of lower chloroform layer was noted as "x" ml. Upper methanol-water layer was removed along with removal of some chloroform layer to assure the complete removal of upper layer and remaining volume of chloroform was considered as "y" ml. Solution was then transferred into a pre-weighed conical flask of "a" g and chloroform was evaporated in desiccator at 45°C. Residue was then weighed and weight of flask was noted as "b" g. Again, residue was dissolved in 5 ml of chloroform and evaporated and final weight of flask noted as "c" g.

Lipid Content was determined as:

$$\text{Lipids weight (g): (b-a) - (c-a)} = \text{"d" g}$$

$$\text{Total lipids (g)} = \textit{Lipids weight (g)} \times \textit{"x" ml/"y" ml}$$

$$\text{Total lipids (\%)} = \textit{Total Lipids (g)/Sample weight (g)} \times 100$$

3.12. Nitrogen starvation for enhanced carbohydrates accumulation

At exponential phase of growth biomass was centrifuged at 5000rpm for 5 minutes to separate the nitrogen containing medium from culture. Supernatant was removed and pellet was shifted to the freshly prepared nitrogen deficient BBM medium. Culture was grown on nitrogen starved condition for 11 days and optical density was recorded regularly with interval of 1 day at 682nm.

3.13. Total carbohydrates, reducing sugars and lipids content determination after starvation

After giving the nitrogen starvation conditions for 11 days, sample was collected, centrifuged and dried overnight at 60°C. Acidic treated sample was evaluated by Phenol-sulfuric acid and DNSA methods to estimate the increase in total carbohydrates and reducing sugars content respectively while, above discussed protocol was followed for lipid content determination.

3.14. Biomass Harvesting

Biomass was harvested by using the centrifugation technique. Microalgae cell culture was centrifuged at 5000rpm for 15 minutes and separated in pellet form. Supernatant was discarded and pellet was dried overnight in a desiccator at 60°C. The dried sample was grinded into powder and preserved in air tight falcons.

3.15. Biomass pre-treatment or hydrolysis optimization

Microalgal biomass was pre-treated with dilute acid hydrolysis in which two parameters were optimized simultaneously that includes biomass loading rate and acid concentration whereas time and temperature maintained as constant. Four different biomass loading rates were evaluated that includes 5g/L, 10g/L, 15g/L and 20g/L while 3 different acid concentrations were estimated including 1% acid, 2% acid and 3% acid.

12 clean glass tubes were taken with 10ml of distilled water in each tube. Biomass powder and 95% concentrated H_2SO_4 were added in these tubes as given in Table No. 3.4.

Table No. 3.4: Biomass hydrolysis optimization

Test tube No.	1% acid		Test tube No.	2% acid		Test tube No.	3% acid	
	Biomass loading rate (g/10ml)	95% conc. H_2SO_4 quantity (µl)		Biomass loading rate (g/10ml)	95% conc. H_2SO_4 quantity (µl)		Biomass loading rate (g/10ml)	95% conc. H_2SO_4 quantity (µl)
1.	0.05g	105 µl	5.	0.05g	210 µl	9.	0.05g	315 µl
2.	0.1g	105 µl	6.	0.1g	210 µl	10.	0.1g	315 µl
3.	0.15g	105 µl	7.	0.15g	210 µl	11.	0.15g	315 µl
4.	0.2g	105 µl	8.	0.2g	210 µl	12.	0.2g	315 µl

All these tubes were autoclaved at constant temperature and time of 121°C for 1 hour respectively. After cooling at room temperature solution in each tube was centrifuged at 10,000rpm for 10 minutes. Hydrolysates were separated in the form of supernatant and their pH was neutralized with NaOH. For the selection of optimum hydrolysis conditions, Phenol sulfuric acid and DNSA methods were further used to estimate total carbohydrates and reducing sugars released in hydrolysates.

3.16. Yeast based fermentation for bioethanol production

3.16.1. Yeast culture preparation

Commercially recognized fermenter yeast known as "Lalvin" was used for the fermentation reaction.

Table No. 3.5: YPD media composition

Composition	Quantity per Liter	Working volume per 100ml
Yeast extract	10g/L	1g
Bacteriological peptone	20g/L	2g
Dextrose	20g/L	2g
Distilled water	1 L	100ml

3.16.2. Broth inoculum preparation

100ml of YPD broth was prepared in 250ml conical flask and autoclaved at 121°C. Small inoculum from already grown lalvin culture plate was transferred into flask aseptically. The flask was incubated in shaking incubator at 30°C and 150 rpm for 24 hours. Optical density of broth culture was recorded at 620nm in UV-visible spectrophotometer with interval of 8 hours and culture allowed to grow until the growth OD was reached 2 units. Flask was then removed from incubator and stored at 4°C until its further use.

3.16.3. Biomass hydrolysis

2g dried and grinded algal biomass was added with 100ml of distilled water and 1% sulfuric acid in 250ml flask. The solution was stirred by magnetic stirrer for 30 minutes, followed up by autoclaving at 121°C for 1 hour and then centrifuged at 10000rpm for 10 minutes. Hydrolysate was separated in the form of supernatant and its pH was neutralized with NaOH and adjusted to 6.5-7.

Table No. 3.6: LB medium preparation

Composition	Quantity per Liter	Working volume per 100ml
Tryptone	10g	1g
Yeast extract	5g	0.5
NaCl	5g	0.5g
Glucose	20g	2g
Distilled water	1L	100ml

3.16.4. Fermentation setup

Fermentation setup was comprised of 3 reagent bottles containing 100ml of LB medium. pH was adjusted to 7 with 1M NaOH and autoclaved at 121°C for 45 minutes. Bottles was filled in following manner given in table no. 3.7.

Table No. 3.7: Fermentation setup

Serial No.	Bottles	LB medium	Sugar source	Yeast inoculum (v/v)
1st	Sample	Present	Microalgal hydrolysate	10% inoculum
2nd	Positive control	Present	Glucose	10% inoculum
3rd	Negative control	Present	Not any	10% inoculum

After cooling at room temperature 10% yeast inoculum was added into each bottle. 1st bottle was containing LB medium in which microalgal hydrolysate was used in replacement of glucose as sugar source. 2nd bottle was taken as positive control having both the yeast culture and glucose as sugar source while, 3rd bottle was holding the LB medium lacking glucose or any other sugar source thus considered as negative control.

3.16.5. Experiment

Bottles were sealed with airtight rubber corks equipped with two pipes passing through each cork. Inside of the bottle, one pipe was dipped into the medium which was used for sample collection while the other pipe was above the surface of medium and connected with 50ml graduated syringe from outside for gas collection. Corks were properly fixed and sealed by using black magic and nitrogen flushing was done to remove oxygen from bottles to ensure the anaerobic condition. Open ends of pipes from outside were locked by using stoppers and bottles were incubated for 72 hours at 30°C with continues agitation at 120rpm.With regular interval of 8 hours, gas production was noted and sample was collected from each bottle. Optical density of samples was recorded at 620nm with UV- visible spectrophotometer and preserved at 4°C.

3.16.6. Quantitative estimation of bioethanol by potassium dichromate method

Quantitative estimation of ethanol using biochemical method was done by potassium dichromate method. Method was based on the complete oxidation of ethanol by dichromate in the presence of sulphuric acid into acetic acid.

Acid dichromate solution: 200 ml of water was added to a 500ml conical flask and 40 grams of potassium dichromate was dissolved in it. Then 275ml of concentrated sulphuric acid was carefully added. The flask was cooled under cold water tap and diluted upto volume of 500 ml with distilled water.

2M Sodium hydroxide solution: 80g of NaOH was dissolved in 1000 ml of distilled water.

Procedure: 0.1-1ml of absolute alcohol was taken in different test tubes and 0.1ml of solution was taken from fermentation sample collected on each day from both positive control and microalgal hydrolysate and volume of each test tube was adjusted to 1ml with distilled water. 1 ml of potassium dichromate reagent and 2 ml of 2M sodium hydroxide solution was added into each tube. Tubes were then incubated at 50°C for 30 minutes and after cooling at room temperature optical density was measured at 600nm by using UV-visible spectrophotometer.

3.17. Biodiesel production

3.17.1. Alkaline Transesterification

Small 25 ml beaker was added with microalgal extracted oil/lipids and pre-heated at 65. Methanol in the ratio of 1:6 with respect to oil along with 1% KOH was added and beaker was covered with foil to avoid methanol evaporation. Reaction was carried out at 65°Cand 600rpm for 2 hours on magnetic stirring heating plate. After reaction completion contents was shifted into test tube and kept static for 24 hours for layers separation. The upper yellow biodiesel layer was collected in glass viol by using micropipette and placed in desiccator at 45°C for methanol evaporation.

3.17.2. Biodiesel or FAME analysis

Fatty acid methyl esters formed after transesterification were analysed using Fourier Transform Infrared Spectrometer (FTIR) of Bruker, model tensor 27 with software version opus 65 equipped with ZnSe ART. 5µl sample of both microalgae oil and biodiesel was loaded at sample injector and 16 scans were performed in range of 400 to 4000 cm^{-1} and average was represented in the form of spectrum with different peaks ranges.

RESULTS

4.1. Microalgae sample collection and physiochemical characterization

Microalgae sampling was carried out from Shahdara stream, located near Quaid-i-Azam University Islamabad. At time of sample collection temperature of location was noted as 29°Cand it was a bright sunny day. Water samples were brought to laboratory and their physiochemical analysis were performed there.

Table No. 4.1: Fresh water samples physicochemical characterization

Sr. No.	Physiochemical characteristics	Quantity
1	TDS	47 mg/L
2	TS	128 mg/L
3	COD	201 mg/L
4	Nitrate	4.9 mg/L
5	pH	8.1
6	Salinity	79 µcm
7	Total Nitrogen	53 mg/L
8	Phosphate	45 mg/L
9	Sulfates	24 mg/L

4.1. Culture enrichment and color appearance

Microalgae samples were inoculated in BBM medium for culture enrichment and incubated at room temperature for 15 days under continuous illumination and aeration. Green color appearance in flasks indicated the microalgal growth as given in figure 4.1.

Figure 4.1: Microalgae culture enrichment

4.2. Growth on agar medium and pure cultures screening

1 ml from each enriched microalgal culture was poured onto BBM agar medium plates and plates were putted under illumination at room temperature for growth development. Figure 4.2(A) and indicates the spread microalgal growth on agar plates. Each unique colony differentiated on its appearance and morphology was streaked and further re-streaked onto BBM agar plates and pure cultures were isolated as shown in figure 4.2(B).

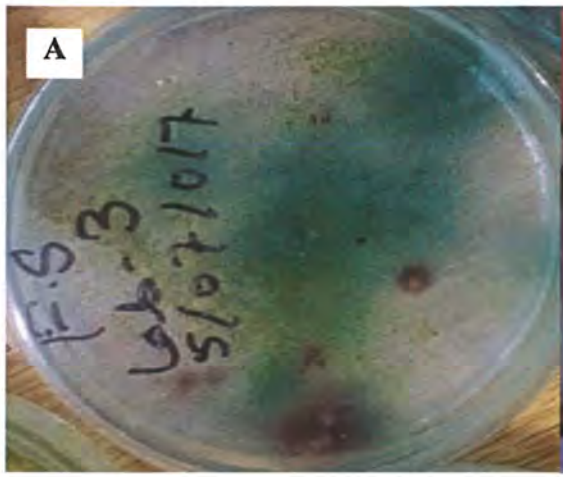

Figure 4.2 (A): Spread growth on agar

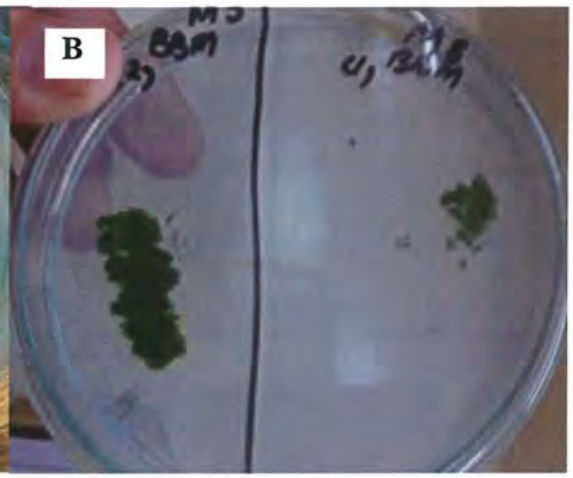

Figure 4.2(B): Pure culture growth

4.3. Pure cultures development in broth

Five different strains were isolated and shifted in BBM broth medium for cultures cultivation. Figure 4.3 A, B and C shows the pure cultures growth in flasks.

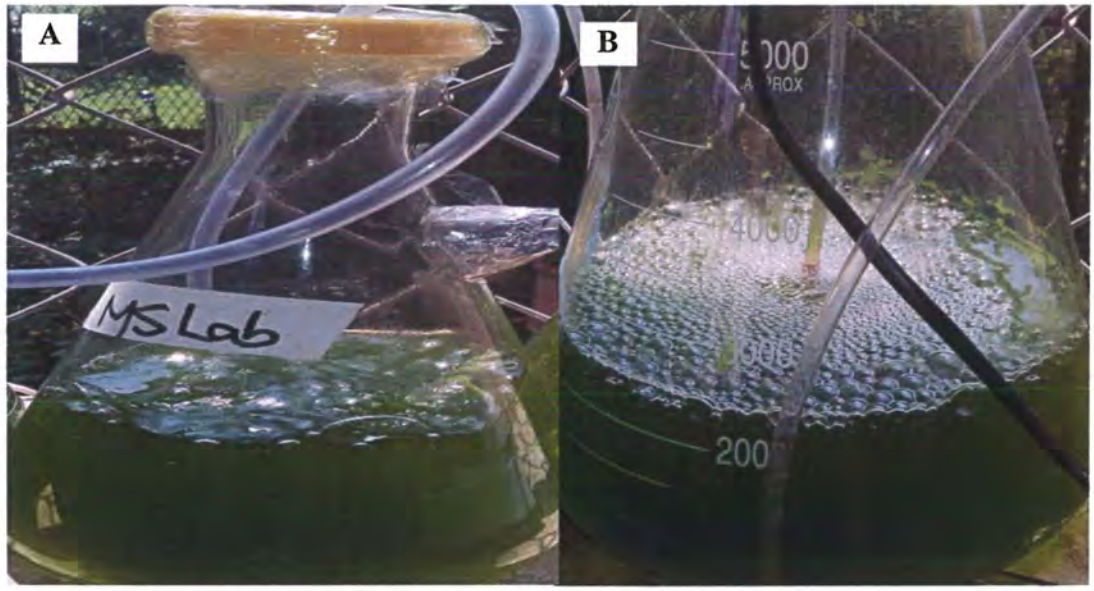

Figure 4.3 (A and B): Cultivation of pure cultures

4.4. Pure cultures growth comparison

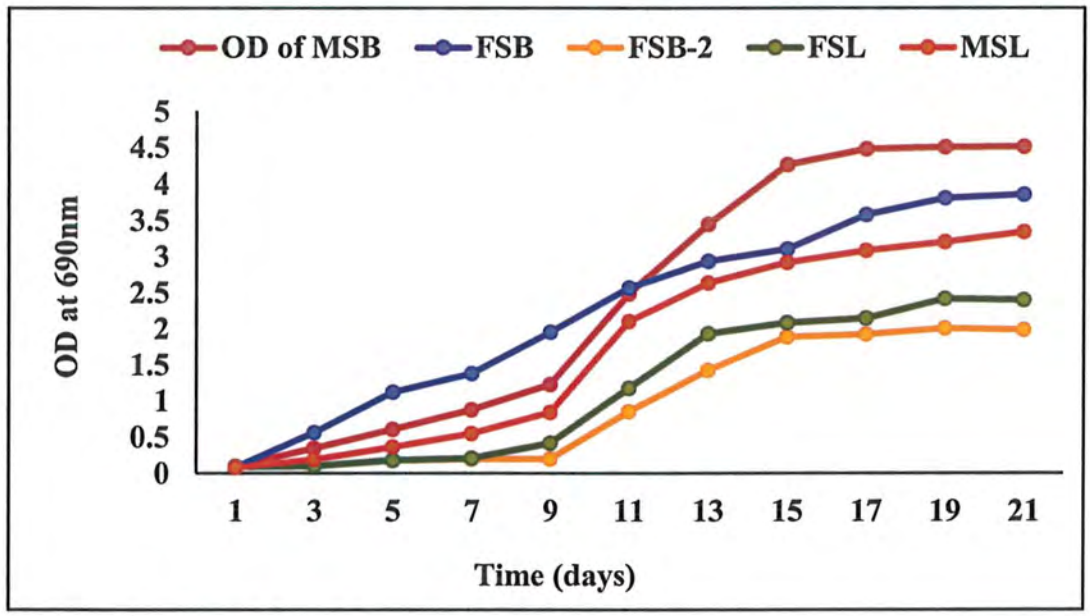

Figure 4.4: Pure cultures growth on BBM medium

4.6. Growth rate and biomass productivity

Table 4.2: Pure culture specific growth rate and biomass productivity

Microalgal strains	Specific growth rate (μ=lnX-lnX◦/No. of days)	Biomass productivity $P_{dwt}(gL^{-1}day^{-1})$
FSB	0.115	0.196
MSB	0.175	0.187
FSL	0.115	0.138
MSL	0.16	0.121
FSB-2	0.083	0.128

4.7. DNA extraction and gel electrophoresis

CTAB method was followed for DNA extraction from samples. To confirm DNA presence after extraction 1% agarose gel electrophoresis technique was performed. Samples was run in gel for 30 minutes and DNA bands was observed under UV illuminator. Figure 4.5 shows the DNA bands of isolated strains.

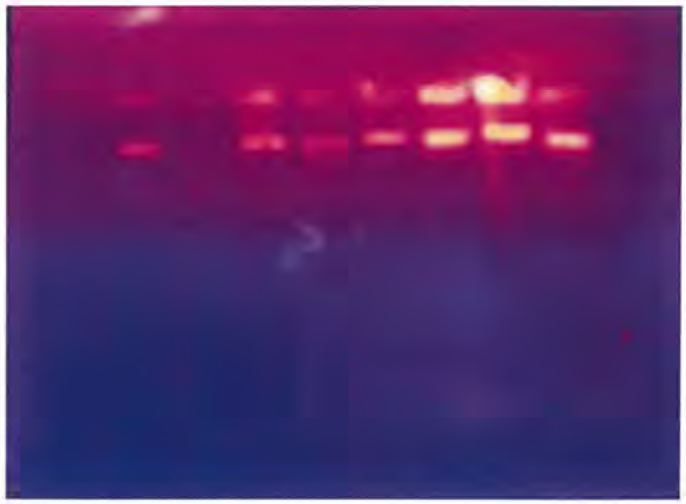

Figure 4.5: Gel electrophoresis

4.8. Carbohydrates content determination

Carbohydrates content of all the 5 isolated microalgal strains was determined for the selection of single strain with higher carbohydrates content. Acid extracted samples were treated with Phenol–Sulfuric acid method and standard curve was drawn. OD values of samples were integrated in trendline equation and total carbohydrates content of samples was determined. Figure 4.6 describes the standard curve of phenol sulfuric acid method.

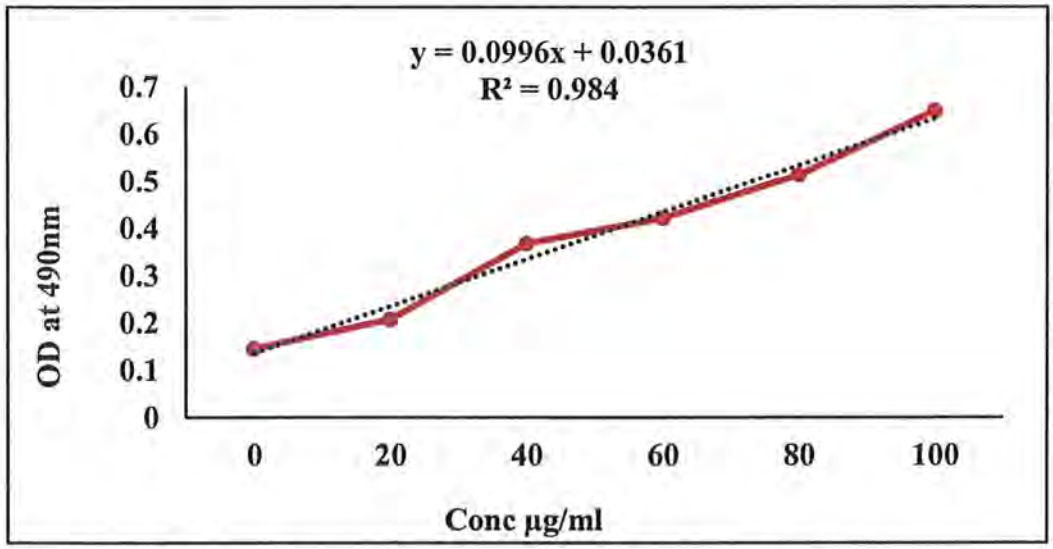

Figure 4.6: Standard curve of phenol-sulfuric acid method

Estimated total carbohydrates content of strains grown in the presence of nitrogen source and after giving nitrogen starvation for 11 days is demonstrated in table No. 4.3.

Table No. 4.3: Total carbohydrates content of isolated strains

Microalgae samples	Pre-starvation carbohydrates Content (mg/100mg)	Pre-starvation carbohydrates content (%)	Post-starvation carbohydrates Content (mg/100mg)	Post-starvation carbohydrates Content (%)
FSB	2.13	2.13	1.93	1.93
MSB	2.23	2.23	2.45	2.45
FSL	2.1	2.1	2.1	2.1

MSL	2	2	1.94	1.94
FSSB-2	1.76	1.76	2.1	2.14

4.9. Reducing sugars estimation

All strains when grown under the supply of nitrogen source and after giving nitrogen source starvation conditions for 11 days, were estimated for their reducing sugars content by dinitrosalicylic acid (DNSA) method. Sugars content was determined by integrating the OD values of the samples in trendline equation of figure 4.7 represents DNSA standard curve while reducing sugars content is given as table No. 4.4.

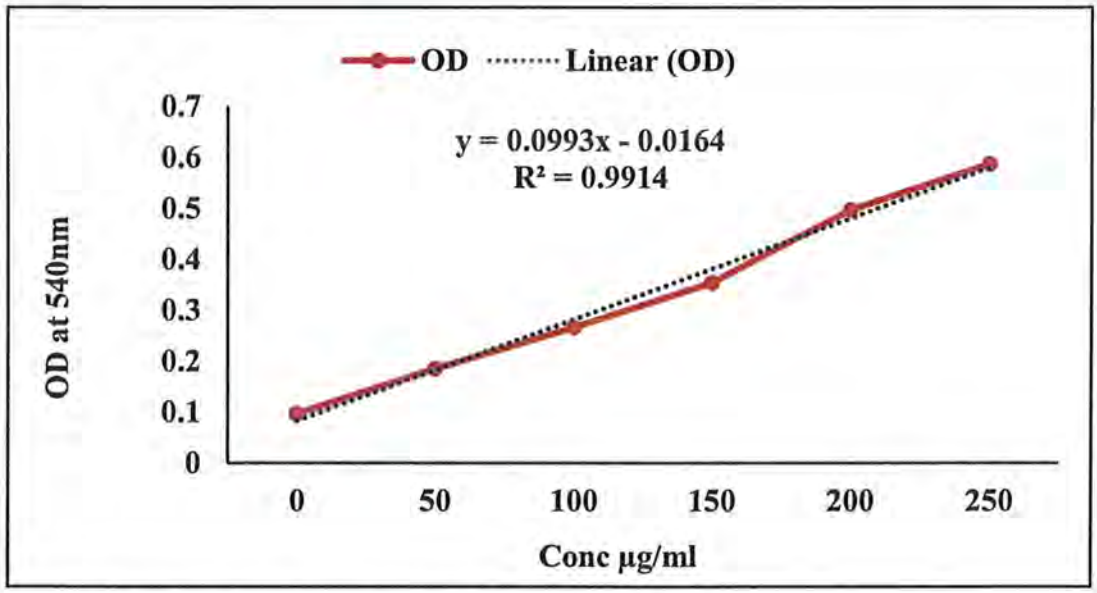

Figure 4.7: Standard curve of DNSA method

Table No. 4.4: Estimated reducing sugars content of isolated strains

Microalgae samples	Pre-starvation reducing Sugars content (mg/100mg)	Pre-starvation reducing sugars content (%)	Post-starvation reducing sugars content (mg/100mg)	Post-starvation reducing sugars content (%)
FSB	3	3		4
MSB	3.14	3.14	4.23	4.23

FSL	3	3	4	4
MSL	2	2	3.5	3.5
FSSB-2	2.45	2.45	3.8	3.87

4.10. Strain Selection

Based on better growth rate, higher biomass productivity and carbohydrates accumulation with respect to other strains, MSB strain was selected for evaluation of its subsequent bioethanol production potential.

4.10.1. Morphological characterization:

Microscopic observation of MSB strain at 40X magnification indicated the greenish colored MSB strain as crescent-shaped cells with colony size of up to 40 µm as shown in figure 4.8 A and B.

Figure 4.8 (A and B): Microscopy

4.10.2. Optimization of MSB strain

4.10.2.1. Nitrogen source optimization

The growth of microalgae strain MSB was determined on four different nitrogen sources like NH_4Cl, Urea, $NaNO_3$, KNO_3 in quantity of 0.25g/L. Best growth and biomass production was observed when used Urea as nitrogen source as shown in figure 4.9 (A) and (B).

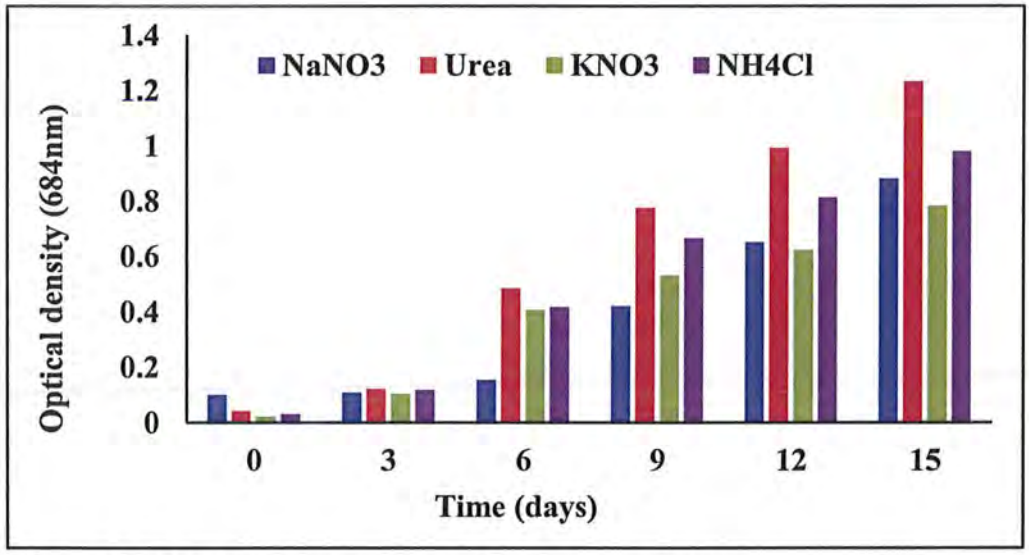

Figure 4.9 (A): Nitrogen sources effect on growth rate

Based on different Nitrogen sources dry biomass of MSB strain after 15 days

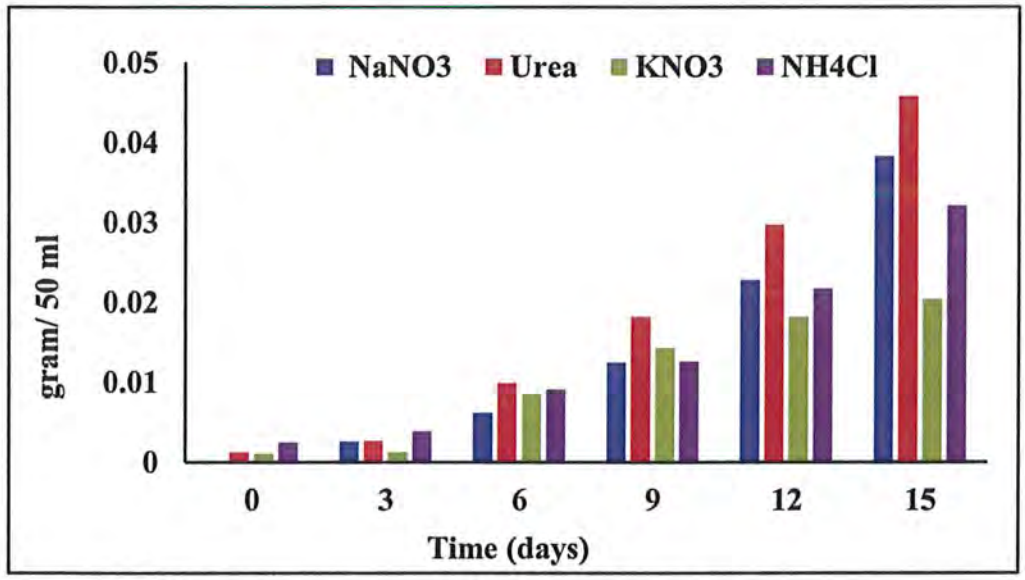

Figure 4.9 (B): Nitrogen sources effect on biomass production

4.10.2.2. pH optimization

MSB strain cultured for 15 days in Urea-modified BBM medium adjusted with five different pH ranges of 5, 6, 7, 8, 9. Higher growth rate and biomass production was recorded at pH 7 as shown in figure 4.10 (A & B).

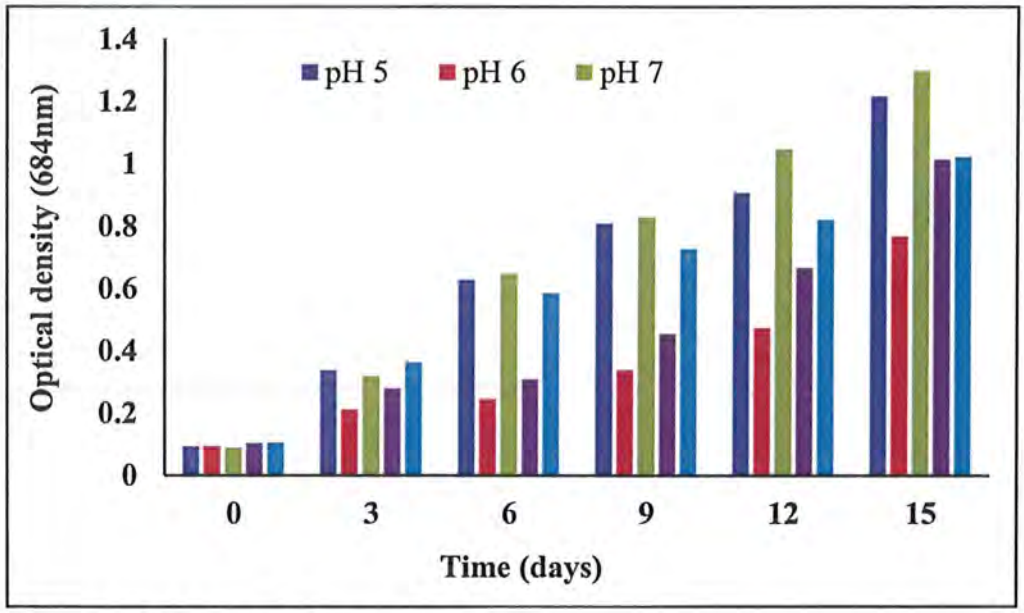

Figure 4.10 (A): pH effect on growth rate

Based on different pH dry biomass of MSB strain after 15 days

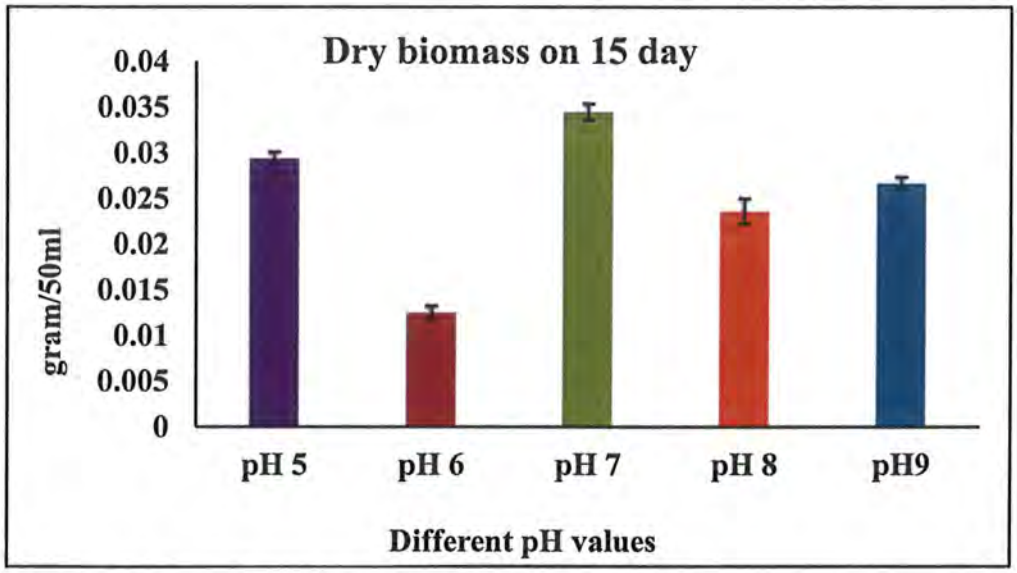

Figure 4.10 (B): pH effect on biomass production

4.10.2.3.Effect of aeration/mixing on microalgal growth

13 days experiment containing optimized conditions with Urea-modified BBM culture medium, adjusted pH 7 and incubated with 12:12 dark light cycle under sunlight showed higher growth rate in proper air bubbles mixing condition supplied through pump aerator as compared to condition without aeration as shown in figure 4.11.

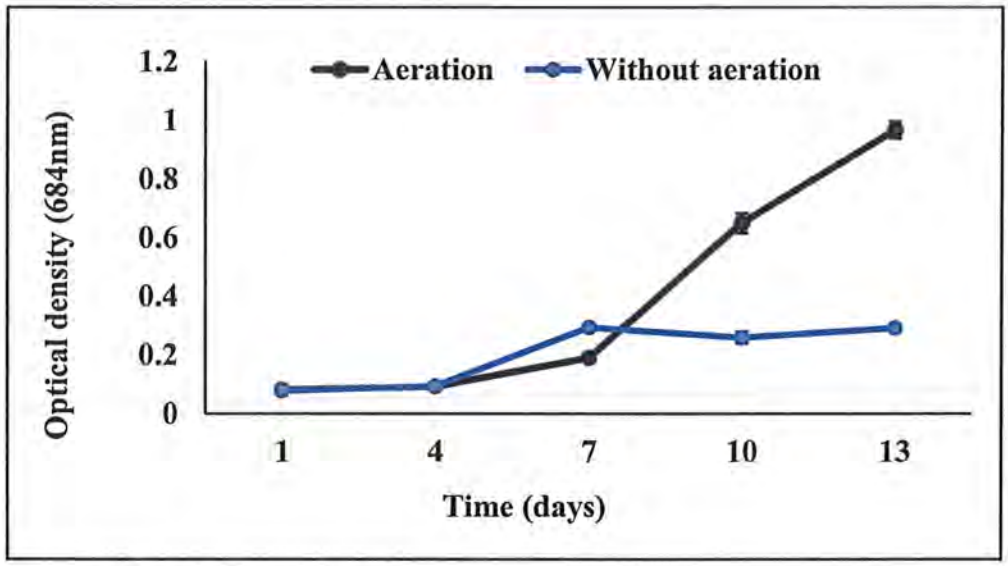

Figure 4.11: Aeration effect on growth

4.10.2.4. Effect of Dark-Light Interval on microalgal growth

Experiment carried out for 13 days containing optimized conditions of medium and pH showed higher growth rate when incubated under 24 hours continuous illumination provided by fluorescent lamps as compared to culture kept under sun light with 12:12 hours dark and light intervals as indicated in figure 4.12.

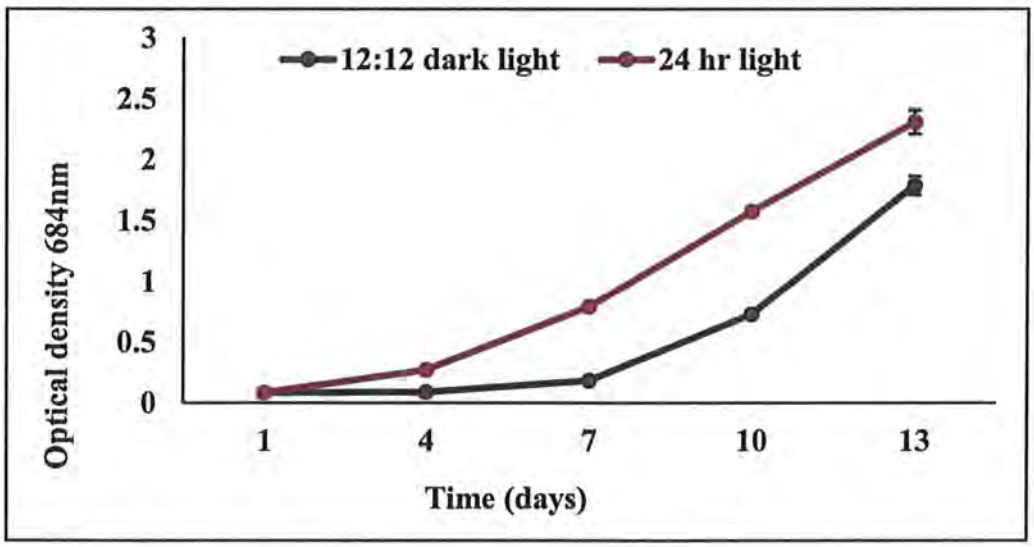

Figure 4.12: Effect of light interval on growth

4.10.3. MSB strain growth under optimized conditions

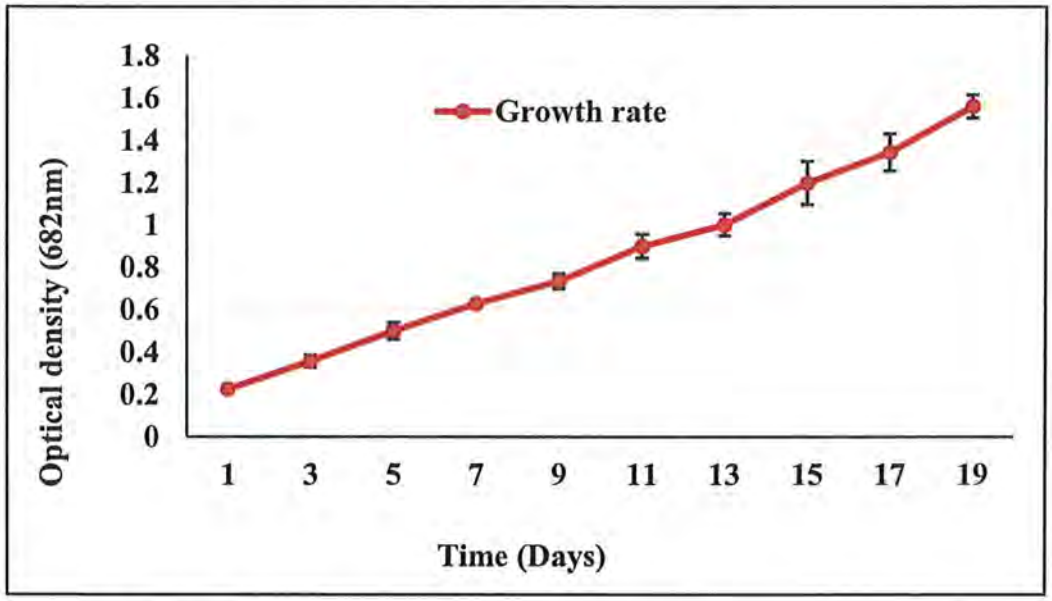

Figure 4.13: MSB growth under optimized conditions

4.10.4. MSB strain growth under nitrogen starvation conditions

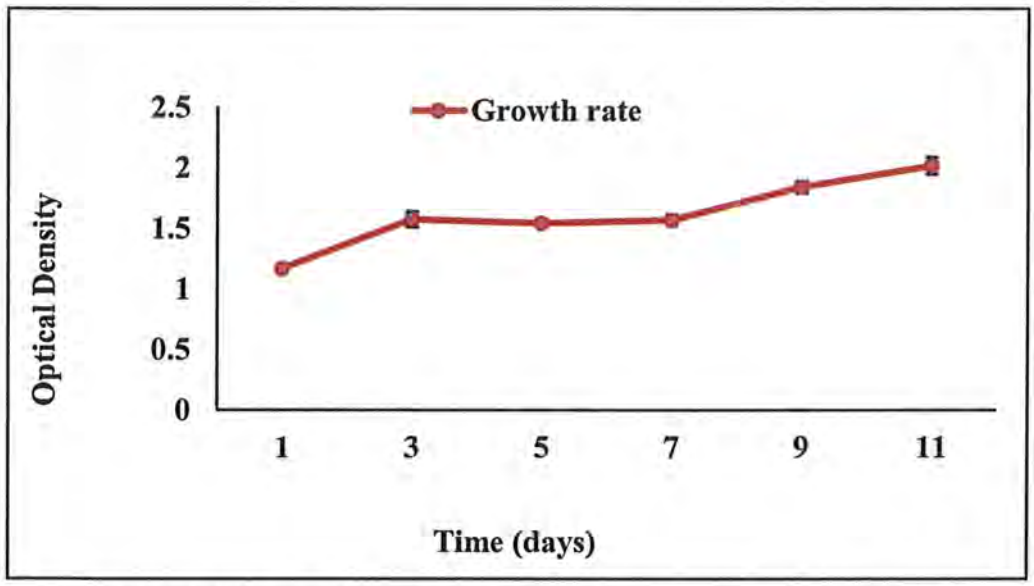

Figure 4.14: MSB growth under nitrogen starvation conditions

4.10.5. Seasonal variation effect on biomass production

Growth and biomass production was significantly affected by temperature variation. Maximum biomass production of 1.75g/L was observed in the month of August at

elevated temperature while lowest production of 0.8g/L was recorded in December shown by figure 4.15.

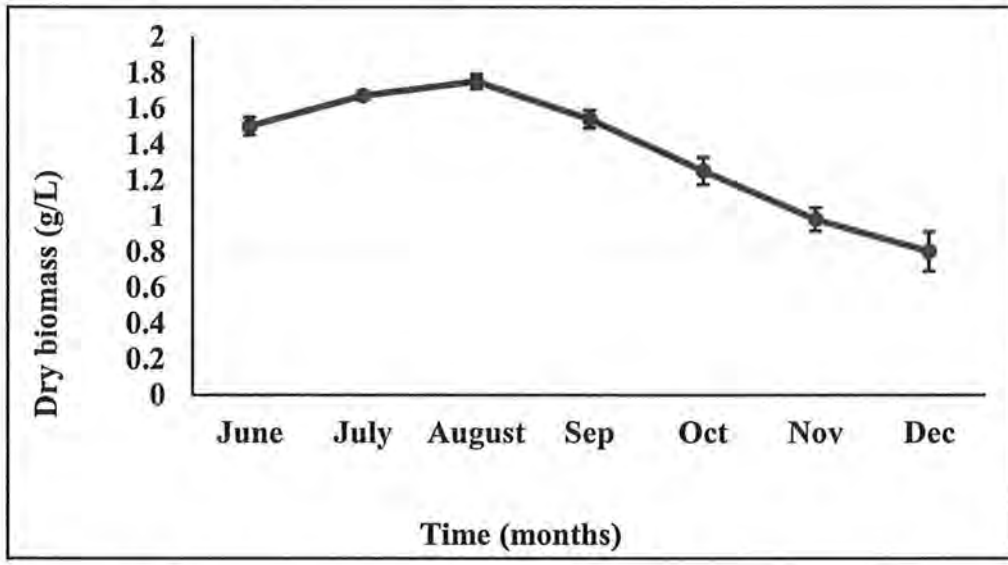

Figure 4.15: Seasonal variation/temperature effect of on biomass

4.10.6. Carbohydrates, reducing sugars and lipids determination before nitrogen starvation

MSB strain cultured in optimized conditions and harvested at its exponential phase when grown in the presence of sufficient nitrogen source. Sample was then treated with respective methods for evaluating its total carbohydrates, reducing sugars and lipids content accumulation accordingly as demonstrated in table No. 4.5.

Table No. 4.5: Pre-starvation estimations

Total carbohydrates (mg/100mg)	Soluble carbohydrates (mg/100mg)	Insoluble Carbohydrates (mg/100mg)	Reducing sugars content (mg/100mg)	Lipids content (%)
7.67	1.6	6.07	7.455	19.6

4.10.7. Carbohydrates, reducing sugars and lipids determination after nitrogen starvation

Culture of MSB strain which was grown in optimized conditions was harvested at its exponential phase and transferred for culturing into nitrogen deficient medium for 11 days. After harvesting and drying sample was treated with respective methods to determining the change in its carbohydrates, reducing sugars and lipids content accumulation as demonstrated in table No. 4.6.

Table No. 4.6: Post-starvation estimations

Total carbohydrates (mg/100mg)	Soluble carbohydrates (mg/100mg)	Insoluble Carbohydrates (mg/100mg)	Reducing sugars content (mg/100mg)	Lipids content (%)
9.78	2.30	7.4	8.95	22.1

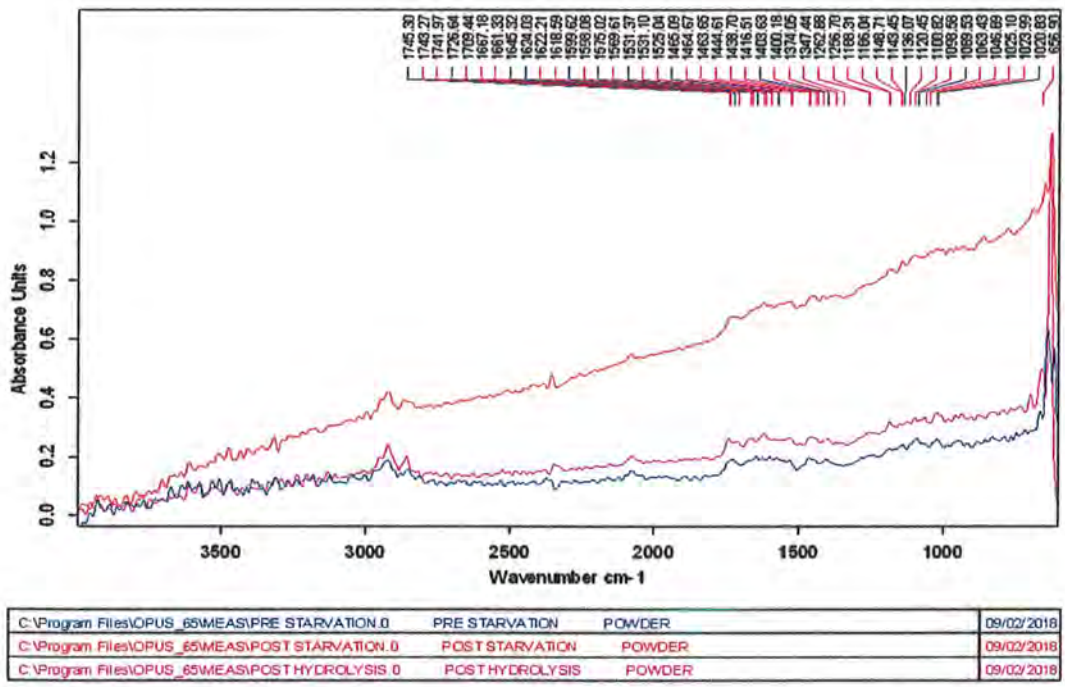

Figure 4.16: FTIR spectra indicating change in microalgal biomass at pre-starvation post-starvation and carbohydrates extracted state

4.10.8. Acid hydrolysis optimization

Two parameters were optimized simultaneously in dilute acid hydrolysis of microalgal biomass that includes biomass loading rate and acid concentration whereas time and temperature were kept constant. Four biomass loading rates (5g/L, 10g/L, 15g/L and 20g/L) were used along with 3 different acid concentrations (1%, 2% and 3% acid). For the selection of optimum hydrolysis conditions, Phenol Sulfuric acid and DNSA methods were further used to estimate total carbohydrates and reducing sugars released in hydrolysates. Maximum biomass hydrolysis was achieved when 5g/L biomass treated with 1% concentrated acid as shown in table No. 4.7.

Table No. 4.7: Hydrolysis optimization

1% Acid Hydrolysis			
Biomass loading rate (g/L)	**Biomass loaded (mg/10ml)**	**Carbohydrates in hydrolysate (mg/10ml)**	**Reducing sugars in hydrolysate (mg/10ml)**
5	50	0.65	1.47
10	100	1	2.3
15	150	1.3	3.51
20	200	1.67	3.9
2% Acid Hydrolysis			
Biomass loading rate (g/L)	**Biomass loaded (mg/10ml)**	**Carbohydrates in hydrolysate (mg/10ml)**	**Reducing sugars in hydrolysate (mg/10ml)**
5	50	0.4	1.23
10	100	0.86	2.65
15	150	1.034	3.45
20	20	1.14	3.56
3%Acid Hydrolysis			
Biomass loading	**Biomass**	**Carbohydrates in**	**Reducing**

rate (g/L)	loaded (mg/10ml)	hydrolysate (mg/10ml)	sugars in hydrolysate (mg/10ml)
5	50	0.4	1.6
10	10	0.74	1.96
15	150	1.2	3.55
20	20	1.5	3.86

4.10.9. Bioethanol production through fermentation

Bioethanol production setup was comprised of 72 hours fermentation experiment consisting of 3 anaerobic reactors with 100ml of LB medium and 10% yeast culture (growth OD 2) in each. Positive control provided with glucose as sugar source instead of microalgal biomass hydrolysate while negative control was without any sugar source. First sample was collected at 15 hours of experiment while next were taken regularly with 8 hours interval and gas production was also noted.

Figure 4.17: Fermentation reactors

4.10.9.3.Evaluating yeast culture growth rate during fermentation

Optical density different intervals were recorded at 620nm for growth rate evaluation of yeast culture during fermentation. Maximum growth rate of yeast culture (in both

positive control and microalgal hydrolysate) was observed at 47 hours as shown in figure 4.18.

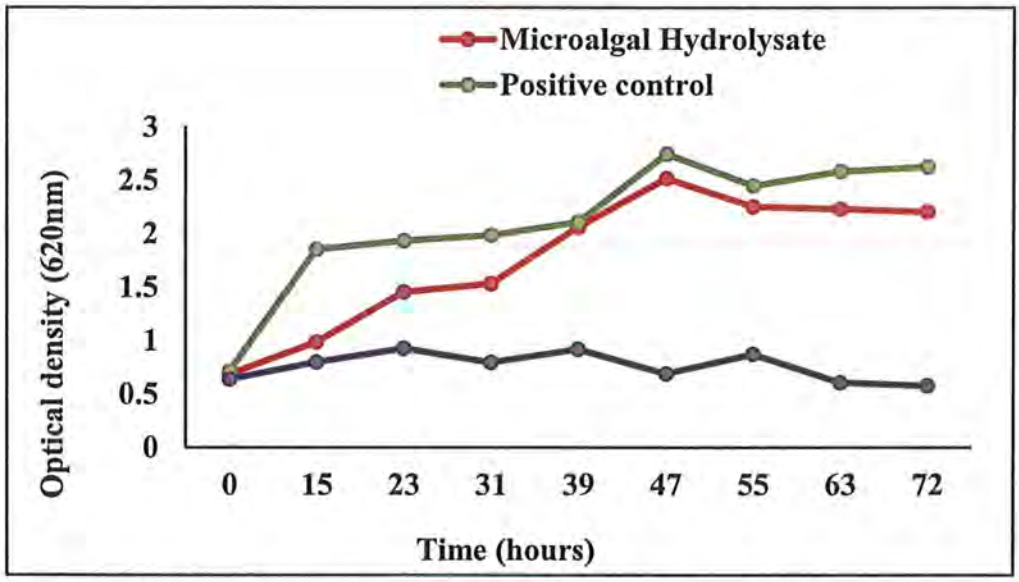

Figure 4.18: Yeast culture growth rate during fermentation

4.10.9.4.Gas collected during fermentation

First gas collection was performed at 15 hours of experiment while next were taken regularly with 8 hours interval. In positive control maximum gas collection was achieved within 15 hours. While in microalgal hydrolysate maximum gas was collected during 39 to 63 hours fermentation as shown in table No. 4.8.

Table No. 4.8: During fermentation gas collected with intervals

Time (hour)	Biomass hydrolysate gas production (ml)	Positive control gas production (ml)	Negative control gas production (ml)
15	3	68	0
23	0.8	4	0
31	0	0	0
39	4	0	0
47	13	0	0
55	2	0	0
63	10	0	0
72	0	0	0

4.10.9.5. Quantitative estimation of bioethanol by potassium dichromate method

Biochemical technique used for the quantitative estimation of bioethanol was potassium dichromate method. The process was based the complete oxidation of ethanol into acetic acid by dichromate in the presence of sulphuric acid. Standard curve of potassium dichromate is shown as figure 4.20, while bioethanol content was estimated by integrating the OD values of samples in trendline equation of the standard curve and given as table No. 4.9.

Figure 4.19: Potassium dichromate test

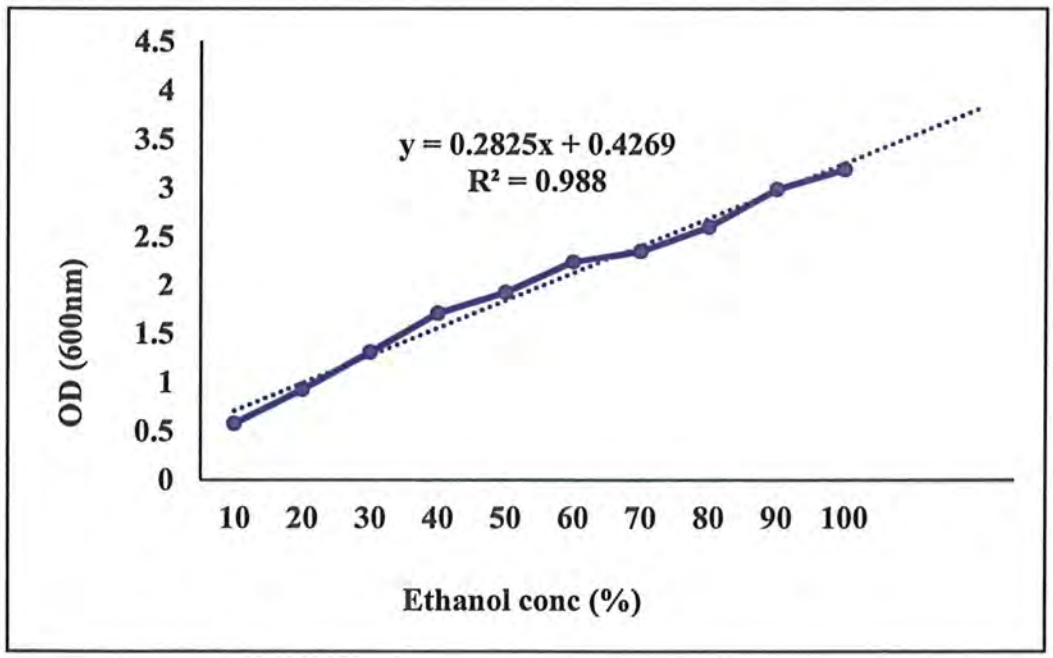

Figure 4.20: Ethanol estimation standard curve

Table No. 4.9: Bioethanol produced during fermentation

Days	Bioethanol from microalgal hydrolysate (%)	Bioethanol from positive control (%)
Day 1	4.8	21.7
Day 2	18.36	22
Day 3	18.75	22

4.10.10. FT-IR Analysis of microalgae oil and its biodiesel

FTIR analysis was performed for biodiesel production confirmation by identification of functional groups and their various stretching and bending existing in oil and biodiesel. Microalgal oil FTIR spectrum was compared with standard FTIR frequency range proposed by Hsieh *et al.*, (2015) as shown in table No. 4.10. FTIR spectra of microalgal oil is shown in figure 4.21, while FTIR spectrum of its biodiesel is given as figure 4.23 and their blend as figure 4.25.

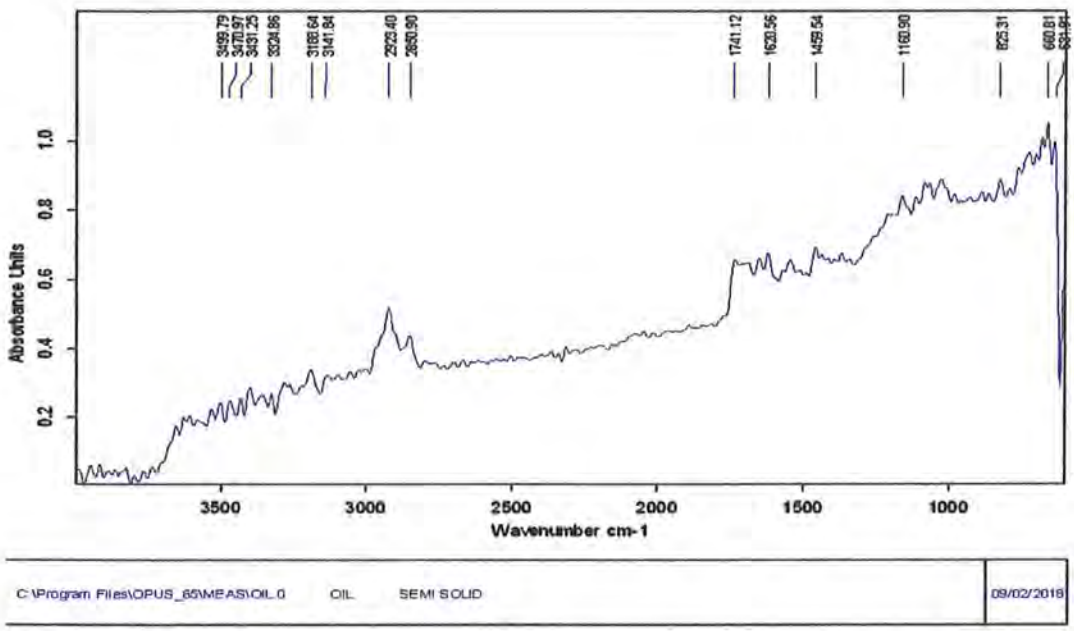

Figure 4.21: FTIR spectra of microalgal oil

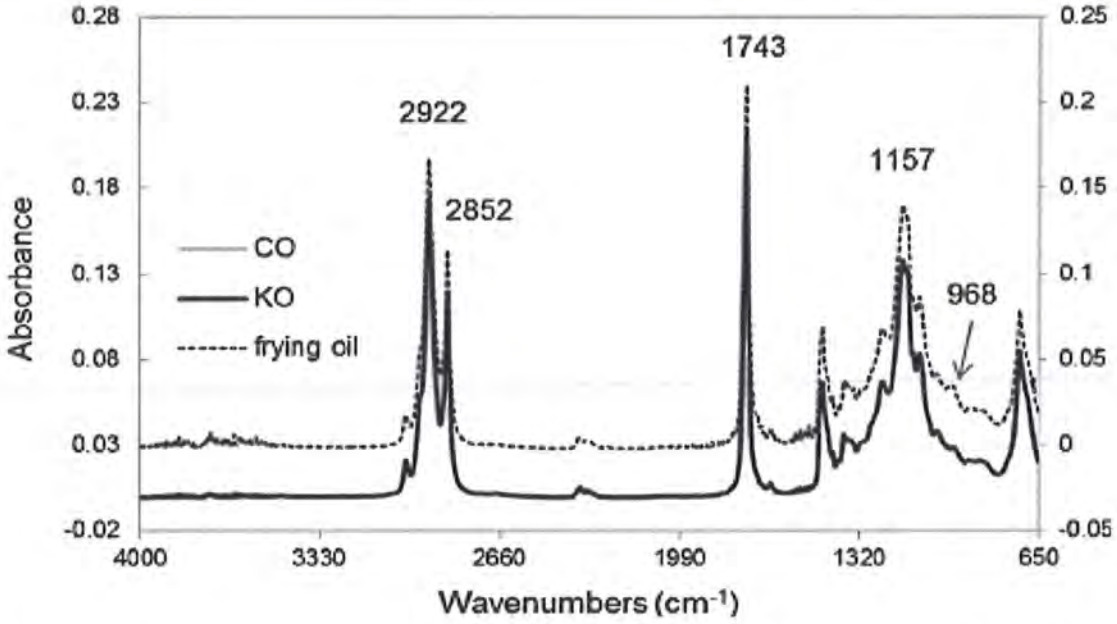

Figure 4.22: FTIR spectrum of different bio-oils (CO: Canola oil, KO: rapeseed Kizakinonatane oil and used Canola frying oil) for microalgal oil comparison (Zhang *et al., 2015*)

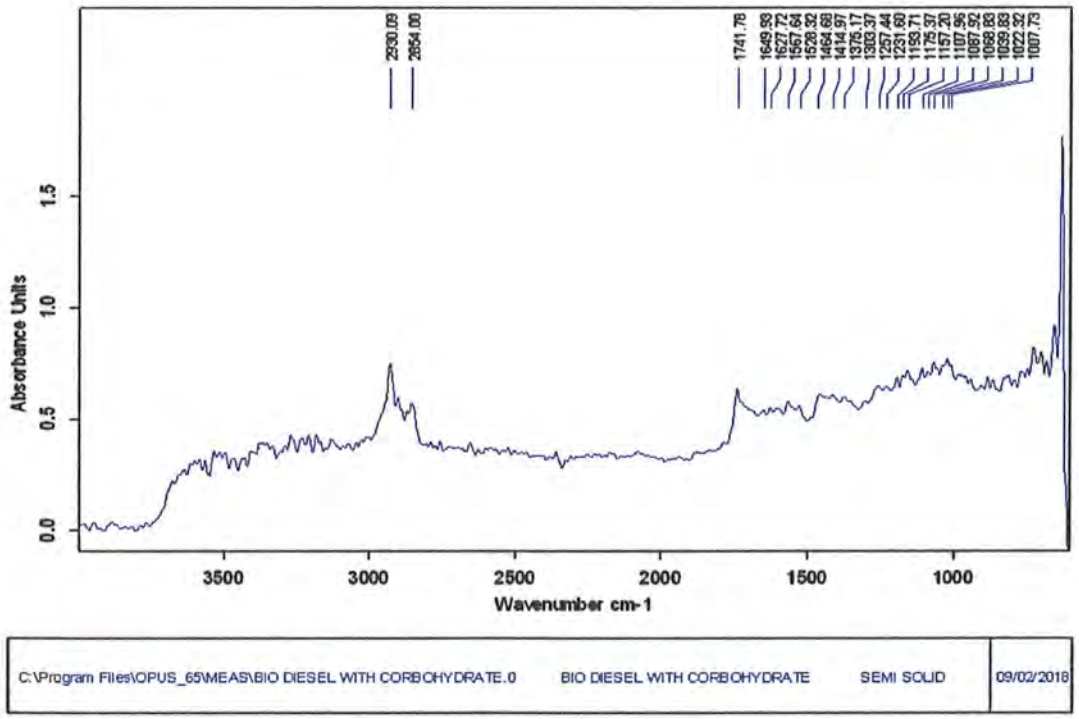

Figure 4.23: FTIR spectra of microalgal biodiesel

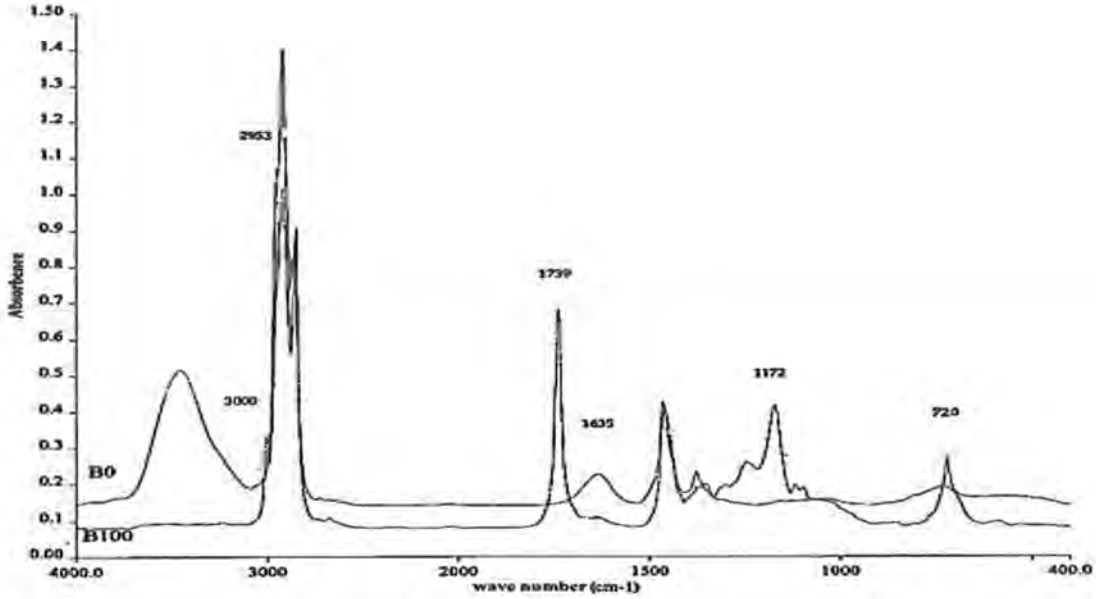

Figure 4.24: FTIR spectra of petro and microalgae *Spirulina platensis* bio-diesel (B0 and B100, respectively), for microalgal biodiesel comparison (Mostafa and El-Gendy., 2017)

Table No. 4.10: Description of FTIR bends and stretches of microalgal oil and biodiesel

Functional groups	Class of compound	Band assignments (cm^{-1}) (Microalgal oil)	Band assignments (cm^{-1}) (Microalgal biodiesel)	Frequency range (cm^{-1})
O-H stretching	Phenols, polymeric O-H, water, impurities	3324.86		3200-3600
C-H stretching	Alkanes	2850.90, 2923.40, 3141.84, 3188.64	2854.00, 2930.09	2800-3200

C=O stretching	Ketones, aldehydes, esters	1741.12	1649.93, 1741.78	1650-1750
C=C stretching	Alkenes, aromatic compounds	1620.56	1627.72	1580-1650
C-H deformation	Alkanes	1459.54	1375.17, 1414.97, 1464.68	1350-1470
C-O stretching,	Primary secondary and tertiary alcohols, phenols	1160.90	1157, 1175, 1231.60.,1257.44	950-1300
O-H bending	Mono, polycyclic, substituted aromatic rings	825.31		675-900

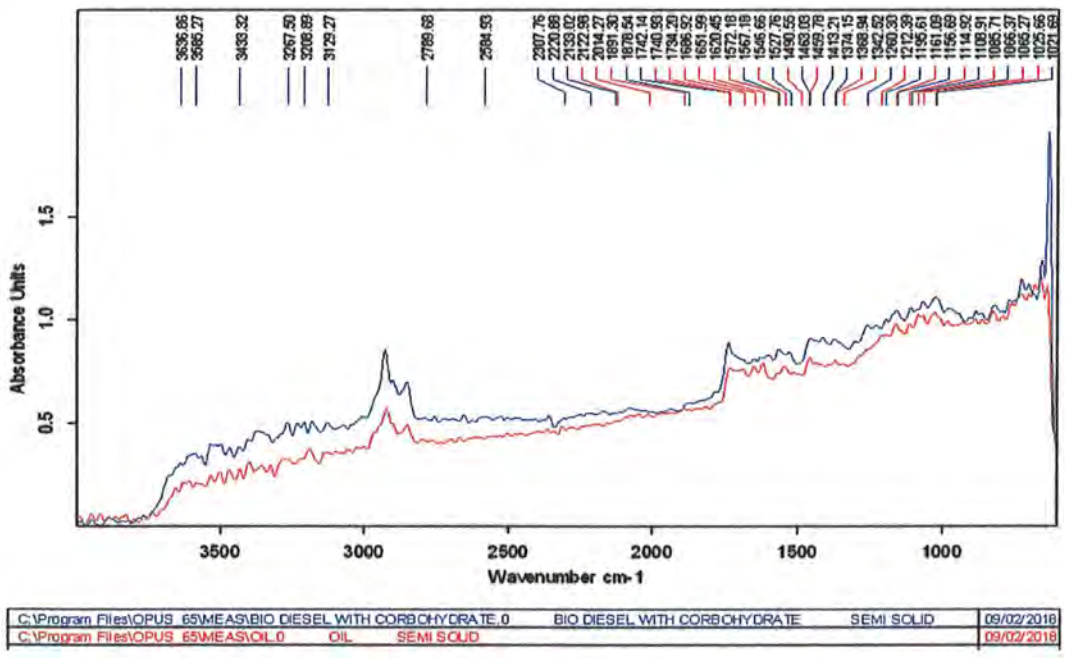

Figure 4.25: Integrated FTIR spectra of Microalgal oil and Biodiesel

DISCUSSION

The current study was primarily focused on isolation, molecular characterization and optimization of indigenous microalgae strain and evaluation as potential source for biofuels production. The strain was analyzed for best growth at different Nitrogen sources, pH, dark-light intervals aeration effects and nutritional starvation. Afterward the optimized strain was cultivated at work station green house for large scale production firstly on nutrition sufficient and then on starvation conditions. Harvested culture was evaluated for biofuels production specifically focused on bioethanol and up to some extent for biodiesel production. The results detail and comparison with literature is discussed below.

Microalgal samples were firstly enriched in BBM broth medium and then through standard plating methods 5 microalgal pure strains were isolated on BBM agar medium after 7 to 10 days incubation in green house. In comparison Lee *et al.*, (2014) used BG11 agar as growth medium and isolated pure cultures by using standard spread and streak plate methods and incubating in green house for 14 days.

Specific growth rate, biomass productivity, total carbohydrates and reducing sugars content of all the 5 strains was evaluated for strain selection with higher biomass productivity and carbohydrates content. Growth rate of MSB strain was calculated as 0.175μ/day with 0.187gL^{-1} day^{-1} biomass productivity described in table No. 4.2. Result evsaluated by Nascimento *et al.*, (2013) reported specific growth rate of *Botryococcus braunii* and *Botryococcus terribilis* as 0.13μ/day while for *Chlamydocapsa bacillus* found as 0.75μ/day. Indigenous strain MSB also exhibited higher biomass productivity of 0.187gL^{-1} day^{-1} than earlier reported 0.13 to 0.25 gL^{-1} day^{-1} (Nascimento *et al.*, 2013; Liang *et al.*, 2009). Phenol-sulphuric acid method and DNSA method respectively exposed that MSB strain was having 2.23% total carbohydrates and 3.14% reducing sugars which was the highest accumulation among all the 5 isolated strains as shown in table No. 4.3 and 4.4. On the other hand, 1.76% accumulation in FSB-2 strain was indicated as the lowest accumulation of carbohydrates. With respect to literature, Brown and Jeffrey., (1992) determined the carbohydrates content of 10 green microalgae species collected from marine water in the range of 10.8–16.7% except *Chlorella* sp. CS-195 that accounts for only 5.9% of carbohydrates. Main reasons for these differences in compositional ratios can be species diversity, mechanisms of retraction, nutrients availability and environmental

conditions. Based on these biochemical evaluations MSB strain was selected for further its optimization and bioethanol production evaluation.

Macronutrients specifically nitrogen is one of the most important factors effecting microalgal growth. Strain MSB growth was determined on 0.25g/L quantity of four different nitrogen sources that includes KNO_3, $NaNO_3$, NH_4Cl and Urea. Highest growth rate biomass productivity was achieved when used Urea as nitrogen source as shown in figure 4.9 (A and B). Similar study was conducted by Ho *et al.*, (2013) as they used four common nitrogen sources like NH_4NO_3, urea, NH_4Cl and KNO_3 for the cultivation of *C. vulgaris* FSP-E and achieved highest specific growth rate 1.206 d^{-1} and biomass productivity of 210.48 mg $L^{-1}d^{-1}$. On the other hand, leaded to maximum glucose and total carbohydrates content accumulation of 46.6 ± 0.6% and 51.0 ± 0.7% respectively.

According to (Bajhaiya *et al.,* 2010), for microalgal growth pH is the most protuberant factor next to illumination and temperature. MSB strain growth was examined in BBM medium adjusted with range of pH 5, 6, 7, 8 and 9. Optical density of growth was observed at 684 nm with UV-visible spectrophotometer after regular time interval. Highest strain growth and biomass productivity was recorded in BBM medium adjusted with pH 7 as shown in figure 4.10 (A and B). Spolaore *et al.*, (2006) optimized the growth conditions for *Nannochloropsis oculata* by using RSM (the response surface method) approach. Along with other factors they checked the effect of pH range from 7 to 9.4 on specie growth and found 8.2 as the most optimum pH for *Nannochloropsis oculata* growth.

Proper mixing and illumination duration is necessary for better microalgal growth. 13 days experiments was conducted to check the effect of mixing through air bubble and dark:light intervals on MSB strain growth. Much higher growth rate was observed in condition with proper air bubble mixing through pump aerator as compared to culture without mixing as shown in figure 4.11. Mixing is proved as another crucial factor influencing microalgal growth as it helps in gaseous exchange and responsible for homogenization of cell culture, metabolites and temperature (Barbosa., 2003). Experiment regarding light interval observation showed higher growth rate when incubated under continuous illumination for 24 hours provided by fluorescent lamps as compared to culture kept under sun light in green house with 12:12 hours dark and

light intervals as described in figure 4.12. Jacob-Lopes *et al.*, (2009) investigated the effect of light cycles (from 0:24 to 24:0 night:day intervals) on biomass production and CO_2 fixation. He found a linear reduction in growth and carbon-fixation was reduced up to 99.69% when compared to cultures gained under continuous illumination.

Effect of seasonal variation (temperature) on microalgal biomass production was evaluated and highest biomass production was noted as 1.75g/L in month of August (having plenty of rain) with average temperature of 29°C. While in winter lowest biomass production of 0.8 g/L was recorded in December with average temperature of 15°C as defined in figure 4.15. Temperature could be considered as the most prominent factor influencing microalgal cell growth and its biochemical pathways. Though average optimum growth temperatures for microalgae ranges between 20 to 25°C but it is usually specie specific. Mesophilic species gives maximum growth between 20 and 25 °C while for psychrophilic strains it can be decreased to 17 °C and increased up to 40 °C for thermophilic strains (Ras *et al.*, 2013). De Oliveira *et al.*, (1999) evaluated the increase in growth and biomass production along with stimulated carbohydrates synthesis when cultivated *S. maxima* on temperature between 30°C and 35 °C.

To analyze the biofuels production potential MSB strain was cultivated with optimized conditions on large scale. In optimized conditions strain was observed with increase in total carbohydrates content that reached up to 8% along with sugar content of 7.5% while the lipids content of strain was estimated as 19.6% as shown in table 4.5. At exponential growth phase culture was shifted to nitrogen starved condition for 11 days resulting in increased of total carbohydrates content up to 10% while reducing sugar content also reached up to 9%. On the other hand, starvation condition also resulted as increase in lipid content to 22.1% mentioned in table No 4.6. Similarly, Ho *et al.*, (2013) investigated Chlorella vulgaris FSP-E under nitrogen starved conditions for 11 days and reported the increase in carbohydrates content from 14.76 to 51.32% on 4rth day of starvation and 93% of these carbohydrates were comprised of glucose. Nitrogen starvation reduces the protein content of the cell and leads toward the enhanced accumulation of high energy products like carbohydrates or lipids (Ho *et al.*, 2012; Siaut *et al.*, 2011). Some microalgal strains could store high

amount of carbohydrates under stressed conditions either in cell wall as cellulose (Sørensen *et al.*, 2012) or within plastids in the form of starch (Rismani-Yazdi *et al.*, 2011).

Dried biomass of the MSB culture was treated with dilute acid hydrolysis for carbohydrates extraction. Two parameters were optimized simultaneously that includes biomass loading rate and acid concentration whereas time and temperature were kept constant. Four biomass loading rates (5g/L, 10g/L, 15g/L and 20g/L) were used along with 3 different acid concentrations (1%, 2% and 3% acid). Maximum hydrolysis was achieved at 5g/L biomass loading rate with 1% acid concentration as shown in table No. 4.7. Ho *et al.*, (2013) conducted Similar study in which different loadings rates of *C. vulgaris* FSP-E dry biomass ranging from 10 to 80 g/L was hydrolyzed with 1 to 5% (v/v) of sulfuric acid concentration for 20 min at 121°C. They found 50 g L^{-1} biomass loading rate with 1% acid as the most suitable combination for hydrolysis which resulted in above 95% glucose yield. In my study possible reason for lower hydrolysis achievement with increased biomass loading could be the high biomass to acid ratio resulting in less exposure of biomass to hydrolyzing reagent.

Bioethanol production setup was comprised of 72 hours fermentation experiment consisting of 3 anaerobic reactors with 100ml of LB medium and 10% yeast culture (growth OD 2) in each. Positive control provided with glucose as sugar source instead of microalgal biomass hydrolysate while negative control was without any sugar source. Bioethanol production in both positive control and microalgal hydrolysate was strongly evidenced by the gas collection in both reactors while, there was no gas production noticed in negative control. There was also observed the increase in yeast culture growth in both the sample and positive control while in negative control yeast culture count was remained same throughout the reaction with slight decrease in growth rate rather than increase, this also proved the bioethanol production in sample reactor. In positive control maximum gas collection was achieved within 15 hours. While in microalgal hydrolysate maximum gas was collected during 39 to 63 hours fermentation as shown in table No. 4.8. This indicated the slower bioethanol production in sample as compared to positive control and maximum growth rate of yeast culture was observed at 47 hours in both positive control and microalgal

hydrolysate as indicated in figure 4.18. Potassium dichromate method was used for the quantitative estimation of bioethanol as biochemical technique and maximum 18.75% bioethanol produced from microalgal hydrolysate as shown in table No. 4.9.

MSB strain biomass was also evaluated for biodiesel production and solvent extraction method was used for lipids extraction. Lower layer of chloroform with dissolved lipids in it was taken and oil was separated. During the transesterification reaction alcohol reacts with long chain fatty acids and converts them into biodiesel (mono alkyl esters of long chain fatty acids) thus reaction is also known as alcoholysis (Ma and Hanna., 1999). In this study methanol was used as alcohol and alkali catalyzed transesterification was performed to convert microalgae oil into FAME with conditions (1:6 molar ratio of oil and methanol, 65°C temperature, 1% KOH catalyst at 600rpm for 2 hours). 95–96% biodiesel yield could be achieved by using methanol/oil in molar ratio of 6:1 with catalyst concentration of 1.0% potassium hydroxide at 65°C through proper stirring at 600 rpm (Rashid and Anwar., 2008).

FTIR analysis was performed for biodiesel production confirmation by identification of functional groups and their various stretching and bending existing in oil and biodiesel. There was slight alteration observed between microalgae oil and its biodiesel spectra. C=O of ester stretch was observed at 1741.12 in microalgae oil while this stretch was located at 1649.93 and 1741.78 in biodiesel and C-O in microalgal oil was observed at 1160.90 while these ester band stretches were observed at 1157, 1175, 1231.60.,1257.44 in biodiesel and there were no such bands indicated in oil in this region. The presence of these ester groups indicated the production of biodiesel from the algal oil as shown in figure 4.21, 4.23. C=O derived strong bands of esters are located within 1650-1750 cm^{-1} on spectra which confirms the existence of carbonyl groups of esters whereas, weaker bands of C-O can also appear in the range 900-1300 cm^{-1} (Stehfest *et al.*, 2005).

All these above discussed factors proved that by growing the MSB strain with optimized parameters and giving nitrogen stress effectively enhances its biomass productivity and carbohydrates accumulation thus can be used as potential source for biofuels (bioethanol and biodiesel) production.

CONCLUSIONS

Pakistan being a developing country undergoing through severe energy crisis and environmental concerns related to fossil fuels, therefore, biofuels production from microalgae could be the most possible solution for all these issues.

- Depending upon growth rate, biomass productivity and carbohydrates accumulation, MSB was shown as the finest one from all the 5 isolated strains and selected for further study.
- Urea (0.25g/L) as nitrogen source, pH 7, proper aeration supply and 24 hours illumination were proved to be the optimized conditions for maximum MSB strain growth and biomass productivity.
- Optimized growth conditions and nitrogen starvation proved to have better impact on carbohydrates and lipids accumulation in MSB strain.
- 20g/L biomass treated with 1% concentrated sulfuric acid at 121°C for 1 hour was proved as the optimum dilute acid hydrolysis conditions for carbohydrates extraction from microalgal biomass.
- In 72 hours fermentation experiment, maximum growth activity and gas collection was recorded during 39 to 63 hours of fermentation, and potassium dichromate method turning microalgal hydrolysate color from brown to bottle green similar to positive control clearly indicated bioethanol production.
- Biodiesel production potential was also evaluated for making the process economically more feasible and esters peaks of FTIR results clearly indicated the bio-oil and biodiesel production.

FUTURE PROSPECTIVES

Present study prospects for future are given as:

- Complete use of carbohydrates extracted biomass for biodiesel and biogas production for making the biofuels production process more economical.
- Screening and optimization of bacterial/fungal strains other than yeast, capable of hydrolyzing pentose sugars for enhanced bioethanol production as Saccharomyces cannot metabolize these sugars.
- Development of feasible, economic and harmless microalgal biomass harvesting method for large scale cultivation.
- Effective carotenoids production from MSB strain for their applications in cosmetics food and pharmaceuticals.
- Pilot scale study on use of microalgae as potential feedstock for biofuels (biodiesel, bioethanol and biogas) production.
- Microalgae co-digestion with other organic substrates for the enhancement of biogas production.
- Lab scale continuous bioreactor for bioethanol production from MSB strain.
- Temperature and media optimization for higher biomass and carbohydrates productivity.
- Microalgae digestate can be effectively used as fertilizer for crops cultivation.
- Enzymatic transesterification of microalgae oil by using immobilized enzyme

REFERENCES

1) Adenle, A. A., Haslam, G. E., & Lee, L. (2013). Global assessment of research and development for algae biofuel production and its potential role for sustainable development in developing countries. *Energy Policy*, *61*, 182-195.
2) Ajit, A., Sulaiman, A. Z., & Chisti, Y. (2017). Production of bioethanol by Zymomonas mobilis in high-gravity extractive fermentations. *Food and Bioproducts Processing*, *102*, 123-135.
3) Akoh, C. C., Chang, S. W., Lee, G. C., & Shaw, J. F. (2007). Enzymatic approach to biodiesel production. *Journal of agricultural and food chemistry*, *55*(22), 8995-9005.
4) Alabi, A. O., Bibeau, E., & Tampier, M. (2009). *Microalgae Technologies & Processes for Biofuels-bioenergy Production in British Columbia: Current Technology, Suitability & Barriers to Implementation*. British Columbia Innovation Council.
5) Alam, F., Date, A., Rasjidin, R., Mobin, S., Moria, H., & Baqui, A. (2012). Biofuel from algae-Is it a viable alternative?. *Procedia Engineering*, *49*, 221-227.
6) Alfani, F., Gallifuoco, A., Saporosi, A., Spera, A., & Cantarella, M. (2000). Comparison of SHF and SSF processes for the bioconversion of steam-exploded wheat straw. *Journal of Industrial Microbiology & Biotechnology*, *25*(4), 184-192.
7) Al-Zuhair, S. (2007). Production of biodiesel: possibilities and challenges. *Biofuels, Bioproducts and Biorefining*, *1*(1), 57-66.
8) Amberg, S. M., & Meints, R. H. (1991). NUCLEOTIDE SEQUENCE OF THE GENES FOR RIBULOSE-1, 5-BISPHOSPHATE CARBOXYLASE/OXYGENASE LARGE SUBUNIT AND RIBOSOMAL PROTEIN S14 FROM A CHLORELLA-LIKE GREEN ALGA. *Journal of phycology*, *27*(6), 753-758.
9) Antczak, M. S., Kubiak, A., Antczak, T., & Bielecki, S. (2009). Enzymatic biodiesel synthesis–key factors affecting efficiency of the process. *Renewable energy*, *34*(5), 1185-1194.
10) Asif, M. (2009). Sustainable energy options for Pakistan. *Renewable and Sustainable Energy Reviews*, *13*(4), 903-909.

11) Asif, M., & Muneer, T. (2007). Energy supply, its demand and security issues for developed and emerging economies. *Renewable and Sustainable Energy Reviews*, *11*(7), 1388-1413.

12) Bajhaiya, A. K., Mandotra, S. K., Suseela, M. R., Toppo, K., & Ranade, S. (2010). ALGAL BIODIESEL: the next generation biofuel for India. *Asian J. Exp. Biol. Sci*, *4*, 728-739.

13) Barbosa, M. J. (2003). *Microalgal photobioreactors: scale-up and optimisation.*

14) Bibi, R., Ahmad, Z., Imran, M., Hussain, S., Ditta, A., Mahmood, S., & Khalid, A. (2017). Algal bioethanol production technology: A trend towards sustainable development. *Renewable and Sustainable Energy Reviews*, *71*, 976-985.

15) Borowitzka, M. A. (1992). Algal biotechnology products and processes—matching science and economics. *Journal of applied phycology*, *4*(3), 267-279.

16) Bozbas, K. (2008). Biodiesel as an alternative motor fuel: production and policies in the European Union. *Renewable and Sustainable Energy Reviews*, *12*(2), 542-552.

17) Brennan, L., & Owende, P. (2010). Biofuels from microalgae—a review of technologies for production, processing, and extractions of biofuels and co-products. *Renewable and sustainable energy reviews*, *14*(2), 557-577.

18) Brown, M. R., & Jeffrey, S. W. (1992). Biochemical composition of microalgae from the green algal classes Chlorophyceae and Prasinophyceae. 1. Amino acids, sugars and pigments. *Journal of experimental marine biology and ecology*, *161*(1), 91-113.

19) Cantrell, K. B., Ducey, T., Ro, K. S., & Hunt, P. G. (2008). Livestock waste-to-bioenergy generation opportunities. *Bioresource technology*, *99*(17), 7941-7953.

20) Carballa, M., Regueiro, L., & Lema, J. M. (2015). Microbial management of anaerobic digestion: exploiting the microbiome-functionality nexus. *Current opinion in biotechnology*, *33*, 103-111.

21) Chen, C. C., Liu, Y. J., & Yao, D. J. (2015). based device for separation and cultivation of single microalga. *Talanta*, *145*, 60-65.

22) Chen, C. Y., Yeh, K. L., Aisyah, R., Lee, D. J., & Chang, J. S. (2011). Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: a critical review. *Bioresource technology*, *102*(1), 71-81.

23) Chen, C. Y., Zhao, X. Q., Yen, H. W., Ho, S. H., Cheng, C. L., Lee, D. J., ... & Chang, J. S. (2013). Microalgae-based carbohydrates for biofuel production. *Biochemical Engineering Journal*, *78*, 1-10.

24) Chisti, Y. (2007). Biodiesel from microalgae. *Biotechnology advances*, *25*(3), 294-306.

25) Clark, J. H., & Macquarrie, D. J. (Eds.). (2008). *Handbook of green chemistry and technology*. John Wiley & Sons.

26) Dahnum, D., Tasum, S. O., Triwahyuni, E., Nurdin, M., & Abimanyu, H. (2015). Comparison of SHF and SSF processes using enzyme and dry yeast for optimization of bioethanol production from empty fruit bunch. *Energy Procedia*, *68*, 107-116.

27) Daroch, M., Geng, S., & Wang, G. (2013). Recent advances in liquid biofuel production from algal feedstocks. *Applied Energy*, *102*, 1371-1381.

28) de Farias Silva, C. E., & Bertucco, A. (2016). Bioethanol from microalgae and cyanobacteria: a review and technological outlook. *Process Biochemistry*, *51*(11), 1833-1842.

29) De Oliveira, M. A. C. L., Monteiro, M. P. C., Robbs, P. G., & Leite, S. G. F. (1999). Growth and chemical composition of Spirulina maxima and Spirulina platensis biomass at different temperatures. *Aquaculture international*, *7*(4), 261-275.

30) Dębowski, M., Zieliński, M., Grala, A., & Dudek, M. (2013). Algae biomass as an alternative substrate in biogas production technologies. *Renewable and Sustainable Energy Reviews*, *27*, 596-604.

31) Demirbas, A. (2008). Importance of biomass energy sources for Turkey. *Energy Policy*, *36*(2), 834-842.

32) Demirbas, A. (2010). Social, economic, environmental and policy aspects of biofuels. *ENERGY EDUCATION SCIENCE AND TECHNOLOGY PART B-SOCIAL AND EDUCATIONAL STUDIES*, *2*(1-2), 75-109.

33) Demirbas, M. F. (2011). Biofuels from algae for sustainable development. *Applied Energy*, *88*(10), 3473-3480.

34) Dincer, I. (1998). Energy and environmental impacts: present and future perspectives. *Energy sources*, *20*(4-5), 427-453.

35) Dincer, I. (2000). Renewable energy and sustainable development: a crucial review. *Renewable and Sustainable Energy Reviews*, *4*(2), 157-175.

36) Dincer, I., & Dost, S. (1996). Energy analysis of an ammonia-water absorption refrigeration system. *Energy Sources*, *18*(6), 727-733.

37) Dincer, I., & Rosen, M. A. (1998). A worldwide perspective on energy, environment and sustainable development. *International Journal of Energy Research*, *22*(15), 1305-1321.

38) Dincer, I., & Rosen, M. A. (1999). Energy, environment and sustainable development. *Applied energy*, *64*(1), 427-440.

39) Dismukes, G. C., Carrieri, D., Bennette, N., Ananyev, G. M., & Posewitz, M. C. (2008). Aquatic phototrophs: efficient alternatives to land-based crops for biofuels. *Current opinion in biotechnology*, *19*(3), 235-240.

40) DuBois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. T., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical chemistry*, *28*(3), 350-356.

41) Ediger, V. Ş., & Akar, S. (2007). ARIMA forecasting of primary energy demand by fuel in Turkey. *Energy Policy*, *35*(3), 1701-1708.

42) Ediger, V. Ş., Akar, S., & Uğurlu, B. (2006). Forecasting production of fossil fuel sources in Turkey using a comparative regression and ARIMA model. *Energy Policy*, *34*(18), 3836-3846.

43) Farooq, M., & Shakoor, A. (2013). Severe energy crises and solar thermal energy as a viable option for Pakistan. *Journal of Renewable and Sustainable Energy*, *5*(1), 013104.

44) Ghirardi, M. L., Zhang, L., Lee, J. W., Flynn, T., Seibert, M., Greenbaum, E., & Melis, A. (2000). Microalgae: a green source of renewable H 2. *Trends in biotechnology*, *18*(12), 506-511.

45) Ghose, T. K. (1987). Measurement of cellulase activities. *Pure and applied Chemistry*, *59*(2), 257-268.

46) Ghosh, A., Khanra, S., Mondal, M., Halder, G., Tiwari, O. N., Saini, S., ... &Gayen, K. (2016). Progress toward isolation of strains and genetically engineered strains of microalgae for production of biofuel and other value added chemicals: a review. *Energy Conversion and Management*, *113*, 104-118.

47) Gill, S. S., Mehmood, M. A., Ahmad, N., Ibrahim, M., Rashid, U., Ali, S., & Nehdi, I. A. (2016). Strain selection, growth productivity and biomass characterization of novel microalgae isolated from fresh and wastewaters of upper Punjab, Pakistan. *Frontiers in Life Science*, *9*(3), 190-200.

48) Godoy-Hernández, G., & Vázquez-Flota, F. A. (2006). Growth measurements: estimation of cell division and cell expansion. *Plant cell culture protocols*, 51-58.

49) Goldemberg, J. (2006). The promise of clean energy. *Energy policy*, *34*(15), 2185-2190.

50) Gonzalez-Fernandez, C., Sialve, B., & Molinuevo-Salces, B. (2015). Anaerobic digestion of microalgal biomass: challenges, opportunities and research needs. *Bioresource technology*, *198*, 896-906.

51) Goyal, H. B., Seal, D., & Saxena, R. C. (2008). Bio-fuels from thermochemical conversion of renewable resources: a review. *Renewable and sustainable energy reviews*, *12*(2), 504-517.

52) Gray, K. A., Zhao, L., & Emptage, M. (2006). Bioethanol. *Current opinion in chemical biology*, *10*(2), 141-146.

53) Gupta, V. K., & Tuohy, M. G. (2013). Biofuel technologies. *Recent Developments. Editorial Springer.*

54) Harun, R., Danquah, M. K., & Forde, G. M. (2010). Microalgal biomass as a fermentation feedstock for bioethanol production. *Journal of Chemical Technology and Biotechnology*, *85*(2), 199-203.

55) Harun, R., Yip, J. W., Thiruvenkadam, S., Ghani, W. A., Cherrington, T., & Danquah, M. K. (2014). Algal biomass conversion to bioethanol–a step-by-step assessment. *Biotechnology journal*, *9*(1), 73-86.

56) Helwani, Z., Othman, M. R., Aziz, N., Fernando, W. J. N., & Kim, J. (2009). Technologies for production of biodiesel focusing on green catalytic techniques: a review. *Fuel Processing Technology*, *90*(12), 1502-1514.

57) Ho, S. H., Chen, C. Y., & Chang, J. S. (2012). Effect of light intensity and nitrogen starvation on CO2 fixation and lipid/carbohydrate production of an indigenous microalga Scenedesmus obliquus CNW-N. *Bioresource technology*, *113*, 244-252.

58) Ho, S. H., Chen, C. Y., Lee, D. J., & Chang, J. S. (2011). Perspectives on microalgal CO 2-emission mitigation systems—a review. *Biotechnology advances*, *29*(2), 189-198.

59) Ho, S. H., Huang, S. W., Chen, C. Y., Hasunuma, T., Kondo, A., & Chang, J. S. (2013). Characterization and optimization of carbohydrate production from an indigenous microalga Chlorella vulgaris FSP-E. *Bioresource Technology*, *135*, 157-165.

60) Ho, S. H., Huang, S. W., Chen, C. Y., Hasunuma, T., Kondo, A., & Chang, J. S. (2013). Bioethanol production using carbohydrate-rich microalgae biomass as feedstock. *Bioresource technology*, *135*, 191-198.

61) Hsieh, D., Capareda, S., & Placido, J. (2015). Batch Pyrolysis of Acid-Treated Rice Straw and Potential Products for Energy and Biofuel Production. *Waste and Biomass Valorization*, *6*(3), 417-424.

62) Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., & Darzins, A. (2008). Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *The plant journal*, *54*(4), 621-639.

63) Jacob-Lopes, E., Scoparo, C. H. G., Lacerda, L. M. C. F., & Franco, T. T. (2009). Effect of light cycles (night/day) on CO2 fixation and biomass production by microalgae in photobioreactors. *Chemical Engineering and Processing: Process Intensification*, *48*(1), 306-310.

64) Jambo, S. A., Abdulla, R., Azhar, S. H. M., Marbawi, H., Gansau, J. A., & Ravindra, P. (2016). A review on third generation bioethanol feedstock. *Renewable and sustainable energy reviews*, *65*, 756-769.

65) Jankowska, E., Sahu, A. K., & Oleskowicz-Popiel, P. (2017). Biogas from microalgae: Review on microalgae's cultivation, harvesting and pretreatment for anaerobic digestion. *Renewable and Sustainable Energy Reviews*, *75*, 692-709.

66) Jones, C. S., & Mayfield, S. P. (2012). Algae biofuels: versatility for the future of bioenergy. *Current opinion in biotechnology*, *23*(3), 346-351.

67) Kelly-Yong, T. L., Lee, K. T., Mohamed, A. R., & Bhatia, S. (2007). Potential of hydrogen from oil palm biomass as a source of renewable energy worldwide. *Energy Policy*, *35*(11), 5692-5701.

68) Khan, H. A., & Pervaiz, S. (2013). Technological review on solar PV in Pakistan: Scope, practices and recommendations for optimized system design. *Renewable and Sustainable Energy Reviews*, *23*, 147-154.

69) Kim, J. K., Um, B. H., & Kim, T. H. (2012). Bioethanol production from micro-algae, Schizocytrium sp., using hydrothermal treatment and biological conversion. *Korean Journal of Chemical Engineering*, *29*(2), 209-214.

70) Kim, J., Lee, J. Y., & Lu, T. (2014). Effects of dissolved inorganic carbon and mixing on autotrophic growth of Chlorella vulgaris. *Biochemical Engineering Journal*, *82*, 34-40.

71) Kim, K. H., Choi, I. S., Kim, H. M., Wi, S. G., & Bae, H. J. (2014). Bioethanol production from the nutrient stress-induced microalga Chlorella vulgaris by enzymatic hydrolysis and immobilized yeast fermentation. *Bioresource technology*, *153*, 47-54.

72) Kim, M. S., Baek, J. S., Yun, Y. S., Sim, S. J., Park, S., & Kim, S. C. (2006). Hydrogen production from Chlamydomonas reinhardtii biomass using a two-step conversion process: anaerobic conversion and photosynthetic fermentation. *International Journal of Hydrogen Energy*, *31*(6), 812-816.

73) Kröger, M., & Müller-Langer, F. (2012). Review on possible algal-biofuel production processes. *Biofuels*, *3*(3), 333-349.

74) Lee, J. Y., Yoo, C., Jun, S. Y., Ahn, C. Y., & Oh, H. M. (2010). Comparison of several methods for effective lipid extraction from microalgae. *Bioresource technology*, *101*(1), S75-S77.

75) Lee, K., Eisterhold, M. L., Rindi, F., Palanisami, S., & Nam, P. K. (2014). Isolation and screening of microalgae from natural habitats in the midwestern United States of America for biomass and biodiesel sources. *Journal of Natural Science, Biology, and Medicine*, *5*(2), 333–339. http://doi.org/10.4103/0976-9668.136178

76) Liang, Y., Sarkany, N., & Cui, Y. (2009). Biomass and lipid productivities of Chlorella vulgaris under autotrophic, heterotrophic and mixotrophic growth conditions. *Biotechnology letters*, *31*(7), 1043-1049.

77) Ma, F., & Hanna, M. A. (1999). Biodiesel production: a review. *Bioresource technology*, *70*(1), 1-15.

78) Mata, T. M., Martins, A. A., & Caetano, N. S. (2010). Microalgae for biodiesel production and other applications: a review. *Renewable and sustainable energy reviews*, *14*(1), 217-232.

79) McKendry, P. (2002). Energy production from biomass (part 2): conversion technologies. *Bioresource technology*, *83*(1), 47-54.

80) Meher, L. C., Sagar, D. V., & Naik, S. N. (2006). Technical aspects of biodiesel production by transesterification—a review. *Renewable and sustainable energy reviews*, *10*(3), 248-268.

81) Menetrez, M. Y. (2012). An overview of algae biofuel production and potential environmental impact. *Environmental science & technology*, *46*(13), 7073-7085.

82) Milano, J., Ong, H. C., Masjuki, H. H., Chong, W. T., Lam, M. K., Loh, P. K., & Vellayan, V. (2016). Microalgae biofuels as an alternative to fossil fuel for power generation. *Renewable and Sustainable Energy Reviews*, *58*, 180-197.

83) Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical chemistry*, *31*(3), 426-428.

84) Mostafa, S. S., & El-Gendy, N. S. (2017). Evaluation of fuel properties for microalgae Spirulina platensis bio-diesel and its blends with Egyptian petro-diesel. *Arabian Journal of Chemistry*, *10*, S2040-S2050.

85) Mussgnug, J. H., Klassen, V., Schlüter, A., & Kruse, O. (2010). Microalgae as substrates for fermentative biogas production in a combined biorefinery concept. *Journal of biotechnology*, *150*(1), 51-56.

86) Mythili, R., Venkatachalam, P., Subramanian, P., & Uma, D. (2014). Production characterization and efficiency of biodiesel: a review. *International journal of energy research*, *38*(10), 1233-1259.

87) Naik, S. N., Goud, V. V., Rout, P. K., & Dalai, A. K. (2010). Production of first and second generation biofuels: a comprehensive review. *Renewable and sustainable energy reviews*, *14*(2), 578-597.

88) Nascimento, I. A., Marques, S. S. I., Cabanelas, I. T. D., Pereira, S. A., Druzian, J. I., de Souza, C. O., ... & Nascimento, M. A. (2013). Screening microalgae strains for biodiesel production: lipid productivity and estimation of fuel quality based on fatty acids profiles as selective criteria. *Bioenergy research*, *6*(1), 1-13.

89) Nigam, P. S., & Singh, A. (2011). Production of liquid biofuels from renewable resources. *Progress in energy and combustion science*, *37*(1), 52-68.

90) Nikolić, S., Mojović, L., Rakin, M., Pejin, D., & Nedović, V. (2009). Effect of different fermentation parameters on bioethanol production from corn meal hydrolyzates by free and immobilized cells of Saccharomyces cerevisiae var. ellipsoideus. *Journal of chemical technology and biotechnology*, *84*(4), 497-503.

91) Noureddini, H., Gao, X., & Philkana, R. S. (2005). Immobilized Pseudomonas cepacia lipase for biodiesel fuel production from soybean oil. *Bioresource technology*, *96*(7), 769-777.

92) Patil, V., Tran, K. Q., & Giselrød, H. R. (2008). Towards sustainable production of biofuels from microalgae. *International journal of molecular sciences*, *9*(7), 1188-1195.

93) Pragya, N., Pandey, K. K., & Sahoo, P. K. (2013). A review on harvesting, oil extraction and biofuels production technologies from microalgae. *Renewable and Sustainable Energy Reviews*, *24*, 159-171.

94) Rafique, M. M., & Rehman, S. (2017). National energy scenario of Pakistan–Current status, future alternatives, and institutional infrastructure: An overview. *Renewable and Sustainable Energy Reviews*, *69*, 156-167.

95) Raheem, A., Abbasi, S. A., Memon, A., Samo, S. R., Taufiq-Yap, Y. H., Danquah, M. K., & Harun, R. (2016). Renewable energy deployment to combat energy crisis in Pakistan. *Energy, Sustainability and Society*, *6*(1), 16.

96) Rajkumar, R., Yaakob, Z., & Takriff, M. S. (2013). Potential of micro and macro algae for biofuel production: a brief review. *Bioresources*, *9*(1), 1606-1633.

97) Ranganathan, S. V., Narasimhan, S. L., & Muthukumar, K. (2008). An overview of enzymatic production of biodiesel. *Bioresource technology*, *99*(10), 3975-3981.

98) Rao, A. R., Dayananda, C., Sarada, R., Shamala, T. R., & Ravishankar, G. A. (2007). Effect of salinity on growth of green alga Botryococcusbraunii and its constituents. *Bioresource technology*, *98*(3), 560-564.

99) Ras, M., Lardon, L., Bruno, S., Bernet, N., & Steyer, J. P. (2011). Experimental study on a coupled process of production and anaerobic digestion of Chlorella vulgaris. *Bioresource technology*, *102*(1), 200-206.

100) Ras, M., Steyer, J. P., & Bernard, O. (2013). Temperature effect on microalgae: a crucial factor for outdoor production. *Reviews in Environmental Science and Bio/Technology*, *12*(2), 153-164.

101) Rashid, U., & Anwar, F. (2008). Production of biodiesel through optimized alkaline-catalyzed transesterification of rapeseed oil. *Fuel*, *87*(3), 265-273.

102) Rathore, V., & Madras, G. (2007). Synthesis of biodiesel from edible and non-edible oils in supercritical alcohols and enzymatic synthesis in supercritical carbon dioxide. *Fuel*, *86*(17), 2650-2659.

103) Rismani-Yazdi, H., Haznedaroglu, B. Z., Bibby, K., & Peccia, J. (2011). Transcriptome sequencing and annotation of the microalgae Dunaliellatertiolecta: pathway description and gene discovery for production of next-generation biofuels. *BMC genomics*, *12*(1), 148.

104) Rizza, L. S., Smachetti, M. E. S., Do Nascimento, M., Salerno, G. L., & Curatti, L. (2017). Bioprospecting for native microalgae as an alternative source of sugars for the production of bioethanol. *Algal Research*, *22*, 140-147.

105) Robles-Medina, A., González-Moreno, P. A., Esteban-Cerdán, L., & Molina-Grima, E. (2009). Biocatalysis: towards ever greener biodiesel production. *Biotechnology advances*, *27*(4), 398-408.

106) Rodolfi, L., ChiniZittelli, G., Bassi, N., Padovani, G., Biondi, N., Bonini, G., & Tredici, M. R. (2009). Microalgae for oil: Strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. *Biotechnology and bioengineering*, *102*(1), 100-112.

107) Salameh, M. G. (2003). Can renewable and unconventional energy sources bridge the global energy gap in the 21st century?. *Applied Energy*, *75*(1), 33-42.

108) Samson, R., & LeDuy, A. (1983). Influence of mechanical and thermochemical pretreatments on anaerobic digestion of Spirulina maxima algal biomass. *Biotechnology letters*, *5*(10), 671-676.

109) Santos-Ballardo, D. U., Rossi, S., Hernández, V., Gómez, R. V., del Carmen Rendón-Unceta, M., Caro-Corrales, J., & Valdez-Ortiz, A. (2015). A simple spectrophotometric method for biomass measurement of important microalgae species in aquaculture. *Aquaculture*, *448*, 87-92.

110) Schenk, P. M., Thomas-Hall, S. R., Stephens, E., Marx, U. C., Mussgnug, J. H., Posten, C., ... & Hankamer, B. (2008). Second generation biofuels: high-efficiency microalgae for biodiesel production. *Bioenergy research*, *1*(1), 20-43.

111) Searchinger, T., Heimlich, R., Houghton, R. A., Dong, F., Elobeid, A., Fabiosa, J., ... & Yu, T. H. (2008). Use of US croplands for biofuels increases

greenhouse gases through emissions from land-use change. *Science*, *319*(5867), 1238-1240.

112) Shafiee, S., & Topal, E. (2009). When will fossil fuel reserves be diminished?. *Energy policy*, *37*(1), 181-189.

113) Shakeel, S. R., Takala, J., & Shakeel, W. (2016). Renewable energy sources in power generation in Pakistan. *Renewable and Sustainable Energy Reviews*, *64*, 421-434.

114) Sharma, Y. C., & Singh, B. (2009). Development of biodiesel: current scenario. *Renewable and Sustainable Energy Reviews*, *13*(6), 1646-1651.

115) Sheehan, J., Dunahay, T., Benemann, J., & Roessler, P. (1998). *Look back at the US department of energy's aquatic species program: biodiesel from algae; close-out report* (No. NREL/TP-580-24190). National Renewable Energy Lab., Golden, CO.(US).

116) Siaut, M., Cuiné, S., Cagnon, C., Fessler, B., Nguyen, M., Carrier, P., ... & Peltier, G. (2011). Oil accumulation in the model green alga Chlamydomonas reinhardtii: characterization, variability between common laboratory strains and relationship with starch reserves. *BMC biotechnology*, *11*(1), 7.

117) Singh, J., & Gu, S. (2010). Commercialization potential of microalgae for biofuels production. *Renewable and Sustainable Energy Reviews*, *14*(9), 2596-2610.

118) Sivasamy, A., Cheah, K. Y., Fornasiero, P., Kemausuor, F., Zinoviev, S., & Miertus, S. (2009). Catalytic applications in the production of biodiesel from vegetable oils. *ChemSusChem*, *2*(4), 278-300.

119) Sørensen, I., Rose, J. K., Doyle, J. J., Domozych, D. S., & Willats, W. G. (2012). The Charophycean green algae as model systems to study plant cell walls and other evolutionary adaptations that gave rise to land plants. *Plant signaling & behavior*, *7*(1), 1-3.

120) Spolaore, P., Joannis-Cassan, C., Duran, E., & Isambert, A. (2006). Commercial applications of microalgae. *Journal of bioscience and bioengineering*, *101*(2), 87-96.

121) Spolaore, P., Joannis-Cassan, C., Duran, E., & Isambert, A. (2006). Optimization of Nannochloropsisoculata growth using the response surface method. *Journal of Chemical Technology and Biotechnology*, *81*(6), 1049-1056.

122) Stehfest, K., Toepel, J., & Wilhelm, C. (2005). The application of micro-FTIR spectroscopy to analyze nutrient stress-related changes in biomass composition of phytoplankton algae. *Plant Physiology and Biochemistry*, *43*(7), 717-726.

123) Sulaiman, A. Z., Ajit, A., Yunus, R. M., & Chisti, Y. (2011). Ultrasound-assisted fermentation enhances bioethanol productivity. *Biochemical Engineering Journal*, *54*(3), 141-150.

124) van Maris, A. J., Abbott, D. A., Bellissimi, E., van den Brink, J., Kuyper, M., Luttik, M. A., ... & Pronk, J. T. (2006). Alcoholic fermentation of carbon sources in biomass hydrolysates by Saccharomyces cerevisiae: current status. *Antonie Van Leeuwenhoek*, *90*(4), 391-418.

125) Vandamme, D., Foubert, I., & Muylaert, K. (2013). Flocculation as a low-cost method for harvesting microalgae for bulk biomass production. *Trends in biotechnology*, *31*(4), 233-239.

126) Walters, L. C., Porter, D. L., & Crawford, D. C. (2002). Nuclear fuel considerations for the 21st century. *Progress in Nuclear Energy*, *40*(3-4), 513-521.

127) Wan, C., Alam, M. A., Zhao, X. Q., Zhang, X. Y., Guo, S. L., Ho, S. H., ... & Bai, F. W. (2015). Current progress and future prospect of microalgal biomass harvest using various flocculation technologies. *Bioresource technology*, *184*, 251-257.

128) Wang, Y., Wu, H., & Zong, M. H. (2008). Improvement of biodiesel production by lipozyme TL IM-catalyzed methanolysis using response surface methodology and acyl migration enhancer. *Bioresource technology*, *99*(15), 7232-7237.

129) Wei, N., Quarterman, J., & Jin, Y. S. (2013). Marine macroalgae: an untapped resource for producing fuels and chemicals. *Trends in biotechnology*, *31*(2), 70-77.

130) Xin, L., Hong-ying, H., Ke, G., & Ying-xue, S. (2010). Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga Scenedesmus sp. *Bioresource technology*, *101*(14), 5494-5500.

131) Yamada, T., & Sakaguchi, K. (1982). Comparative studies on Chlorella cell walls: Induction of protoplast formation. *Archives of Microbiology*, *132*(1), 10-13.

132) Yoon, M., Choi, J. I., Lee, J. W., & Park, D. H. (2012). Improvement of saccharification process for bioethanol production from Undaria sp. by gamma irradiation. *Radiation Physics and Chemistry*, *81*(8), 999-1002.

133) Zamalloa, C., Boon, N., & Verstraete, W. (2012). Anaerobic digestibility of Scenedesmus obliquus and Phaeodactylum tricornutum under mesophilic and thermophilic conditions. *Applied Energy*, *92*, 733-738.

134) Zhang, H., Ma, J., Miao, Y., Tuchiya, T., & Chen, J. Y. (2015). Analysis of carbonyl value of frying oil by fourier transform infrared spectroscopy. *Journal of oleo science*, *64*(4), 375-380.

135) Zhao, G., Chen, X., Wang, L., Zhou, S., Feng, H., Chen, W. N., & Lau, R. (2013). Ultrasound assisted extraction of carbohydrates from microalgae as feedstock for yeast fermentation. *Bioresource technology*, *128*, 337-344.

136) Ziolkowska, J. R., & Simon, L. (2014). Recent developments and prospects for algae-based fuels in the US. *Renewable and Sustainable Energy Reviews*, *29*, 847-853.