CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

In Silico Construction of Hybrid ORF Protein to Enhance Algal Oil Content for Biofuel

by

Atia Liaqat

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

in the

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Dedicated to Allah Almighty, Hazrat Muhammad (S.A.W.W) and my father Liaqat Ali. My mother prayers have always enlightened my way throughout my life. It's also dedicated to my brother and all other family members who taught me that the best kind of knowledge to have is that which is learnt for its own sake. They taught me that even the largest task can be accomplished if it is done one step at a time.

CERTIFICATE OF APPROVAL

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My first word of thanks to the one who taught words to Adam, Who blessed man with knowledge, who is the sublime without whose "Kun" nothing is possible. Secondly my humblest thanks from the core of my heart, to our beloved **Prophet Hazrat Mohammad (S.A.W.W)** who is the eternal fountain of knowledge and guidance for the whole mankind. Then I would like to pay my deepest gratitude to my admirable teacher and supervisor Dr. Arshia Amin Butt and Biosciences, Capital University of science and Technology Islamabad. I am grateful to her for approving research topic, her disciplinarian attitude, and strictness in punctuality and working in organized manner makes possible to complete this work.

I now turn to those who were and are first on my mind but are mentioned last, my father (Liaqat Ali) my role model, my mother, sisters, and all teachers of my career. I believe their most sincere and innocent prayers which were a constant source of strengthen and inspiration to me that resulted in the completion of this work. I dedicate this work to my beloved parents at the end. I pray to Allah Almighty to give me the strength and resources to serve them to the best of my efforts best and wisdom not to neglect them in future; Ameen.

(Atia Liaqat)

Abstract

The current energy demands and depletion of fossil fuels urges us to develop some alternative energy resources. In recent years Algae has gained much attention as a third generation biofuel feedstock. Among Algae, Microalgae is being considered as good source of biofuel because of their relatively more oil concentration and rapid production of biomass. The main benefit of using microalgae is that it does not interrupt food chain or food crops. Microalgae have the capability to produce a wide variety of biofuels including bio-oil, bio-diesel, bio-syngas, and bio-hydrogen. Commercial production of biofuel from microalgae is presently not in use because of high expense of production. From cultivation to oil extraction, the whole process is costly. Hence, there is need to enhance the oil content of microalgae, so that it can meet the expense of production and the process proves to be fruitful. This research was planned to produce a hybrid ORF that might enhance the oil yielding capability of microalgae on its translation into a working protein. By using intensive literature survey 6 genes from 3 microalgae species were selected. Further the ORFs of selected genes were identified and Hybrid ORF was constructed by combining those ORFs. Afterwards restriction enzyme analysis and thermodynamic analysis of Hybrid nucleotide sequence was carried out which showed that the Hybrid sequence was stable. The Hybrid ORF nucleotide sequence was translated into protein sequence. This protein sequence was used for homology modeling of Hybrid ORF protein. The protein conformation predicted by homology modeling was further verified by Ramachandran plot. The metabolic pathways were analyzed by KEGG which showed that all the selected genes were functioning in biosynthesis of lipids. This showed that the Hybrid ORF constructed could be used in a way to enhance oil content of microalgae for producing biofuel particularly biodiesel. These results can be validated by further in vitro analysis and can be used for construction of genetically engineered microalgae.

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Abbreviations

CABC Creating Algae Biofuel Commercialization Consortium

DAG Diacylglycerol

GHG Greenhouse gases

Kegg Kyoto Encyclopedia of Genes and Genomes

MAG Monoacylglycerol

NCBI National Centre of Biotechnology Information

NNFCC National Non-Food Crops Centre

ORF Open Reading Frame

PBRs Photobioreactors

PubMed Public/Publisher MEDLINE

SABC Sustainable Algal Biofuel Consortium

SIB Swiss Institute of Bioinformatics

TAG Triacylglycerol

Chapter 1

Introduction

1.1 Global Energy Crises

Energy protection and demand is amongst one of the key issues that are being investigated and explored in this age because there is an immense increase in world energy demands and the fossil fuels are depleting with the passage of time [1] [2] [3]. The unsystematic extraction and over usage of petroleum-based fuels due to rapid growth of population, construction of buildings and industrial development globally is leading to a decline in petroleum-based fuel resources [4] [5].
Fuels that are based on petroleum are being taken from very limited reserves that are found in particular localities of world. Moreover, the quality and amount of petroleum-based fuels are reducing with the passage of time; therefore, states which don't have resources will soon face serious energy crisis [6] [7] [8].

- Today, our main energy sources are petroleum, coal, natural gas, hydroelectric and atomic power. And the need for energy is going up day by day. The fossil fuels are now considered as unsustainable because of the limited supplies and addition of Carbon dioxide CO_2 in atmosphere, which aids in warming of environment and change of climate [9]. The burning of fossil fuels emits lots of CO_2 in the surrounding atmosphere.

1.2 Greenhouse Gas Reduction

The burning of fossil fuels emits lots of CO_2 in the surrounding atmosphere. Consequently, the concentration of CO_2 in atmosphere has reached alarmingly high, at level of 400 ppm. Industries and their effluents also contribute to atmospheric hazards, which increase greenhouse gases (GHG) accumulation that contribute in global warming and leads to climate change [10]. Subsequently, Scientists from overall world are working to discover the solutions of problems. They are working to make proper measures, mainly removal of CO_2 from environment which is emitted by different sources [8] [3].

Recent enticement to eliminate greenhouse gasses, particularly CO_2 , has enriched the interest of scientists in fuels derived from plants, since plants possess an innate capability to absorb sunlight energy by using photosynthetic pigments (in light process), while effectively restoring CO_2 from the environment as their main carbon supplier (in dark process [16].

This carbon obtained from absorption of CO_2 is next chemically transformed into high-energy celluloses, starches, proteins and oils/lipids as storage and structural compounds found in plants. Selected species of algae are recognized to have ability to effectively convert CO_2 to approximately 60-70% of their dry weight into oils and lipids useful for different purposes shown in 1.1

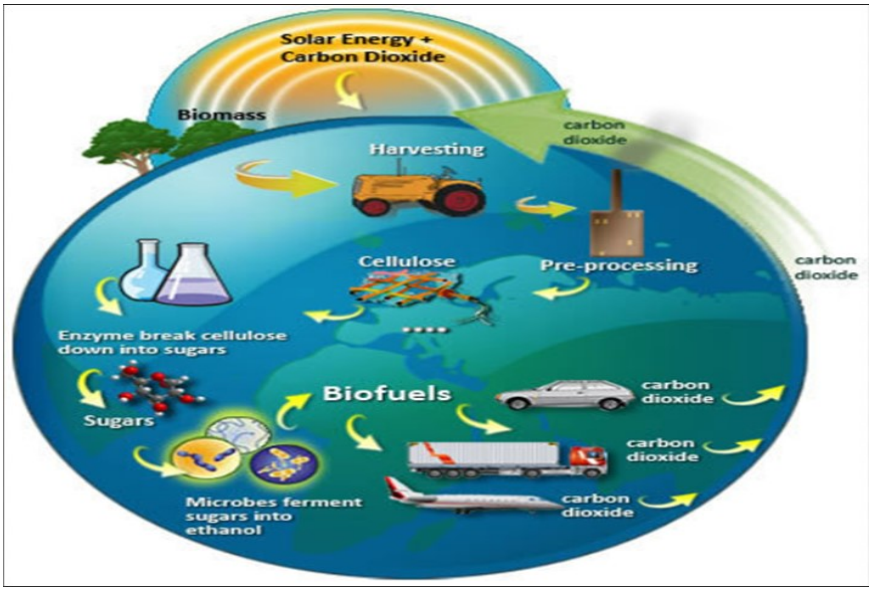

FIGURE 1.1: Sustainable biofuel production [29]

1.3 Biofuels

Biofuels are all those fuels which are obtained from plant biomass by physical and chemical processing with low CO_2 production [18] [19]. Biomass is any living (mainly plant-based) material that has stored energy by the process of photosynthesis; including wood, plants, remains of plants and agronomic waste products [20] [21]. Increased in energy expenditure and usage along with the extensively increasing energy prices are reflecting adversely upon natural energy reserves. This has triggered researchers and high ups to find out some alternate, sustainable, carbon-free and non-traditional energy sources that will be ecologically and commercially maintainable.

- Biofuel is the most well-known alternative fuel for its potential to provide sustainable and renewable energy, and biodiesel is the most popular in the market today, along with bioethanol and biogas [22] [23] .

Biofuels have been categorized into three major classes based on their biomass source i.e. first, second and third generation of biofuels; the highly accepted among these are bio-diesel, bio-gas and bio-ethanol [24] [25].

1.4 Microalgae

Microalgae are a category of diverse prokaryotic and eukaryotic microscopically visible autotrophic microorganisms that survive in variety of environments . These have chlorophyll pigment which are further efficient in photosynthesis than other terrestrial plants and can be found in every environment on the planet. They don't possess rooting, main stalk, leaves, conducting tubes, and sophisticated reproductive tissues, unlike higher plants [26]. They can be as small as a few micrometres (µm) or as large as hundreds of micrometres. Depending on the species, they can

develop in a variety of ways (independently, in form of chain or colonies, or in the form of filaments). Macroalgae and microalgae are the two types of algae. Brown algae, red algae, and green seaweed are examples of macroalgae. Microalgae include *chlorella, spirulina, and green algae*. In the marine and freshwater environment, there are about 20,000 different types of microalgae. However, just a few types of microalgae have been discovered thus far for bioenergy conversion. Microalgae offer more advantages than macroalgae, like simple structure, rapid reproduction rates, and very high amount of oil, among others. As a result, most industrial businesses have a preference to use microalgae as their feed-stocks for biofuel production [26].

Microalgae are classified in different groupings which include *cyanophyta* (which includes cyanobacteria), *chlorophyta* (mainly green algae), *phaeophyta* (known as brown algae), rhodophyta (known as red algae), *bacillariophyta* (including diatoms), and more based on their structural, functional, life cycle, biochemical ability, and genomic conditions [27] . Only 40,000–50,000 species have been described

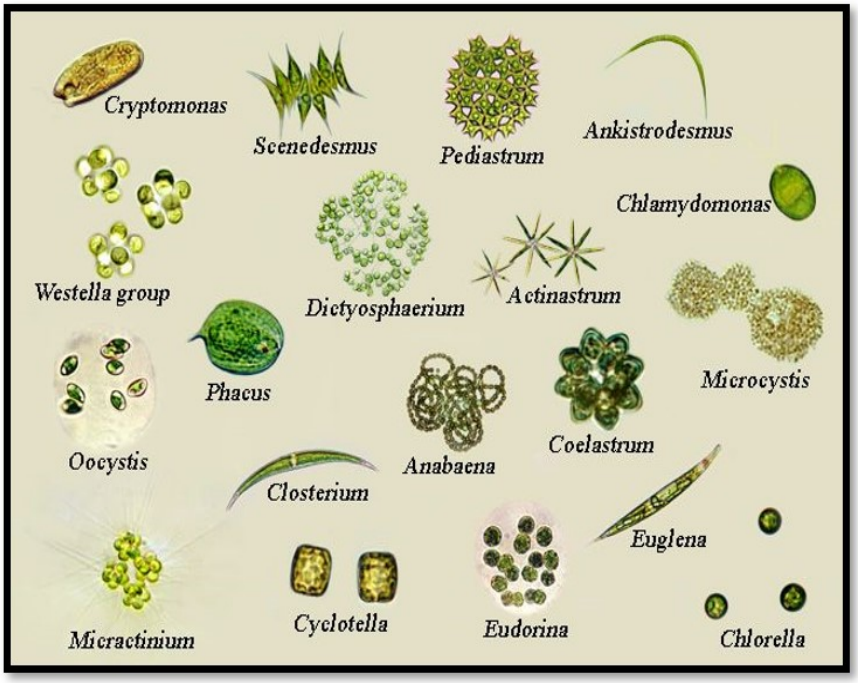

FIGURE 1.2: Different species of Microalgae [29].

and explored for various reasons out of an estimated 200,000–800,000 species. Many algae strains have been found to produce biofuels, including *Chlamydomonas, Chlorella, Botryococcus braunii*, and others.

1.5 Biodiesel

Biodiesel is a kind of sustainable biofuel containing fatty acids and methyl esters which originated from plant oils, animal fats and oils found in microalgae. It has a solid capability to take place of petroleum based biodiesel. For affordable production, the choice of feedstock for biofuel is very critical. For production of biodiesel from microalgae, two steps are required. In first step lipids are separated by extraction method from cells of microalgae and in second step the isolated oil is transformed into biodiesel [28].

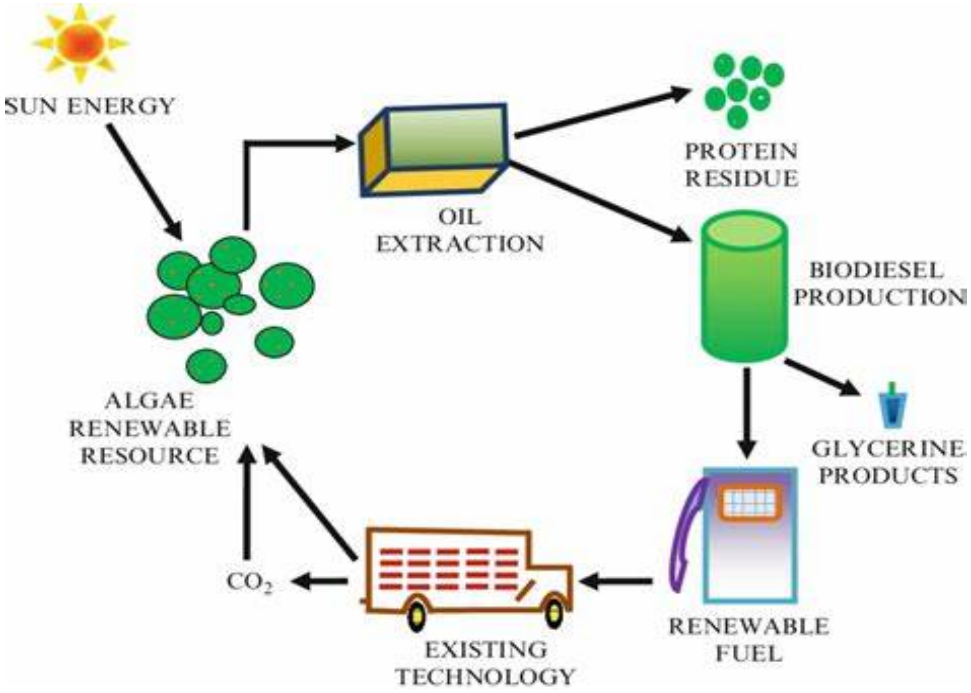

FIGURE 1.3: Oil extraction from microalgal biomass [28].

Biodiesel is made through trans-esterification process of triacylglycerols (TAGs). The TAGs are reacted with alcohol, most often methanol or ethanol, at room temperature with the help of a catalytic agent such as Potassium Hydroxide (KOH), Sodium hydroxide (NaOH), or Sulfuric acidH_2SO_4. Mono-alkyl fatty acid esters or biodiesel are produced as a result of the procedure [29].

Three steps are involved in the trans-esterification reaction of TAGs shown in 1.1. TAG would be first transformed into a diacylglycerol (DAG) and a fatty acid ester molecule. After that, the DAG is transformed into a monoacylglycerol (MAG) and

a fatty acid ester moity. Finally, the MAG is broken up into glycerol and one fatty acid ester. As a result, one molecule of glycerol and three molecules of fatty acid ester are produced as byproducts [29].

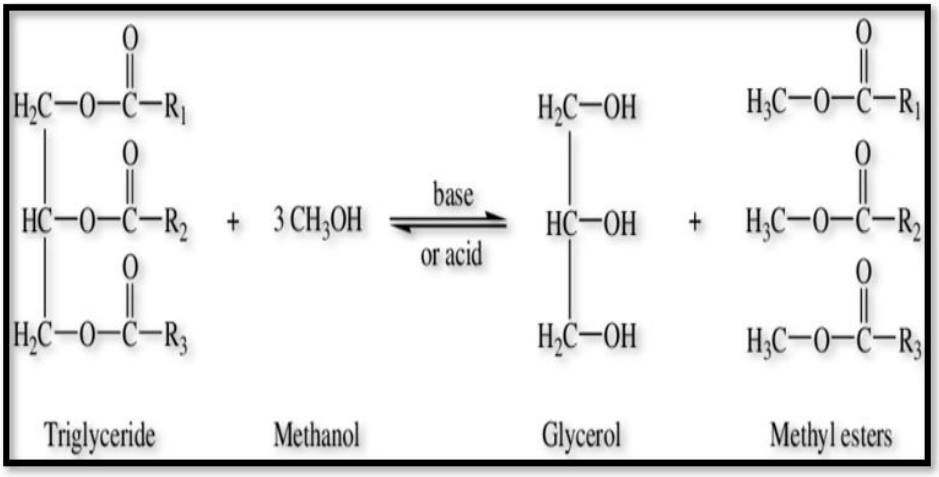

FIGURE 1.4: Process of Trans-esterification: One mole of Triacylglycerol reacts with three moles of alcohol. The alcohol is mostly methanol or ethanol and reacts in the presence of catalyst [44]

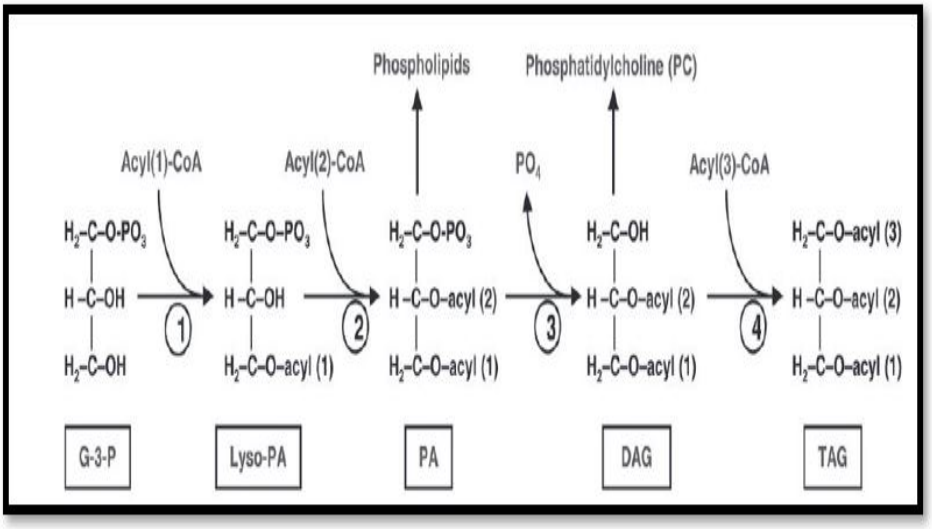

FIGURE 1.5: TAG biosynthesis [42].

Biodiesel has gotten a lot of interest in recent years because it's known to be eco-friendly, renewable, recyclable, CO_2 -free source of energy and no toxicity at all. Biodiesel emits less gaseous pollutants and emits no net CO_2 or sulphur to the atmosphere than regular diesel (petroleum-based diesel). It possess similar

physic-chemical qualities as petro-diesel, such as a high volatitility, lower sulphur content, and higher lubrication and cetane number [30].

1.6 Biodiesel Production Using Microalgae as Feedstock

Through the Hill reaction which is the light phase in photosynthesis, microalgal species possess the intrinsic capability to acquire energy from sunlight from the surroundings and change it in to biochemical energy and redox compounds.
Dark phase of photosynthesis is responsible for the efficient fixation of CO_2 from atmosphere as their main carbon source which is called Calvin cycle. Then the fixed carbon is transformed into compounds having high energy such as carbohydrates, proteins, and lipids.

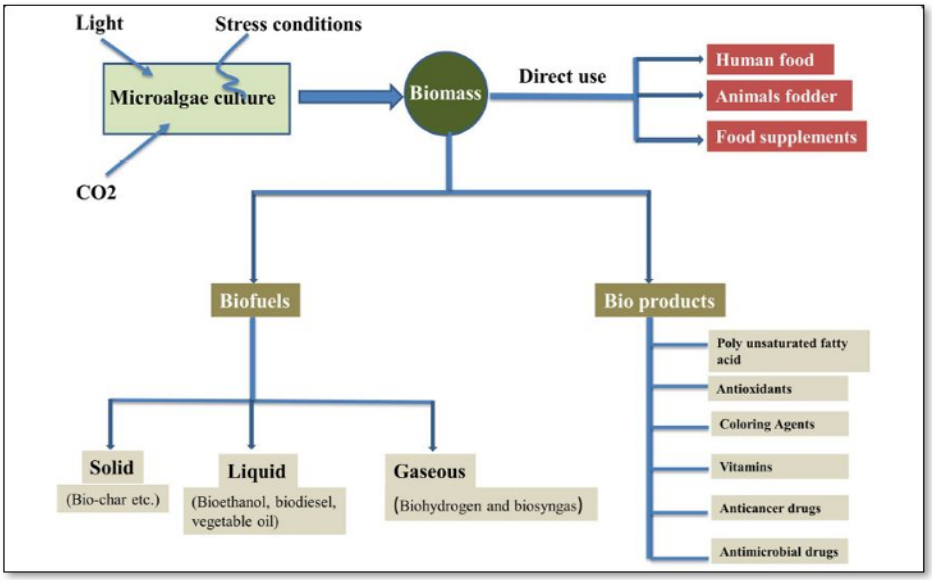

FIGURE 1.6: Procedure of biomass and biofuel production from microalgae [55].

Numerous scientific studies have confirmed the possibility and practicality of biodiesel production from microalgae [31] [32]. In contrast to other possible feedstocks, Microalgae has received extensive research as a profitable and long-term source of biodiesel. Microalgae is simple for cultivation, able to grow in harsh

environments, uses waste water for its growth, is able to be used in treatment of wastewater to make it available for irrigation, has no threat of food scarcity, and reduce GHG emissions through CO_2 usage. The biomass of microalgae increases two folds in 24 hours and can complete the whole growth cycle in few days [42]. Various microalgae strains have ability to adapt in an extensive variety of climatic states. As a result, we can identify strains that are suitable for the indigenous environmental conditions or have some particular characteristics of growth. This is unattainable with existing feed-stocks for biodiesel (including soybean, rapeseed, sunflower, and palm oil) . In comparison to the typical forest, agriculture crops, and plants which grow in water, growth rate and production yield of microalgae is much higher. Microalgae demands significantly less area of land than other agricultural feed-stocks for biodiesel, approximately 49 times reduced as compared to Brassica napus and 132 times lesser than soya bean yield. The oil content of algae is 30 percent (w/w) [33]. Furthermore, microalgae has proven as a feedstock for a

TABLE 1.1: Comparison of other Feed-stocks of biodiesel with microalage [23]

Type of Feed-stock	**Amount of oil**	**Yield litre**	**Area used biodiesel**	**Water foot-print**	**Expense of production**	**Acid value of oil**	**Total production**
R. communis	48	1307	9	24700	0, 92-1	4, 6	89%
B. napus	41	974	12	4300	0, 99	2, 0	87%
Glycine max	18	636	18	4200	0, 40-0	0, 2	90%
L. chinensis	36	5366	2	5000	0, 68	6, 1	95%
Helianthus	40	1070	11	6800	0, 62	0, 1 ,4	90%
Microalgae	50	97800	1	591-3276	96-10	8, 9	60%

variety of sustainable fuels, including bio-hydrogen, methane (biogas), biodiesel, and bioethanol; microalgae biodiesel does not contain any sulphur and works similarly to petroleum diesel [34].

Recently, Many International firms are financing in biofuel production by microalgal biomass, with many projects: Green Fuel Technologies, Algenol, Origin Oil

in USA, AlgaFuel in Portugal, Varicon Aqua Solutions in the UK, Neste Oil in Finland, Euglena in Japan, Algae Link in Netherlands.

Biodiesel made with agricultural crops is inefficient and unsustainable [35]. Because oil constitutes less than 5% of the overall biomass in oil crops, many of crops are necessary to yield a considerable quantity of oil for biodiesel manufacturing. Furthermore, large agricultural regions are required to produce the requisite yield of crops for biomass.

FIGURE 1.7: Cultivated land area

It has detrimental consequences for the ecosystem. The reason is a lot of people in the world depend on the food grown on reasonably small agriculture land, this circumstance could pit food against fuel [56]. Furthermore, extensive production of biofuel crops necessitates a lot of insecticide, organic and inorganic fertilizers, and irrigation.

Only by reducing the quantity of land necessary for the growing of a suitable amount of feedstock can sustainability be accomplished. "Biodiesel is a sustainable energy source only if feed-stocks are generated sustainably," the researchers concluded.

Microalgae offer a sufficient supply of oil required for making biodiesel. Microalgae show most potential as bioenergy prospects amongst the several other land crops which produce oil and utilized as feed-stocks for biodiesel since these have more oil, may develop in short time, and be easy to grow. The time it takes for their growth to double is usually approximately 24 hours. Under perfect temperature, light,

TABLE 1.2: Dry biomass's oil content of different strains of microalgae [48] [8] [6] [23]

Sr.No	Microalgal Strains	Amount of Oil (% of dry weight)
1	*B. braunii*	25-75
2	*C. vulgaris*	14-22
3	*C. pyrenoidosa*	46.7
4	*C.protothecoides*	57.9
5	*C. emersonii*	28-32
6	*C. cohnii*	20
7	*D. tertiolecta*	35.6
8	*S. dimorphus*	16-40
9	*P. parvum*	22-38
10	Nitzschia sp.	45-47
11	Cylindrotheca sp.	16-37
12	*D. primolecta*	*23*
13	*D. salina*	*6*
14	*Nannochloropsis sp.*	*31-68*
15	*Hormidium. sp.*	*38*
16	*Isochrysis sp.*	*25-33*
17	*M. salina*	*20*
18	*Nannochloris sp.*	*20-35*
19	*S. platensis*	*6-7*
20	*N. soleoabundans*	*6-7*
21	*S. maxima*	*4-9*
22	*P. carterae*	*30-50*
23	*Schizochytrium sp.*	*50-77*
24	*T. suecica*	*15-33*
25	*P. tricornutum*	*20-30*
26	*S. obliqus*	*12-14*

and nutrient circumstances, microalgae may increase by two times the biomass in approximately 120 minutes during exponential growth .
Microalgae bio-oil yields are projected to be 20,000 to 80,000 litres per acre, which is about 7–31 time more than the other prolific crops producing oil [36].

A microalga has the capability to produce many types of biofuels which include bio-hydrogen, bio-oil, biogas, biodiesel and bioethanol. These biofuels can prove to be cheap and sustainable alternative of petroleum fuels. Moreover, the biofuel from microalgae will be Carbon neutral, may recycle CO2 and add O2 to the environment [37].

1.7 Advantages of Microalgae

Selection of microalgae as feedstock for biofuel has many advantages. Some of those advantages are listed below:

1. Microalgae are photosynthetic organisms which utilize sunlight, water and CO_2 to form organic compounds. These organic compounds include carbohydrates, proteins and lipids. Lipids are converted into biodiesel and bio-oil. Carbohydrates produce biohydrogen and bioethanol. Due to absorbance of CO_2 and release of oxygen in the environment, microalgae are considered as harmless for climate.

2. Microalgae have very simple cellular structure and smaller in size. Due to this property their growth on large scale for biofuel production becomes very easy.

3. The method of reproduction of microalgae is simple splitting which makes it easy for multiplying and they increase number of cells very quickly. Also cell cycle of these microalgae is very short which makes it very feasible to

cultivate them on a large scale.

4. Microalgae have the capability to grow in a variety of environmental conditions so they can be grown in sea water, alkali water and also in industrial effluent/sewage water. So it is very easy to produce microalgal biomass in areas where freshwater is not available or land is water deprived.

5. The oil content of microalgal is very much greater than first and second generation sources of feedstock, which is a significant sign for production of biodiesel from microalgae species.

6. Microalgae also play an important role in bioremediation of contaminated waste water or heavy metal containing water. Therefore it is very significant to take advantage of microalgae for both characteristics i.e. producing biofuel along with treatment of heavy metal contamination in watert [63].

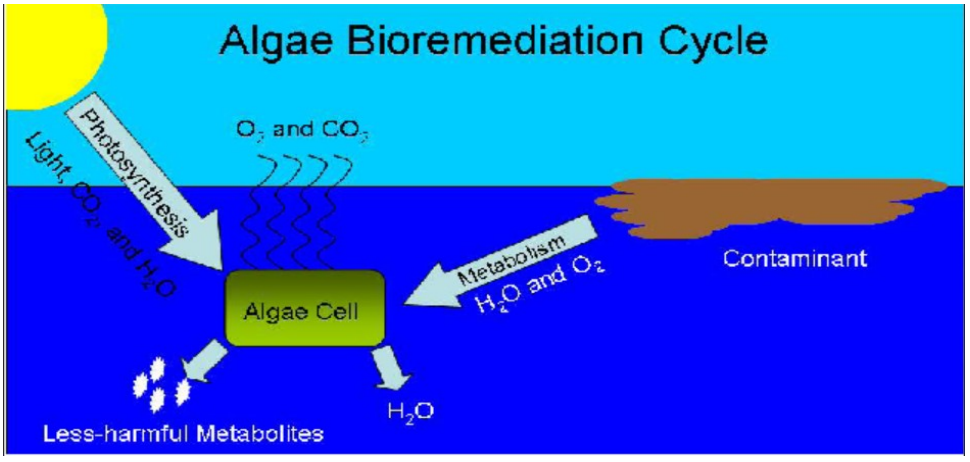

FIGURE 1.8: Removal of heavy metal/contamination by microalgae [63].

Microalgae offer a variety of useful compounds such as source of food, animal feed, provide with many food supplements, and produce antioxidants, pharmaceutical compounds and biofuel. Hence harvesting of microalgae results in giving multiple benefits along with bio-energy [52].

A microalga has the capability to produce many types of biofuels which include bio-hydrogen, bio-oil, biogas, biodiesel and bioethanol. These biofuels can prove to be cheap and sustainable alternative of petroleum fuels. Moreover, the biofuel from microalgae will be Carbon neutral, may recycle CO_2 and add O_2 to the environment.

1.8 Objectives

The main aim of this study is to construct protein hybrid ORF to produce more oil content from microalgae which may help in increased biofuel production. To achieve this aim, the study is designed with following major objectives:

1. To construct hybrid ORF protein of algae for more oil content.

2. To analyze the metabolic pathways of selected genes for lipid biosynthesis.

1.9 Problem Statement

The main challenge in biofuel production is production cost of microalgae, which is very much higher than the amount of oil produced from it. From grow to cultivation, and harvesting, also the process of extracting biofuel, and overall the whole process is costly. Therefore, there is a need to enhance the oil content of microalgae so that it can meet to the expense of production and the process become fruitful.

Chapter 2

Review of Literature

2.1 Global Energy Demand and Climate Change

The current petrochemical resource crisis and environmental pollution are two major concerns that our society must confront. Petroleum oil shortages and rising fuel price are the main issues in constraining the worldwide economy as limited petroleum supplies have been increasingly depleted [61].
Furthermore, according to scientific literature, massive amounts of fossil consumption generate environmental contamination, which leads to global warming and climate-related disasters. To address these issues, social and industrial experts have begun to hunt for sustainable energy resources which can be an alternative for petroleum fuels in order to create a more sustainable society and promote global economic recovery.

2.2 Biofuel

Biomass energy sources are becoming popular as alternatives of conventional fuels, by terrestrial crops and aquatic algae. Energy from biomass is typically generated by continental crops and aquatic algae for use in the production of bio-gas and bio-fuel. The United States and Brazil have been developing biofuels by using

maize for producing bioethanol, from 1970s with outstanding outcomes. Energy production from biomass is not only more environmentally friendly than fossil fuels, but it also helps to solve the energy deficit problem. Terrestrial crops which are, feed-stocks for first (I) and second (II) generations of biofuel present different issues, such as the encroachment on fertile land-area, that can result in a crisis of food. As a result, the biggest pressing challenge about sustainable growth nowadays is the requirement of land to produce food for over-growing world population. According to an FAO statistic, an average of 25,000 individuals die due to lack of food everyday around the planet [40].

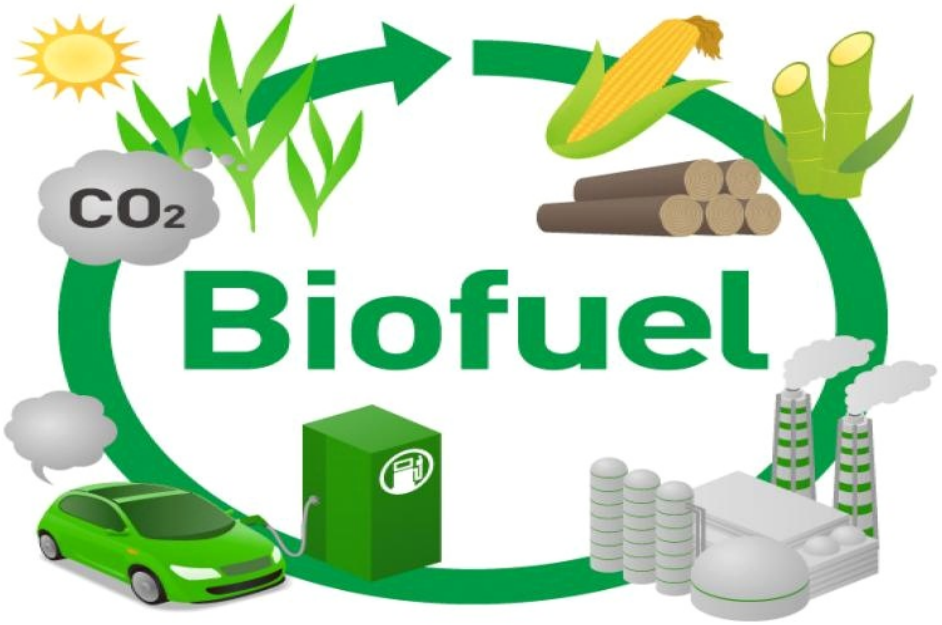

FIGURE 2.1: Conversion of bio-mass into biofuel [76].

The most important alternative of fossil fuel is energy production from biomasss. It means the production of bioenergy from air, water, and soil through photosynthesis. Staple crops, waste of crops, timber, rubbish, animal's dung, also aquatic algae, among other plants, animals, and microorganisms, can all be used to extract it. Biomass has two major advantages:

1. It is renewable and produces less pollution. As an alternative to petroleum fuels, biomass is used to produce biofuel and biogas.

2. Biomass development has now become a significant technique to change energy structure and reduce greenhouse gas emissions on a global scale in pursuance of achieve ecological and financial stability [41].

Biofuels are classified into three generations based on their biomass sources, limits as a sustainable source of energy, and technical progress.

2.3 First Generation of Biofuel

Under the discussion over biofuel, it's important to differentiate between first generation and second generation technology. The first cohort of biofuel includes both liquid and gaseous fuels. The liquid forms of biofuels include bio-diesel, vegetable-oil, bio-ethers, and bioalcohol. Oil producing plant-seeds and food crops, such as starch, sugar cane, fat of animals, sunflower, rapeseed, and palm make up the first generation (G1) biomass. Each oil molecule is distinct due to the complicated triglycerides molecules with varying length of alkyl chain.

Triglycerides contain three units of fatty acids bonded together by an ester molecule, and they are turned into biofuels by the techniques used. Trans-esterification, anerobically decomposition, fermentation, and pyrolysis are different methods utilized to produce fuels. The first generation biofuels are capable to be utilized exclusive of mixing or blended to improve their performance [41].

Oil obtained from castor, sunflower, palm, and jatropha is used in biodiesel plants in Europe, North America, South America, and Asia. In India, the United States, France, China, Germany, and Australia, corn and sugarcane are considered fortune spinners. Lowa State University claims that the annual cost of maize plant producing ethanol in 2013 was about 0.75 USD/litre that include the cost of machinery, feed-stock supply, manufacturing, and shipping. In spite of reductions in greenhouse gases accumulation and contamination, they wreak havoc on food availability and societal unrest. As a result, the inclusion of stakeholder opinions

FIGURE 2.2: Feedstock for first generation a) Corn b) Wheat c) rapeseed d) palm [76]

in ranking and evaluating a certain generation creates concerns for the generation of first generation fuels in future [42].

2.3.1 Second Generation of Biofuel

Because the source is non-food biomass, second generation G_2 fuels are a sure substitute for first generation fuels. Inedible byproducts of the food manufacturing industry and wooden factories, such as dry wood, corn stalks, also wheat husk, comprise the biomass for second generation biofuel [45].

The products range from cellulose-derived ethanol to bio-syngas (BioSNG), which is the blend of carbon mono-oxides (CO) and hydrogen H_2 produced using various specialized methods.

The major processes involved in G_2 are saccharide fermentation, gasifying the dried

FIGURE 2.3: Feedstock for seconf generation. a) switchgrass b) miscanthus (c)coppice willow (d) corn stalks and (e) wood parts.

bio-mass, BtL (biomass-to-liquid) technology, and HTL (hydrothermal liquification) of oils obtained from plants. Gasification of dried biomass produces bio-hydrogen, cellulose and syngas fermentation produces ethanol, butanol CH_9OH, and methanol, and Fischer-Tropsch (FT) synthesis along with biomass-to-liquid produces C(5-18) hydrocarbons fuel. In comparison, these obtained biofuels prove to be in compliance along EU-RED (EU-Renewable Energy Directive), are environmental safe, unpolluted ignition, without corrosion, don't cause desertification due to removal of trees, and cannot be used as animal feed because of their low toxicity [36].

This advanced energy perspective has attracted investors from all over the world to invest in biodiesel and bioethanol production. Biodiesel made from reclaimed oil (food, animal fat, and vegetal oil) by Australian Renewable Fuels Limited, and bio-ethanol made by husk of Triticum, waste of agriculture, wooden-pieces, and sugarcane biomass by BlueFire Ethanol Fuels, Inc., US, Cosan, Brazil, and Coskata, US, are examples. Although, second generation biofuel output is yet inferior to first generation, with the previous generating 500 million gallons and

the second producing 15 billion gallons. Furthermore, the absence of appropriate skill and high saturated fatty acid content in G_2 feed-stocks drives the option as a short-term and not permanent. So the quest for development of a sustainable and efficient biofuel led to the development of G_3 biofuels [47].

2.3.2 Third Generation of Biofuel

Algae are the non-flowering plants that contain chlorophyll but are dissimilar to other plants and range from small to large size, are shiny or dark-green covers present in shady and moist areas. The nutritional industry, bio-plastics, pharmaceuticals, special chemical manufacturing, biological nutrition, and the thriving biomass-based fuel production all benefit from the processing of these microorganisms. Algae have unique characteristics like:

a They absorb CO_2 for their growth which reduces the greenhouse effect,

b Algae does not need a large land area for growing in comparison to other plants used for food,

c Algae are able to grow in saline water, and d) they have a high lipid content [48].

During the early nineteenth century's energy crisis, methane production from algae gained a lot of traction. Half a century ago, Harder and Von Wiltsch recommended alga for an alternative of food as well as power source. During World War II, Japan, England, and Israel started large-scale culturing of *Chlorella sp.*
Because of the abundant supply of petroleum-based fuel, this concept algae to be used as alternative source for fuel was relegated to food merchandises. The authorized drive "Aquatic Species" has been launched by the United States 18 years ago with a budget of 25 million . In modern times, there has begun more

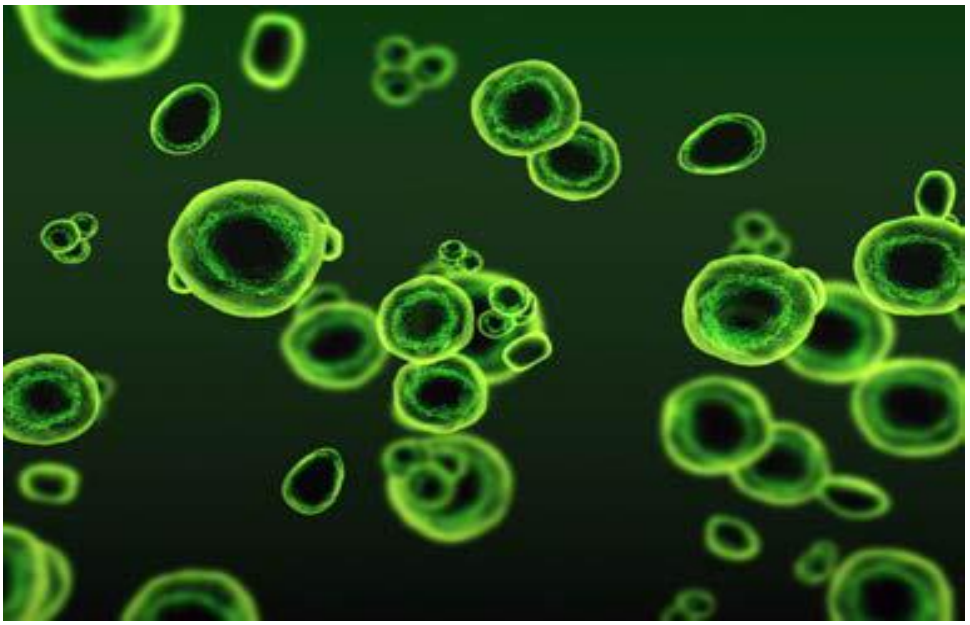

FIGURE 2.4: Sample of *Chlorella sp* [87].

interest to use algae to synthesize alternative fuels, and algae can be an alternative for first and second generation biofuel. The generalised method for converting vegetable oil into biodiesel can be used to convert the lipids in algae to biodiesel. Bioethanol and biobutanol, on the other hand, are made from algae carbohydrates. Other biofuels have a market value of only half that of algal biofuel (420 million US dollars) [46]. This figure would rise with the right technological practice in the near future. *Spirulina platensis* contains 8% oils and 60% sugar, *Chlorella species* contain 19% oils, 56% sugar) are a few algae of research interest. Today, almost ten countries are keen on algae biomass biofuel production. A number of techniques for microalgal biofuels treatment are presented in 2.4.

2.4 Need for Biofuel Production from Microalgae

As a feedstock for 3rd generation biomass, algal biomass was identified. Reported yields are at least 10 times greater than those obtained from other crops rich in lipids. Its main advantage is that it is possible to modify and process different

products on a commercial scale in a biorefinery approach. The third generation of biomass from algal biofuels is divided into macroalgae and microalgae, and is to some degree associated with the usage of CO_2 in feed-stock manufacture. In academia as well as industry, fluid and gaseous biofuels produced from algae have been topical with a majority of the research focused on microalgae [46].

Most species of algae are unique and are commonly known as microalgae. The microalgae are diversified in ancient civilizations and still used as food with a taxonomic classification.

As a result of petro-oil distress at the beginning of 1970s, the US Aquatic Species Program started a number of renewable energy programmes, including microalgae biofuels (i.e., ASP, currently NREL). The production of biofuel micro-algae sector was supported by opportunities to mitigate GHG by capturing micro-algae by CO_2 and by developing global biofuel policies. However, the major problem for the processing of microalgae is the demand for pre-conversion water-removal, which needs high amount of energy and large amount for production expense, representing twenty to thirty percent of the whole production expenditure. The mentioned and other many production limitations are keeping microalgae biofuels in terms of technology and cost-effectiveness. Research has again began in recent years to the multiple product bio-refining idea, therefore enhancing microalgae technology's cost-effectiveness [53] [54] [55]. As a result of the high productivity

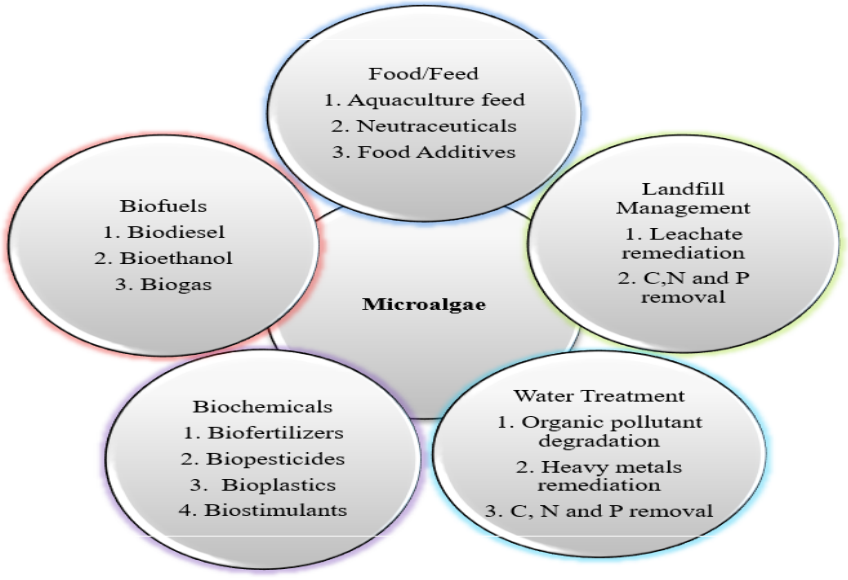

FIGURE 2.5: Uses and Products of Microalgae

of microalgae in a unit area of land plus the capability to cultivate in a land which is not suitable for cultivation of crops, compared to most of oilseed crops with a shorter growing period, this is significantly more efficient.

Microalgae may also obtain the required nutriments from sevage water and may obtain CO_2 released as a result of combustion. For the reason of its adaptableness to different conditions, like treatment of sevage water and aquaculture, a microalga has received significant attention [35]. Microalgae production, as a third generation biofuel feedstock, is presently going through considerable improvement in study as well as industrial area. Because of increased photosynthesis in biomass production, higher rate of growth, no scarcity of edible crops for agriculture and high oil yields, this is the most sustainable and reliable feedstock in comparison with biofuels of the 1st and 2nd generations.

The potential to yield many products from microalgae other than biofuel makes it more valuable. Figure 2.7 shows the uses and products of microalgae.

2.5 Requirements for Culturing of Algae

The cultivation of large-scale microalgae will contribute decisively in the advancement of industrial setup for renewable and efficient biofuel, and the manufacturing of low-cost produce which may have more value. Several microalgae strains have capability for growing on large area, yet insufficient information is available for commercial studies. In order to compete with other feed-stocks, a large amount of microalgae biomass is needed for organic bioethanol manufacture. Efficient technology for cultivating microalgae would have to generate greater quantity of biomass to use the edible crops relatively less attractive for the manufacturing of bio-ethanol. Different methods and conditions can cultivate microalgae. They need sunlight as a supply of energy for photosynthetic processes to transform intake water and CO_2 into food and bio-mass. The accretion of photosynthetic products varies among 20 to 50 percent of overall organic matter in diverse ways, comprising of parts of cells and storing compounds. Nitrogen plus phosphorus are necessary as major nutrients, representing an algae biomass of 10–20 percent. The

macronutrients Na, Mg, Ca, and K, as well as micronutrients like Mo, Mn, B, Co, Fe, and Zn, and other trace elements, are all necessary for growth. Waste-water is a decent resource of the nutritional needs for growth of microalgae. Biological and unrefined sewages from the agriculture and food activities may thus be used to feed microalgae.

Algal cells go into various stages as they grow (e.g., lag-phase, exponential-phase, stationary-phase, death-phase). The growth media requirements of different microalgae species may differ. However, almost all species have the same basic requirements, which may consist of necessary nutritional requirements, supply of carbon, iron, phosphorus, and nitrogen [56].

It is essential to design those technologies for culturing of microalgae which pro-

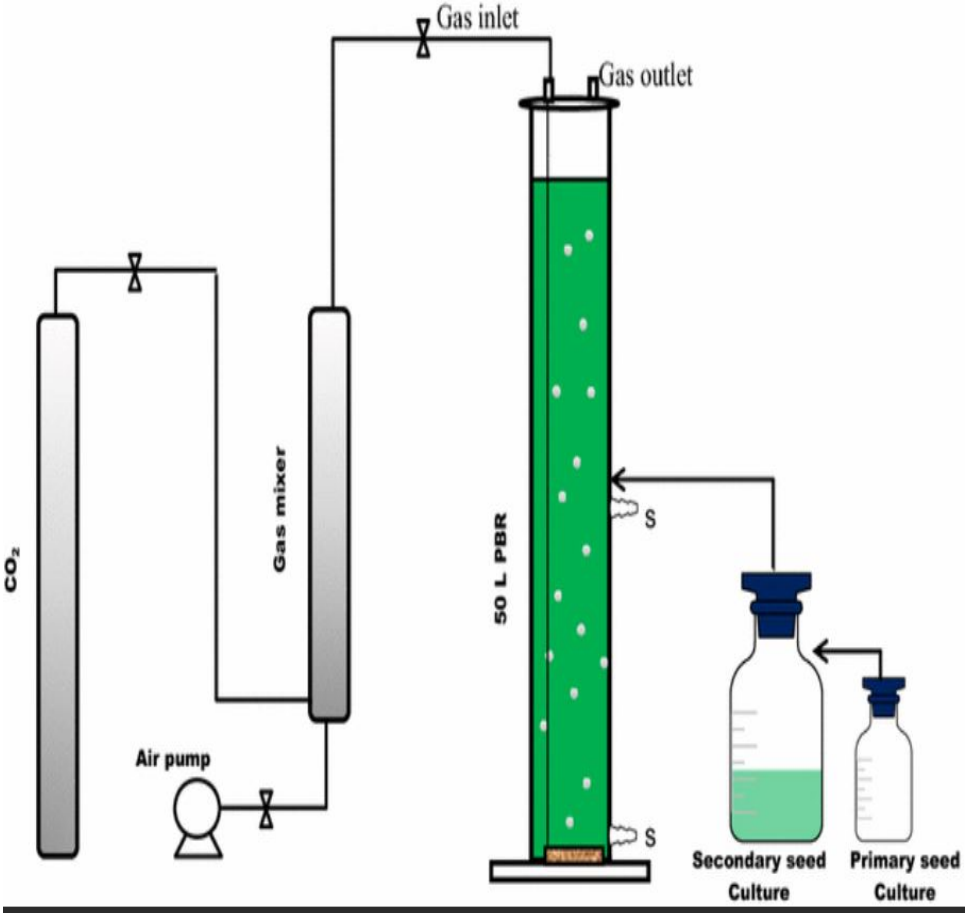

FIGURE 2.6: Microalgae cultivation system [56].

duce targeted bio-mass to make algae production more sustainable, practicable and financially doable. The biomass output for a viable algal culture should be greater than 30 g/m2-day [57] [58]. About forty thousand diverse microalgal types

are identified [59].

The following are the critical growth factors which have a promising effect on the yield of bio-mass and other products from microalgae.

2.5.1 Light Intensity

Light intensity is the main restraining factor in cultivation of microalgae. Light duration and intensity have a direct impact on microalgae photosynthesis, as well as their biochemical composition and amount of biomass produced [60]. Rates of growth and biomass yield are predicted as the result of light in experimental models of algal cultures in open-air or closed-bioreactors. Intensity of light varies inside the medium and reduces downward in the culture medium; this should be considered when modeling a bioreactor or open pond system. The amount of light that algae require for highest rates of growth and biomass varies by species.

Microalgae can only grow in moderate intensity of light, very high or low light intensity is not efficient.

Net growth is zero at the compensation point of light when the rate of intake of CO_2 is equal to the CO_2 release as a result of respiration. When intensity of light increases the rate of photosynthesis also increases up to a highest point, afterward it will reduce till photo-respiration and photo-inhibition balance the photosynthetic rate. As a result, the optimal light intensity in each case must be measured by experiment in order to maximize absorption of CO_2 while minimizing photorespiration and photo-inhibition.

- Algal photosynthesis necessitates a particular length for light and dark intervals. The dark reaction of photosynthesis, that produces carbon compounds, require light for ATP and NADPH synthesis. Up to the saturation point, the growth of microalgae increases directly with intensity and length of light/-dark period [61] [62].

By growing the same algae type in altered intensity of light and for different periods, Khoeyi et al. confirmed the variations of product of biomass and rate of

growth. With decreasing light duration, rate of growth and biomass production also reduced. Many research papers showed that Sixteen hours of sunlight and eight hours of dark period are ideal for algae growth [63]. In order to escape oxidation due to light and inhibition of growth, suitable amount of sunlight and period are necessitated in culture media for microalgae . In order to prevent obstruction of light, when deep tiers are covered by upper tiers, and appropriate light penetration and uniform scattering in medium is also mandatory. Although fluorescent tubes can also be used, LED sources of light are also a better option for this [64] [65].

2.5.2 Temperature

Temperature is also a significant parameter for the growth of microalgae, as it has a direct impact on bio-chemical activities in cellular system of algae, including photosynthetic activity [66] [67]. All strains of microalgae have their particular ideal temperature range for maximum growth. Increases in temperature up to certain optimal scale increases growth in microalgae, however increases or decreases of temperature other than that point reduce or stop algal growth and cellular activities [68] [69] [70]. Many algal strains prefer temperatures between 20 and 30 degrees Celsius [71], though thermophile microalgae for example :-

- Anacystis nidulans,
- Chaetoceros have ability to survive under temperature as high as 40 degrees Celsius,
- Also those which grow in warm water of springs about 80 degrees Celsius.

Those media cultures of microalgae which are grown at non-optimum temperature lose a lot of biomass, especially in open-air growth mediums . Temperature is a significant parameter in large-extent farming, particularly in open-air system, and it requires thorough checking because algae face considerable temperature variation with time [68]. Extremely low temperature reduces photosynthetic activity by

inhibiting carbon integration, while very high temperature may inhibit photosynthetic activity by deactivating proteins involved in photosynthesis and disrupting cell's energy value. Cell size and respiration both shrink as the temperature rises. A decrease in growth rate is caused by a decrease in photosynthesis [79]. The main consequence of temperature on photosynthetic activity is a decrease in the reactivity of the dual-function enzyme ribulose-1,5-bisphosphate (Rubisco). Rubisco reacts as oxygenating agent as well as carboxylation and its role depends on the availability of relative amount of O_2 and CO_2 in chloroplast. Carboxylase or CO_2 fixing action of Ribulose boosts with increasing temperature until a point and after that it decreases [80]. Therefore temperatures has an effect on the activity of Rubisco with CO_2, So it is a rate determining parameter in the multiplication and production of biomass of algae.

1. Temperature can be used as an inducing factor for producing many important metabolites as the result of stress treatment [80].
2. According to Converti et al growing *Chlorella vulgaris* at 25°C produces more carbs and lipids as compared to growing it at 30 °C.
3. According to Kitaya et al. Several microalgae species thrived at temperatures between 27 and 31°C.

2.5.3 Nutrients

Varying microalgae strains have different nutritional requirements, however they all have the same basic required conditions. Microalgal main-structure is made from nitrogen, phosphorus, and carbon, and these macronutrients are needed for growth of algae. Some species of marine microalgae also use silicon as major part of nutrients. Water is used by microalgae to absorb oxygen and hydrogen. Different microalgae species may have varying levels of macronutrients including nitrogen and phosphorus. As the amount of nitrogen and phosphorus was lessened

from 31.5 and 10.5 mg/l, correspondingly, chlorella growth was reported to be reduced [81]. Cell growth is directly influenced by the amount of nitrogen available in the culture. Nitrogen limitation can impair growth and biomass productivity while enhancing carbohydrate and lipid production in microalgae cultivation. The nitrogen concentration that yields 3.43 g/l biomass in *Chlorella vulgaris* has measured to be 0.5 g/l .

- Mo, K, Co, Fe, Mg, Mn, B, and Zn are only required in trace levels, but their impacts on a range of enzymatic activities in algal cells have a substantial impact on microalgae growth.

Nitrates plus phosphates are the most common forms of inorganic nitrogen and phosphorus absorbed. Other inorganic nitrogen sources, such as urea, are also acceptable and cost-effective options. Carbon may be introduced in algal growth medium in the form of organic molecules such as glycerol or acetates, as well as CO_2. Though, for development of microalgae at a big extent, CO_2 from atmosphere should be utilized as resource of carbon, that not only cheap plus also has the added advantage of reducing CO_2, P, N, and C remain the most important mineral nutrients for microalgae development [83]. Nutrition insufficiency has a considerable effect on microalgae development rates, resulting in reduced biomass. Nutrient supply has a significant impact on the manufacture and aggregation of carbs and oil in microalgal cells [88]. For industrial manufacturing of microalgal biomass, the medium should grow quickly; consequently, delivering the correct nutrients is crucial to accelerate algal growth. As growth stimulants, microalgae can benefit from the application of some extremely limiting chemicals. Furthermore, by giving vital nutrients, certain bacteria can aid microalgae growth [91].

2.5.4 Mixing

In microalgae culture, blending and providing air ensure equal dissemination of nutrients, air, and CO_2. They also allow light to penetrate and disperse evenly throughout the culture, preventing biomass from settling and aggregating [92].

Biomass productivity will be drastically lowered if all requisites are provided but blending doesn't happen. As a result, to bring every cell afloat and in exposure to light, microalgae cultures must be regularly mixed. A suitable mixing mechanism in a photo-bioreactor allows for not only nutritional dissolving and dispersion of light in the medium, also gases are exchanged efficiently [93].

2.5.5 pH and Salinity

The pH of the growth medium is also a crucial component that influences microalgae growth. Varied microalgae strains possess special need of pH. The range of pH from 6 to 8.76 is ideal for most plants. The pH of various growth medium sources varies. *Chlorella p.* can grow in a broad pH scale; however growth rates and production of biomass are best around pH 9–10 [72]. As the pH rises, the salt of the growth medium rises, that is extremely toxic to algal cells [94].

2.5.6 Mixotrophic Cultivation

Microorganisms in autotrophic cultivation generate energy from the sunlight, whereas those living as heterotrophs metabolize the sources of carbon for energy. Mixotrophic circumstances combine autotrophic and heterotrophic models, allowing cultivated microbes to use both inorganic and organic carbon sources for photosynthesis, for example sugar, glycerol, and acetate [95]. Microalgae can develop faster and manufacture chemicals using both autotrophs and heterotrophs in mixotrophic cultures. Furthermore, by giving vital nutrients, certain bacteria can aid [96].

2.6 Bio-refinery of Microalgae

A variety of processes are used in microalgae bioprocessing for extraction of bioactive compounds such as lipids, proteins, and carbohydrates. This process of biorefining extracts biofuels and more bioactive compounds from microalgal biomass.

The use of microalgae bio-refining process to reduce warming of climate caused by harmful GHGs such as Carbon Dioxide in the atmosphere is a potential technique. While bio-refining microalgae, isolation of various parts without major losing other parts is critical. Accessible, cost-effective, and energy-efficient isolation methods can be employed to overcome this challenge. Microalgal biomass is a good crude matter for a bio-refining technique since it may create many elements suited for a variety of factories including food, energy, medicines, plus nutraceuticals. Despite the immense potential of microalgal biomass, the existing limitations of an algal bio-refining process must be acknowledged. An annual production of microalgal biomass in industry is currently around 15,000 tonnes. In comparison to current industrial demands this figure is very low. The expensive cost of cultivation, harvesting, and extraction is a significant contributor to this poor production rate.

1. As a result, microalgae are currently being exploited to extract high-value specialty goods.
2. Biofuel manufacturing is at the lowest edge of the range because of stiff battle with fossil fuels.
3. Biofuel costs do not have to be less than nonrenewable fuel costs. On the other side, biofuel manufacturing requires less energy.

This constraint has yet to be overcome. The creation of value-added microalgal products has been the subject of numerous investigations. The two primary steps in processing of microalgal bio-refinery are upstream and downstream. The cultivation of microalgae is the most essential step in the upstreaming process. The upstream process uses water, nutrients, light, and CO_2 as basic materials. Nutrients like phosphorus and nitrogen control the increase in number of microalgae. With the correct volume of nutrient delivery, high biomass output and a faster growth time can be obtained. The source of illumination has an effect on the rate of growth of microalgae accessible, cost-effective, and energy-efficient [89].

2.6.1 Lipids Fraction

Various microalgae species have been observed to collect lipids about 15-40% of their dry mass, including *C. vulgaris, Scenedesmus sp., and Spirogyra sp.* In severe settings, microalgae, on the other hand, can collect lipids up to 70% to 90% of their dry matter [43,44]. The degree of stress applied to the microalgae culture during cultivation has an impact on the lipid content buildup [9]. Because of the lack of nitrogen in the culture broth, microalgae accumulate lipids when the carbon-nitrogen (C/N) percentage in the growth medium is high [45,46]. High salinity, high temperature, and a very high pH of the culture medium, and restricted nitrogen source all affect lipid productivity [90].

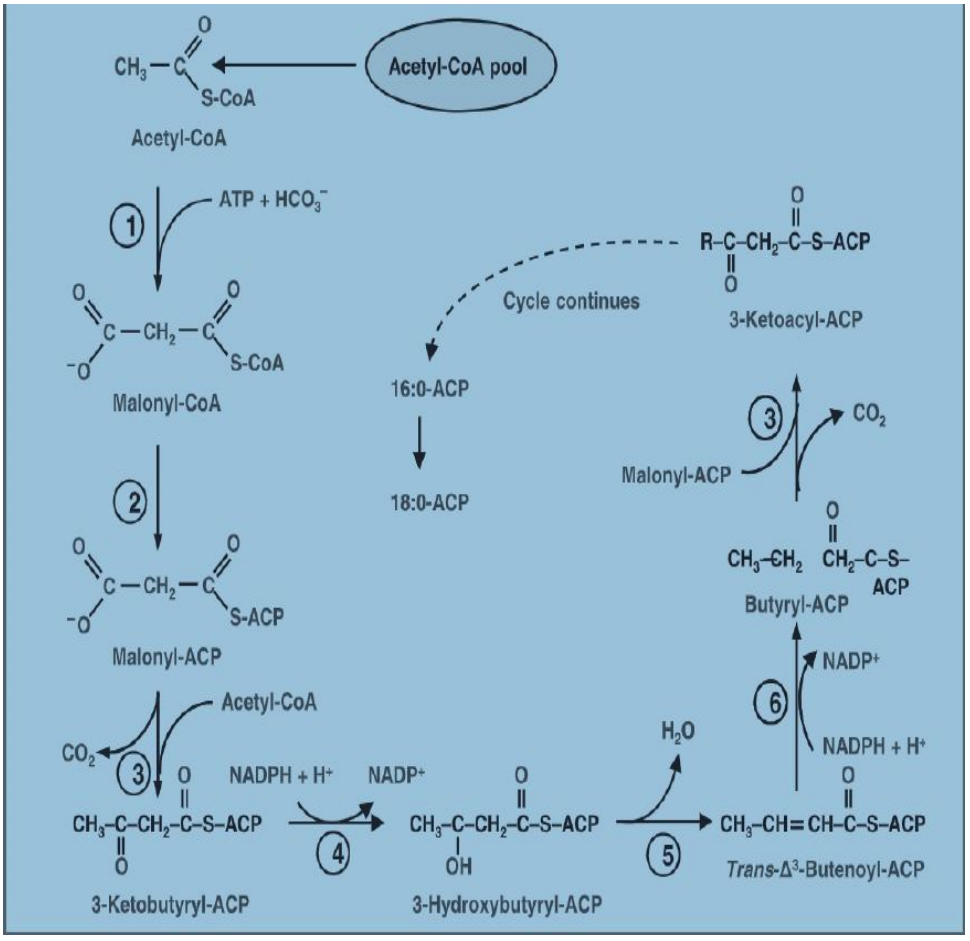

FIGURE 2.7: Pathway of fatty acid synthesis [42]

The lipids found in microalgae are divided into two groups. Fatty acids which have 14–19 carbon atoms in chains make up the first type, while those with more

than 19 carbon atoms in chains make up another type. Because it has a saturated fatty acid with no double bond in the chain of hydrocarbons, former type is usually bio-transformed into biodiesel. The latter is known as poly-unsaturated fatty acids (PUFAs) in the food industry because it is unsaturated and its hydrocarbon chain has double bonds. Microalgae oil production ability is thought to be greater as compared to conventional lipid producing sources. Table 2.1 shows the average amount of oil and the type of sources needed to produce it. Table 2.1 shows that lipids extracted from microalgal biomass are preferred for biodiesel manufacture.

TABLE 2.1: Lipid yield and Resources needed to produce it [89, 90]

Sr.No	**Resource type**	**Biomass have oil**	**Yield**	**Land area required**	**Biodiesel**
1	*Zea maize*	44	172	66	152
2	*L. chinensis*	36	5366	2	4747
3	*Glycine max*	18	636	18	562
4	*Cannabis sativa*	33	363	31	321
5	*Ricinus communis*	48	1307	9	1156
6	*J. curcas*	28	741	15	656
7	*Helianthus*	40	1070	11	946
8	*C. sativa*	42	915	12	809
9	*Brassica napus*	41	974	12	862
10	Microalgae	30	58,700	0.2	51,927
11	Microalgae	50	97,800	0.1	86,515
12	Microalgae	70	136,900	0.1	121,104

Triacylglycerides are a type of lipid that is needed for producing biodiesel (TAGs). Trans-esterification converts these Triglycerides into biodiesel . Trans-esterification is a process that involves reaction of methanol with lipids of microalgae and results in production of glycerol and fatty acid methyl esters (FAME).

1 mole of TAG react with 3 moles of methanol to form 3 moles of FAME and 1 mole of glycerol in the process of trans-esterification. Supplementing, the trans-esterification process with acid catalysis speeds up the process. The reaction catalyzed by alkali is 4000 times more fast when reaction catalyzed by an acid.

Producing biodiesel from the microalgal biomass has several advantages, but it is not as simple as traditional biodiesel production. Extraction and purification are both difficult processes. Several studies have recently been conducted to lessen

the complexities found in harvesting, extracting oil, and subsequent production of biodiesel. When wet microalgal biomass is used which has 50% (w/w) of water content, increased FAME output by up to 84 percent .

As a co-solvent, methanol was used. Another recent study used a 90 percent (w/w) water content microalgae culture in manufacture of biodiesel with excess hexane and methanol as co-solvents.. The approach omitted the separation stage and instead produced FAME by direct trans-esterification. A comparable study achieved a 97.3 percent biodiesel conversion rate using C. vulgaris having 71 percent amount of water [90] .

Hu et al. showed the lipid content of different species of algae shown in Figure.

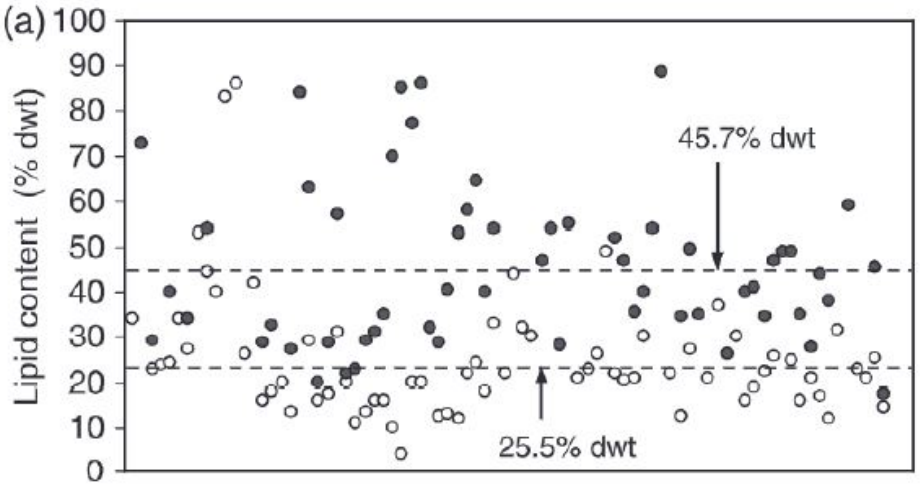

FIGURE 2.8: Lipid content in green microalgae [42].

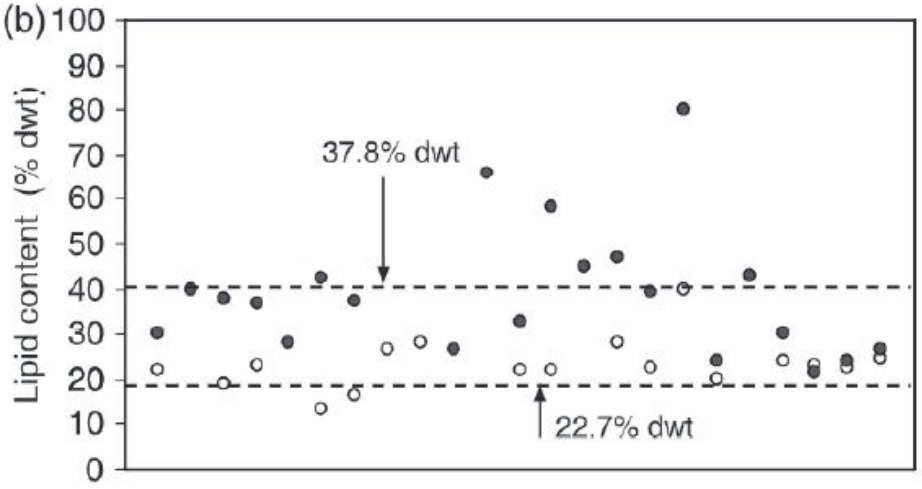

FIGURE 2.9: Lipid content in diatoms [42].

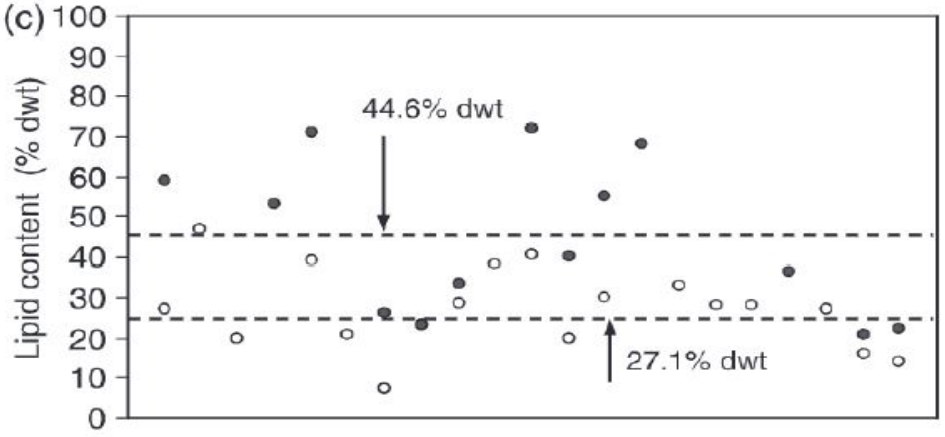

FIGURE 2.10: Lipid content in oleaginous species [42].

The figure 2.8, 2.8, 2.10 are representing the lipid content in various strains of microalgae as stated in literature. Figure 2.8 represents the lipid content of green microalgae which is found to be 25.57% of the dry weight but this value almost increased by two-folds when they were subjected to stress of nutrient deficiency or photo-oxidative. The value increased to 45.7% of the dry weight.Figure 2.8 represents the lipid content in diatoms which is 22.7% of dry weight normally but increased to 37.8% under stress. Figure 2.10 represents the lipid content in oleaginous species which was found to be 27.1% of dry weight in normal conditions but increased to 44.6% in stress. From this figure it can be concluded that for more lipid amount in microalgae can be increased by inducing a stress condition which may double the oil content in some cases.

2.6.2 Carbohydrate Fraction

Carbohydrate content in microalgae has been reported to be as high as 50% dry matter. Mono-saccharides like glucose, fructose, mannose, and galactose, as well as polysaccharides like starch and cellulose, make up the majority of the carbohydrates secreted by microalgae. The glucose and starch extracted from microalgae are used to create biofuels like biohydrogen and bioethanol. Polysaccharides, on the other hand, primarily serve as part of structure and in storage molecules. Polysaccharides of microalgae have been shown to activate macrophage function, stimulate the synthesis of NO, active oxygen species, also numerous cytokines, and

hence modulate the immune system . Along with medicinal applications, carbohydrates from microalage are primarily used in the fermentation of bioethanol. For that purpose, microalgae are hydrolyzed with acids or bases to form monosaccharides during the process of saccharification , that is typically the rate-determining phase in bioethanol production.

Chemical or enzymatic methods are used to hydrolyze complicated polysaccharides molecules including cellulose and starch. Chemical hydrolysis, also known as acid-catalyzed hydrolysis, is faster and less expensive than enzymes; however, it produces a variety of remaining derivatives that may obstruct the later process of fermentation. Enzymatic hydrolysis, however, require relatively less amount of energy but is extremely selective, necessitating a large number of enzymes for successful hydrolytic reation. After saccharification, the monosaccharides are converted to ethanol by fermentation with the usage of yeast, bacteria, or fungus.

Several investigations on the synthesis of bioethanol using hydrolysis and microalgae fermentation have also been undertaken [92].

- Chlamydomonas reinhardtii can create intracellular ethanol. The culture was housed in a dark, anaerobic atmosphere. Although this method omitted the costly step of harvesting microalgae, the amount of ethanol obtained and rate of production were reduced as compared to the normal two-stepped process [80].

2.6.3 Protein Fraction

The protein content of microalgal biomass ranges between 40% and 70 percent, with the quality measurd by the amino acid conformation. The human body needs nine essential amino acids (EAA), that cannot be produced inside the body. Meat, dairy, eggs, pulses, and soybeans are all traditional protein sources. Although, when compared to conventional sources, microalgae has been described to be an above-per resource in terms of EAA conformation. Because it requires

less space while providing a better amount of protein than conventional sources of meat, it has the potential to meet the world's rising population's protein needs. Food items which are based upon microalgae need less than 2.5 square metres of area for every kilogramme of protein, according to a Life-Cycle Assessment (LCA) accompanied by de Vries et al., whereas ham, chick meat, and beef need 47–64, 42–52, also 144–258 square metres of area, respectively.

Furthermore, microalgae are able to be grown on non-cultivated land and can possibly use sewage water or marine water as an alternative of freshwater. Their raw sources requisites are less than those of proteins obtained from plants like Pisum sp. protein and Glycine max protein . Proteins are obtained from microalgae using a variety of techniques. The traditional extraction method used filtration or centrifugation to separate parts of cells from other dissolving complexes in the fluid part. The functional properties of extracted proteins were lost as a result of these processes. Despite this, the use of technique of extracting solvent preserves physical characteristics of proteins. After cell disruption, dissolving proteins are extracted using liquid-liquid extraction. Organic solvents containing surfactants are used to solubilize the proteins. Electrostatic forces among proteins and surfactants transport proteins from the aqueous to the organic phases. pH, salt content and type, and organic solvent type are the variables that control this process.The use of super-critical CO2 extraction to get proteins was attempted [93], which avoided the use of harmful solvents.

2.7 Issues and Future Prospects of Microalagal Biofuels

Although microalgae biofuel has a number of advantages and has the capability to take place of conventional petroleum fuels very soon, the commercialization of algae biofuel is being hampered by a number of obstacles.

1. A central challenge for microalgal bioenergy development is the reduction of production cost of microalgal cultivation. Excessive production costs are an

issue that extends from microalgae cultivation to microalgae harvesting. The primary and basic financing in constructing a pond and purchasing equipment, for example, is substantial. Because microalgae growth necessitates a high degree of cultivation parameters, such as CO2 and fertilizer, which provide microalgae with nutrients, the microalgae culture process's production cost will rise. Wet algae feed-stocks must also be dewatered prior to microalgae energy conversion. The cost of specific equipment is outrageously high. Algae harvesting, as previously said, is the highly costly step in the algae manufacturing procedure. The ability to minimize overall expenses is determined by the question of how to reduce harvesting costs [92].

2. Another significant challenge is the environmental impact of microalgae production, as well as waste treatment. Microalgae as a sustainable bioenergy feed-stock have environmental consequences. For example, the technology for reprocessing and using again the solid, liquid, and gas is not developed much in the pyrolysis process of microalgae biofuel. Because fertilizers must be given to the microalgae culturing pond, the culturing water contains nitrogen and phosphorus, which must be treated once the algae have been harvested. So far, there hasn't been a good way to recycle this waste water. The amount of waste water emitted if microalgae manufacturing were done on a large scale would have major environmental repercussions [90].

3. Other technical issues remain unsolved, such as microalgal seed choice, increasing microalgal oil content, and establishing an effective oil extracting process.

The two graphs below depict energy usage and manufacture in various states, as well as different energy production rate, from 2006 to 2030 2.11, 2.12:

In the foreseeable future, global liquid oil consumption will rise, notably in non-OECD Asia. Second, biofuel produced as a byproduct of liquid oil will account for

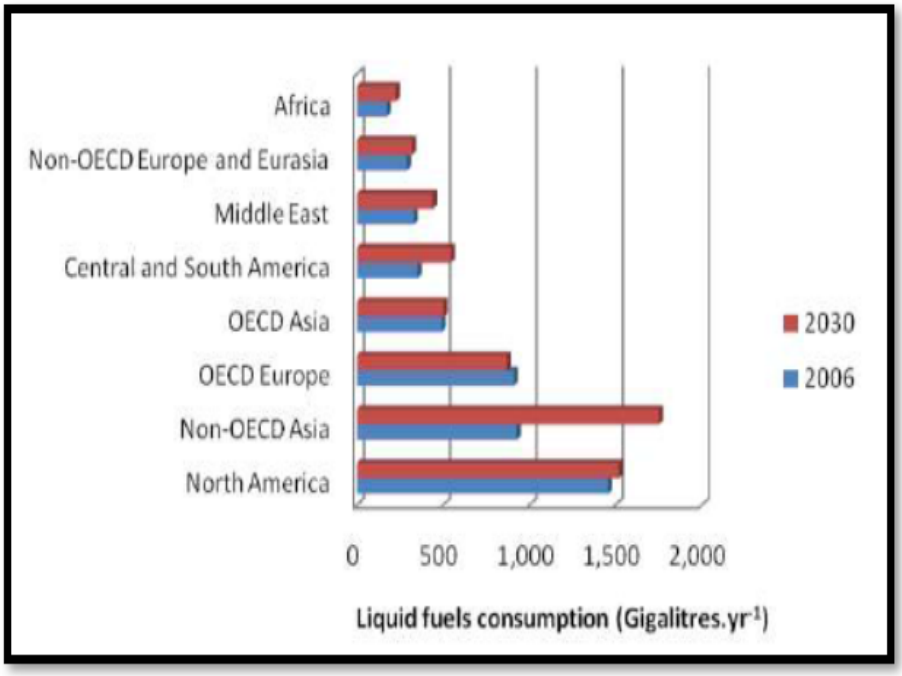

FIGURE 2.11: Liquid Fuel Consumption (Gigalitres/yr). Figure shows a graph of liquid fuel consumption of different regions in year 2006 and expected to be in year 2030.

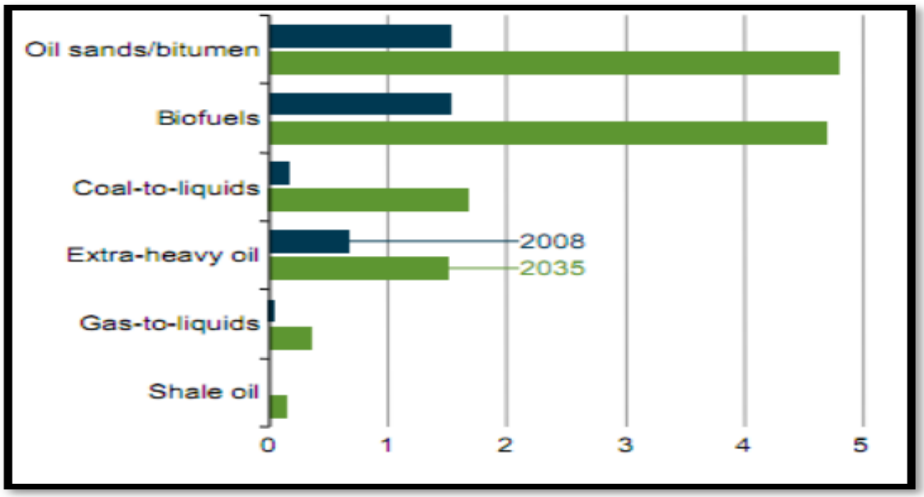

FIGURE 2.12: Different fuels consumption comparison of 2008 and expectation in 2035.

a significant fraction of overall liquid fuel production by the year 2035. Microalgae, as a third-generation biofuel, will offer more positive energy to the energy markets in the near future [94] [93].

Chapter 3

Materials and Methods

3.1 Methodology Flowchart

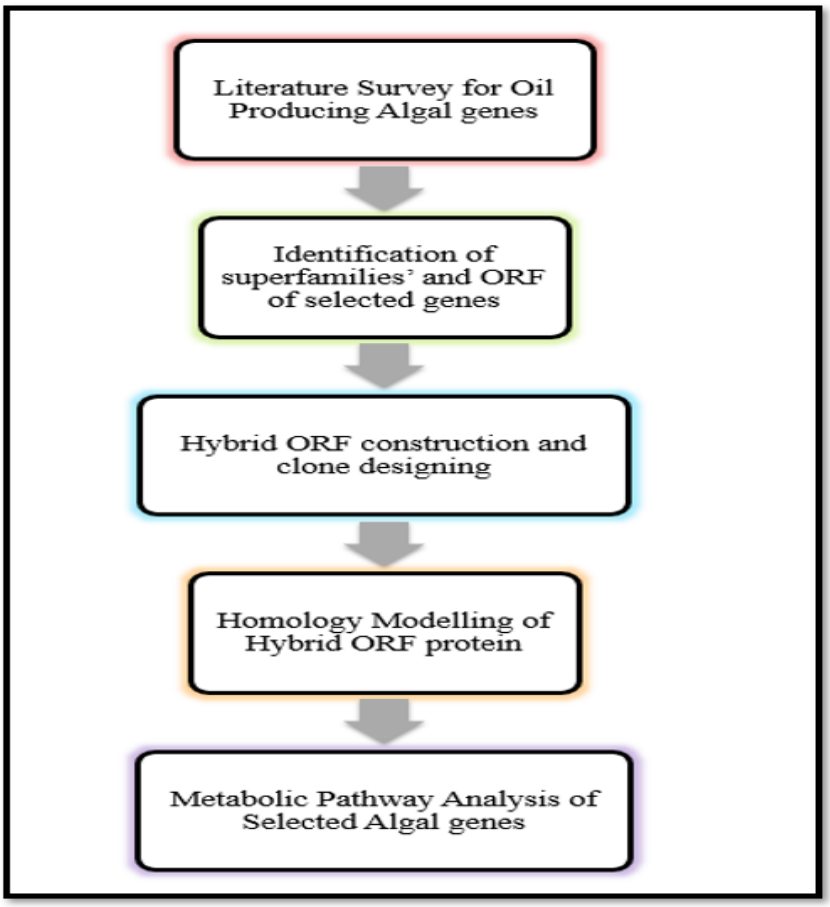

FIGURE 3.1: Methodology of Project

3.2 Literature Survey for Identification of Oil Producing Algal Species

Literature Survey was done for the identification of species of algae which can produce oils/lipids. Literature was searched from National Centre of Biotechnology Information (https://www.ncbi.nlm.nih.gov/) , PUBMED and Google Scholar by using Keywords: Biofuel, Algal Biofuel, and Microalgae as feedstock for biofuel, Lipid Content of Microalgae Species.

The articles related to these keywords were selected. Microalgal species which have high content of lipids were selected for further screening. Only 3 species were selected.

3.3 Identification of Microalgal Genes and Their Functional Protein

Microalgal species which were selected for study were further subjected to screening. And their oil producing genes were explored through literature on internet and NCBI database of nucleotide (https://www.ncbi.nlm.nih.gov/nuccore/?term=) and protein (https://www.ncbi.nlm.nih.gov/protein/) [94]

Overall 6 genes were selected from already chosen species of microalgae. Their Nucleotide sequences were retrieved in FASTA format from NCBI Nucleotide database. Then the functional protein information was collected from NCBI Protein database.

3.4 Identification of Superfamilies and ORFs of Selected Genes

Protein screening of selected genes was based on the superfamilies by using BLASTp tool. Proteins were further classified on the basis of superfamilies to which they

belong. Open Reading Frame (ORF) is that part of gene which has capability to get translated. It consists of codons.

- Which have Start codon and Stop codon in one complete stretch.

Proteins are usually formed from largest ORF of a gene.
The ORFs of selected genes were identified by using ORF finder tool which a graphical analysis tool is given by NCBI. The length of ORFs, conserved region sequence and position of Start and Stop codons was also retrieved [95].

3.5 Construction of Hybrid ORF by Using Conserved Regions of Superfamilies

The ORFs which were retrieved from ORF finder by using Accession number of selected genes. The largest ORFs of all genes were collected in nucleotide sequence format. After that by combining all the ORFs of selected genes, a hybrid ORF was constructed.

3.6 Restriction Enzyme Analysis of Hybrid ORF

Vector NTI® Express designer software was used for Restriction enzyme analysis of Hybrid ORF molecule [89]. This tool gives the visual representation of different restriction sites where different Restriction enzymes can cut the molecule. The restriction enzymes and the restriction sites would be helpful in constructing Hybrid ORF for expanded cloning.

3.7 Hybrid ORF Analysis

Analysis of Hybrid DNA molecule was done by Vector NTI® Express designer. In this software ORF finder is available for verification of Hybrid ORF.

3.8 Thermodynamic Analysis

Thermodynamic analysis of constructed Hybrid ORF was carried out byVector NTI® Express designer. This is used to check the stability of a molecule on various parameters [89].

3.9 Hybrid ORF Clone Designed Using Snap Gene Software

The Hybrid ORF of selected genes was used for designing clone by using Snapgene Software. In-fusion cloning is a versatile method for seamless fusion of desired gene or gene fragment with vector [100].

3.10 Protein Primary Structure Prediction

Hybrid ORF sequence was used to predict the protein primary structure. Expasy (https://web.expasy.org/translate/) is the SIB Bioinformatics resource site that gives approach to science related databanks and many scientific tools for several fields of biological science, one of which is the Translate tool, which converts a given nucleotide sequence into a Primary Protein Sequence [91].

3.11 Homology Modelling of Hybrid ORF Protein by SWISS-MODEL Tool on Expasy Portal

Homology modeling is also known as comparative modelling which means the construction of an atomic model of the aimed protein from the given amino

acid sequence and an experimental three-dimensional structure based on comparison to its homologous proteins which are used as template. SWISS-MODEL (https://swissmodel.expasy.org/) is a web-based service specified for homology modeling of target protein and structure prediction [95]. Constructing a homology model involves four major parts:

1. Firstly a template is identified,
2. Template is aligned with the target sequence,
3. Then model is built, and
4. Then quality evaluation of model is done [99].
5. 3-D structure of protein was obtained by SWISS-MODEL tool and then it was subjected for structure validation through Ramachandran plot by using PROCHECK website (https://saves.mbi.ucla.edu/) [94].
6. 3D structure of protein was also validated ERRAT another tool provided by PROCHECK [100].

3.12 Prediction of Protein Secondary Structure

Secondary structure of protein consists of Alpha helix, Beta sheets and coils. Secondary structure of Hybrid protein was predicted by using NPS webserver from Institute of Biology and Protein Chemistry (https://npsa-prabi.ibcp.fr/.) [92].

3.13 Pathway Analysis of Selected Algal Genes

Kyoto Encyclopedia of Genes and Genomes (KEGG) database contains collection of manual drawn pathways of interaction of molecules, relational network for metabolic reactions, genetic information processing, environmental information

processing, cellular processes, organismal systems, human diseases and drug development [94] . The metabolic pathways of the selected algal genes were studied by using KEGG database [96]. Pathway study was done to identify the function and molecular mechanism of selected genes [98].

Chapter 4

Results and Discussions

4.1 Literature Survey for Identification of Oil Producing Algal Species

Literature survey through NCBI, PubMed, Google scholar and other search engines was done for the identification of those species of Microalgae which are capable of producing oils. A number of species mentioned in Table 4.1 were found which have significant oil content in dry weight. Only 3 species i.e. *Chlorella Vulgaris, Chlamydomonas reinhardtii and Phaeodactylum tricornutum* were selected for further use.

TABLE 4.1: Extent of oil in selected species of Microalgae

Sr.No	Microalgal Species	Oil Content (%dry weight)
1	*Chlamydomonas reinhardtii*	25-80
2	*Chlorella vulgaris*	14-22
3	*Phaeodactylum tricornutum*	20-30

Table 4.1 shows the species and their oil content which were selected for the study. *Chlamydomonas reinhardtii* contains 25-80% of oil in its dry biomass, *Chlorella*

vulgaris contains 14-22% of oil in its dry biomass and *Phaeodactylum tricornutum* contains 20-30-% of oil in its dry biomass. These species were further used for identification of genes.

4.2 Identification of Algal Genes and their Functional Proteins

Oil yielding genes of selected species of Microalgae were identified by using NCBI genomic database. Total 6 genes were selected, 1 gene ACCD was reported in Chlorella vulgaris, 3 genes CHLRE-03g205050v5, CHLRE-02g143000v5 and CHLRE-06g273250v5 were reported in Chlamydomonas reinhardtii, two genes PHATRDRAFT-54926 and PHATRDRAFT-14125 were reported in Phaeodactylum tricornutum. The nucleotide sequences were retrieved in FASTA format for further use. Their functional protein information was collected from NCBI Protein database.

TABLE 4.2: Selected genes of Microalgae and their proteins

S.No	Organism	Gene	Protein	Accession
1	*C. vulgaris*	ACCD	acetyl-CoA	KC436295
2	*C. reinhardtii*	CHLRE06	Glycerol	XP00169
3	*C. reinhardtii*	CHLRE02	Glycerol	MG786474
4	*C. reinhardtii*	CHLRE03	Diacylglycerol	KC788202
5	*P. tricornutum*	PHATR	Monogalactosyldi	XM00218
6	*P. tricornutum*	ACC1	acetyl-CoA	XM00218

Table 4.2 shows the 6 selected genes and their proteins along with gene symbol and accession no. 1 gene ACCD was selected from Chlorella vulgaris which coded for Acetyl-CoA carboxylase carboxyltransferase. CHLRE-06g273250v5 from Chlamydomonas reinhardtii coded for Glycerol acyltransferase family protein, CHLRE-02g143000v5 from Chlamydomonas reinhardtii coded for glycerol 3-phosphate

acyltransferase and CHLRE-03g205050v5 from Chlamydomonas reinhardtii coded Diacylglycerol acyltransferase type 2. PHATRDRAFT-14125 from Phaeodactylum tricornutum coded for Monogalactosyldiacylglycerol synthase and *Phaeodactylum tricornutum* coded for Acetyl-coa carboxylase.

4.3 Identification of superfamilies and ORFs of Selected Genes

BLASTp tool was used for the identification of superfamilies of selected genes. The 6 proteins were further classified on the basis of superfamilies to which they belong, that were Crotonase-like superfamily, PLN02349 superfamily, PycA superfamily, LPLAT superfamily and PLN02605 superfamily. ORF is a single stretch of gene which starts with Start codon and ends with Stop codon. The ORF finder is a graphical assessment tool that was used to find the ORFs of selected genes. Both nucleotide and amino acid sequences were retrieved, along with the ORF length and position of Start/Stop codon.

4.4 Hybrid ORF Construction

ORF sequences of chosen genes which were obtained through ORF finder were used to construct a Hybrid ORF by the use of Vector NTI tool. The length of the constructed Hybrid ORF is 5322 bp.

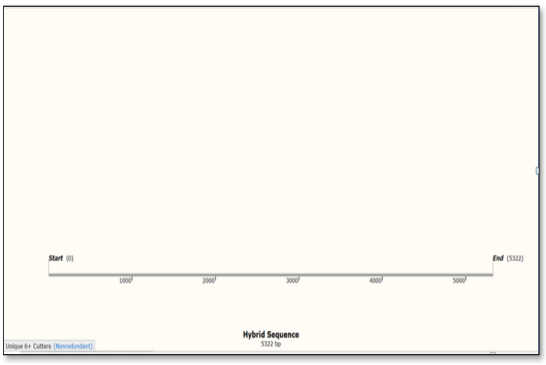

FIGURE 4.1: Restriction Enzyme Analysis of Hybrid ORF

4.5 Restriction Enzyme Analysis of Hybrid ORF

Restriction enzyme analysis of Hybrid ORF molecule was carried out by Vector NTI® Express designer software. This tool gives the visual representation of different restriction sites where different Restriction enzymes can cut the molecule. The restriction enzymes and restriction sites may be helpful while constructing Hybrid ORF for extended insilico or invitro experiments. The restriction sites were identified in Hybrid DNA sequence for many enzymes as shown in figure 4.2.

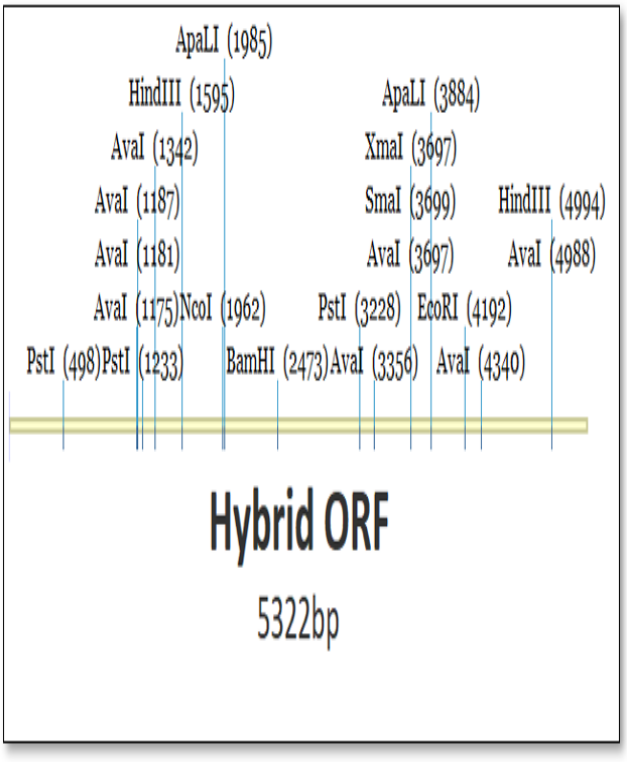

FIGURE 4.2: Restriction Enzyme Analysis of Hybrid ORF

Figure 4.2 shows that PSTI enzyme cuts Hybrid ORF sequence at 498, 1233 and 3288 position of nucleotide.. AvaI cuts at 1175, 1181, 1187, 1342, 3356, 3697 and 4988 position of nucleotide. HindIII cuts at 1595 and 4994 position of nucleotide. ApaLI cuts at 1985 and 3884 position of nucleotide. BamHI cuts at 2473 position of nucleotide. NcoI cuts at 1962 position of nucleotide. EcoRI cuts at 4192 position of nucleotide. XmaI cuts at 3697 position of nucleotide. SmaI cuts at 3699 position of nucleotide.

4.6 Analysis of Hybrid ORF Using ORF Finder

Vector NTI® Express designer was used for the analysis of Hybrid ORF sequence [96]. In this software ORF finder is available for verification of Hybrid ORF. This shows the results with directional arrows as shown in figure 4.3

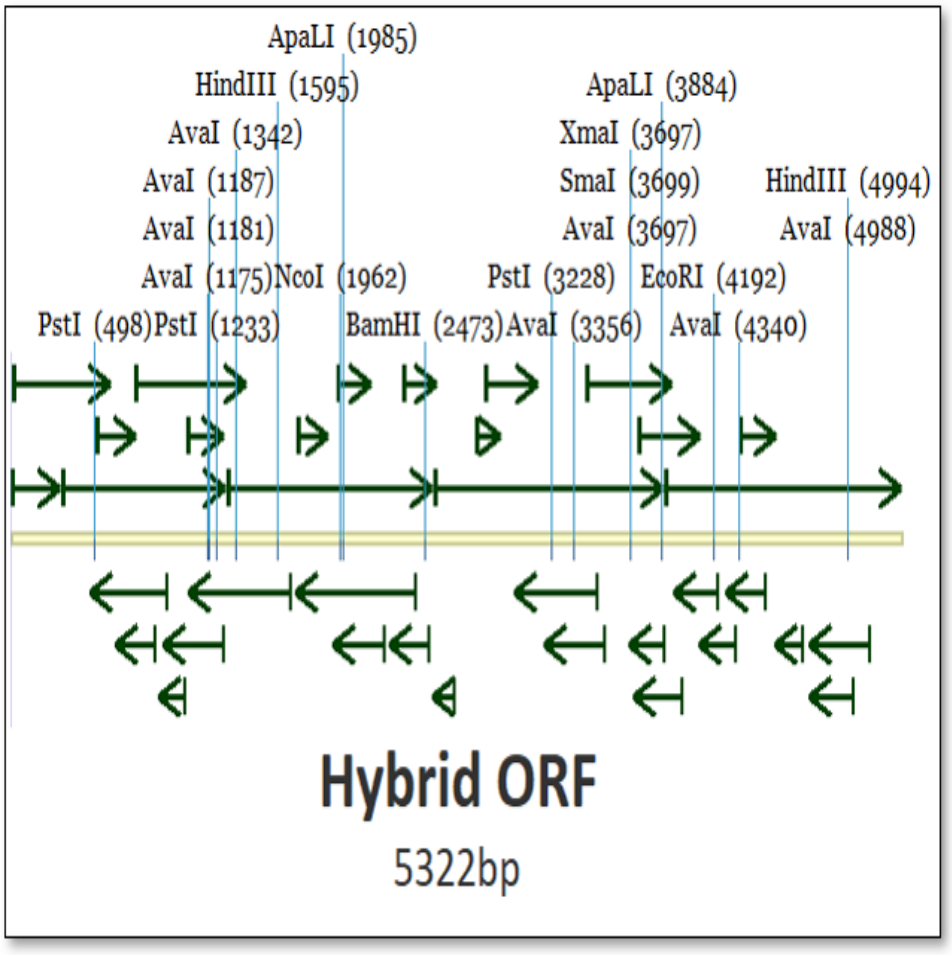

FIGURE 4.3: Verification of Hybrid ORF by ORF finder

Figure 4.3 shows the position of ORFs with directional arrows. ORFs of both forward and Reverese strands are shown in figure.

4.7 Thermodynamic Analysis of Hybrid ORF

Thermodynamic analysis of Hybrid ORF was done by Vector NTI® Express designer. This analysis was used for checking the stability of a molecule shown in

figure 4.4.

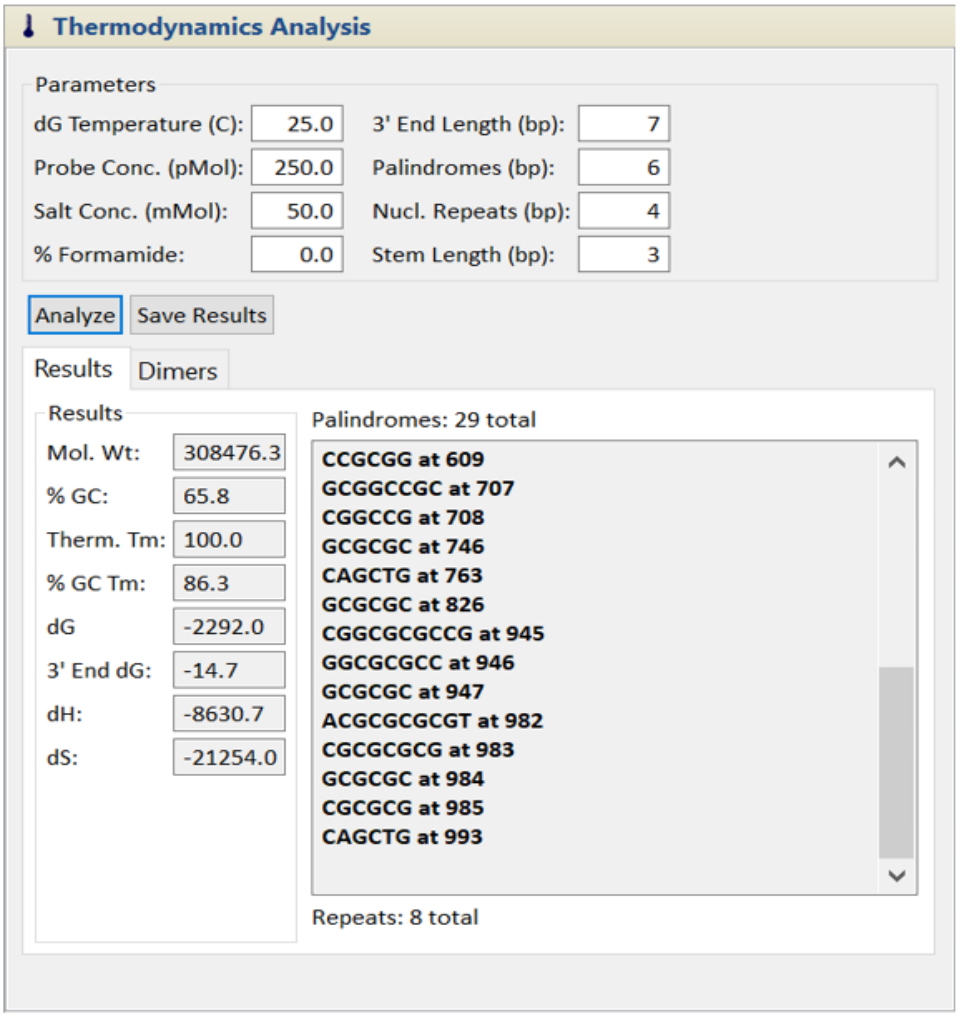

FIGURE 4.4: Thermodynamic Analysis of Hybrid ORF. Default parameters for analysis were dG Temperature (°C), Probe Concentration and Salt Concentration. Analysis showed Molecular weight, % GC, Therm. Temperature,% GC temperature.

Vector NTI® Express designer calculates two different melting temperatures i.e. Thermodynamic Temperature (Therm. Tm.) and %GC Tm for DNA/RNA oligonucleotides. The analysis was carried out at default parameters i.e. Probe Concentration was 250 pM and Salt concentration was 50 mM.

The GC content of designed hybrid ORF was 65.8% and %GC Tm was 86.3, which was higher. GC content greater than 60% is considered as good for gene design, protein expression and Prier designing in PCR. The GC content is important because it affects the stability of DNA due to strong hydrogen bonding of GC pair.

The GC value has also an effect upon the secondary structure of mRNA and annealing temperature of template DNA in PCR experiments.

TABLE 4.3: Thermodynamic analysis of Hybrid ORF sequence by Vector NTI Express designer

S.No.	Parameters of Thermodynamic Analysis	Results
1	dG Temperature(C)	25
2	Probe Conc. (pMol)	250
3	Salt Conc. (mMol)	50
4	% Formamide	0
5	Therm. Tm.	100
6	GC Content	63.60%
7	%GC Tm	85.4
8	Stem Length (bp)	3
9	Palindromes (bp)	6
10	3' End dG	-16.3
11	3' End Length (bp)	7
12	Nucl. Repeats (bp)	4
13	Mol. Wt.	309143.9
14	dH	-8492.1
15	dG	-2248.2
16	dS	-20935.9

This analysis was used for checking the stability of a molecule shown in Table 4.3

4.8 Clone Designing by Using SnapGene Software

The Hybrid ORF of selected genes was used for designing clone by using SnapGene software. Fragment in this case was Hybrid ORF shown in figure 4.5. The length of Hybrid ORF sequence was 6051 bp.

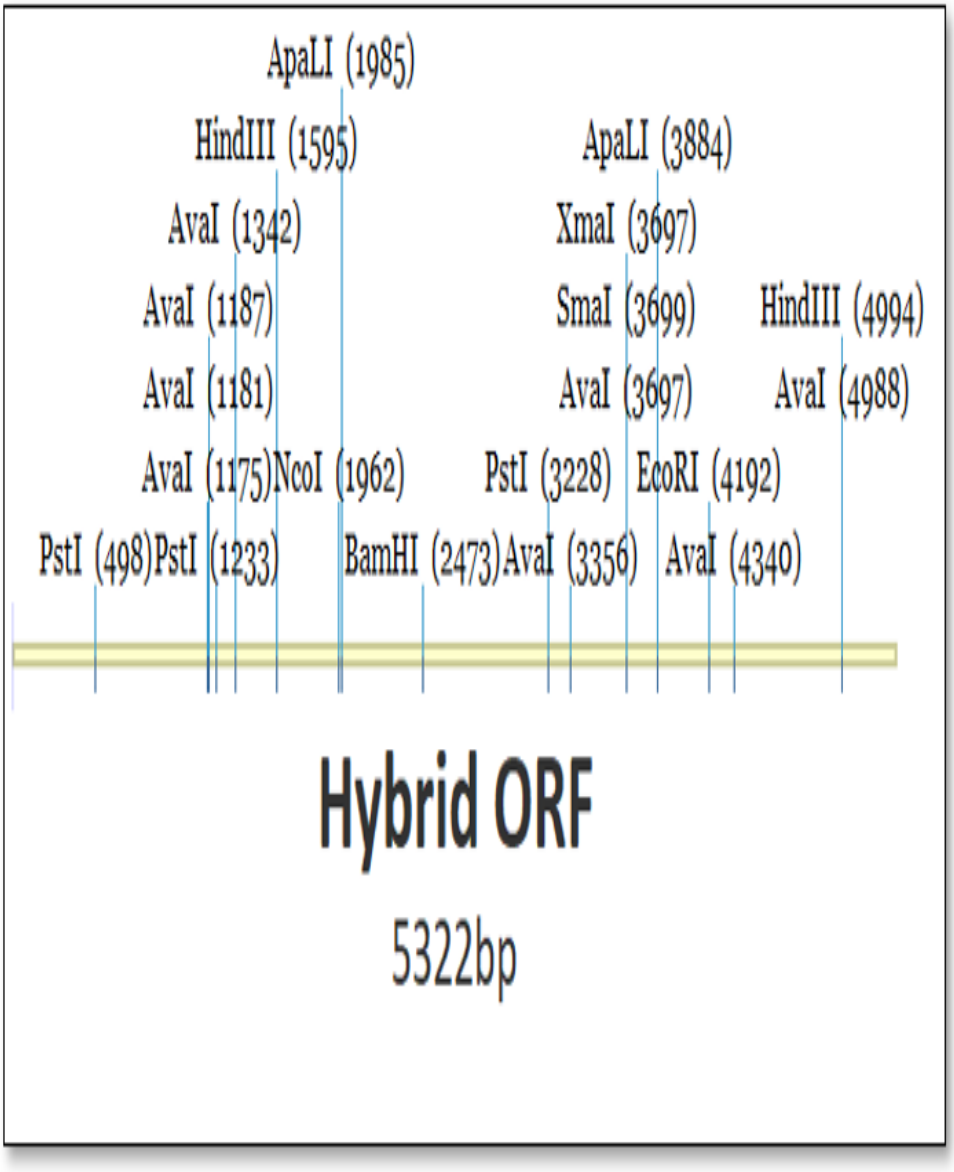

FIGURE 4.5: Hybrid ORF along with its Restriction Enzymes

The vector which was taken for in-fusion cloning was pET-24a(+) shown in figure 4.6.

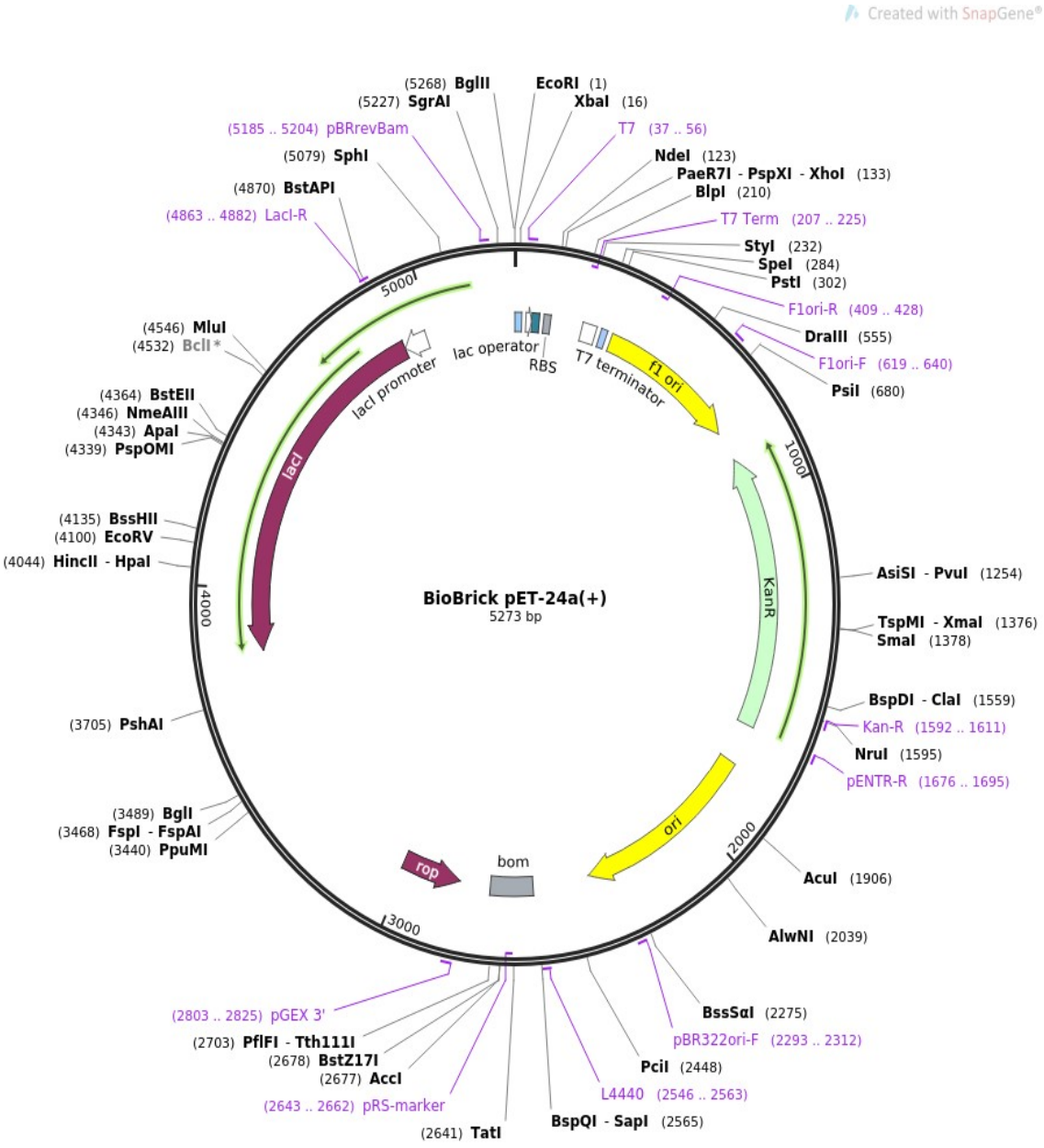

FIGURE 4.6: Cloning Vector pET-24a(+). The size of vector is 5273 bp.

In-fusion cloning method was used for designing clone of Hybrid ORF. In-fusion cloning is a versatile method for seamless fusion of desired gene or gene fragment with vector. For IN-fusion reaction, a linearized vector is mixed with desired DNA fragment that have overlapping ends. Firstly SnapGene adds suitable primers for both vector and fragment automatically, then amplify through PCR. After that the fragment is inserted into the linearized vector and snapGene showed the fused product. The fused Vector had 9593 bp size. Cloning procedure of Hybrid ORF in vector pET-24a(+) by In-fusion cloning method is shown in figure 4.7.

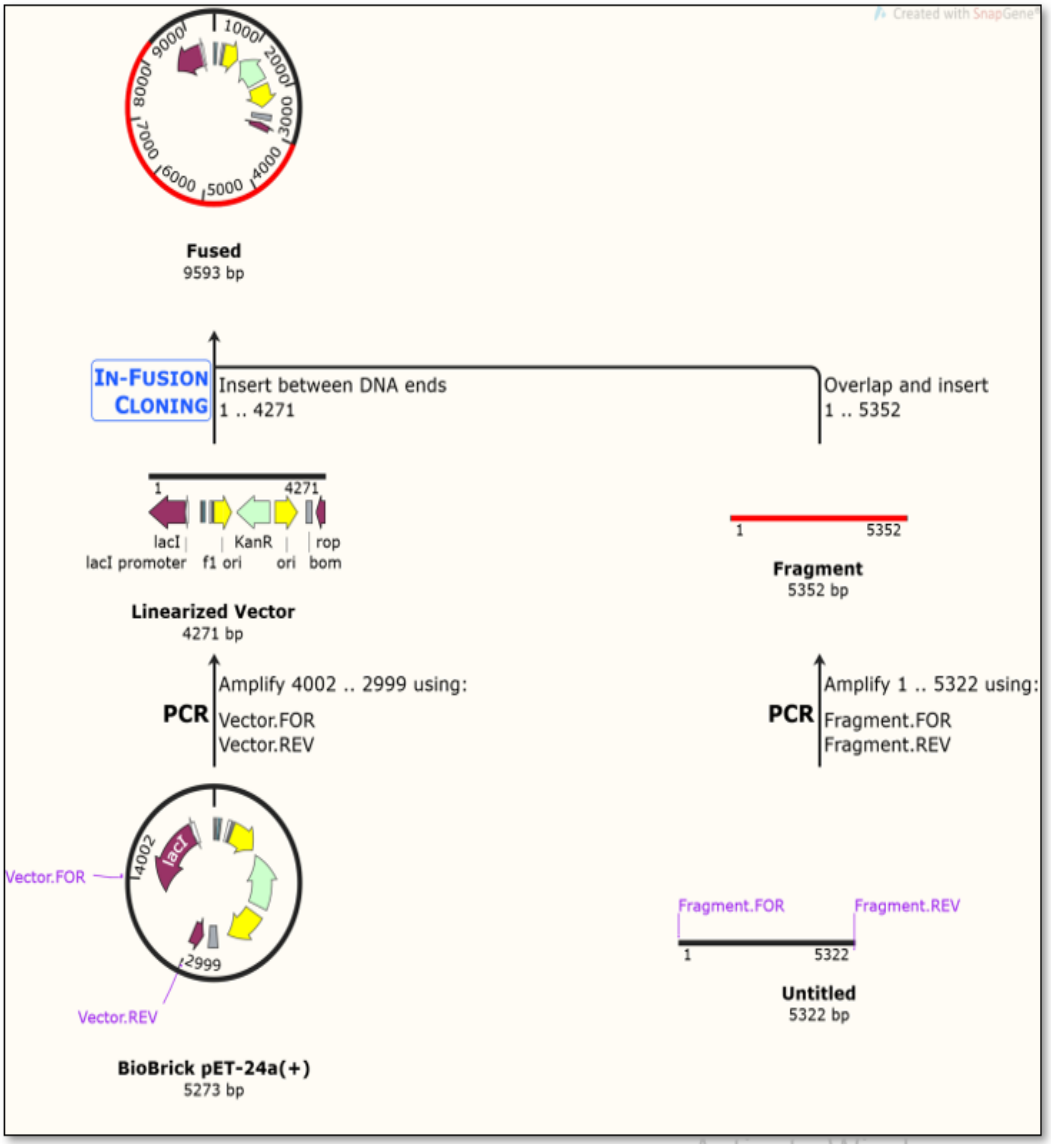

FIGURE 4.7: History of In-fusion Cloning of Hybrid ORF by Snapgene; First both vector and clone were modified by adding Primers and PCR. The Vector was linearized for easy inserting of fragment. Afterward the fragment was inserted into the Vector and fused. The fused vector had 10,376 bp size.

4.9 Protein Primary Structure Prediction

Expasy is a SIB Bioinformatics website which gives easy approach to science related data records and different scientific tools for various fields of biological science, one of which is Translate tool which translates given nucleotide sequence into Primary Protein Sequence. Hybrid ORF sequence was translated into Protein Sequence by using Translate tool of Expasy. The result showed 6 frames; 3

for 5'-3' and 3 for 3'-5'. The frame which had more Open Reading Frames was selected shown in figure 4.8.

FIGURE 4.8: Translation of Hybrid ORF sequence into Protein sequence

Translate tool of Expasy translates the query sequence of nucleotide into amino acid sequence. This will be further used for secondary and tertiary structure prediction.

4.10 Protein Secondary Structure Prediction

Secondary structure of protein is due the folding of polypeptide chain into different folds due the hydrogen bonding. Secondary structure contains two types of

structures which are based on the number of polypeptide chains. First alpha helix, which have only one polypeptide chain, second beta pleated sheets, which have two polypeptide chains. A high percentage of these two structures give stability to the structure of protein. Secondary structure of protein mainly consists of Alpha helices, Beta pleated sheets and coils/loops. Secondary structure of Hybrid protein was predicted by using NPS webserver from Institute of Biology and Protein Chemistry. (https://npsa-prabi.ibcp.fr/.). The input is in the form of amino acid sequence and results show the number and position of Alpha helix, beta-sheets and random coils which are presented in table 4.4.

TABLE 4.4: GOR3 results of Secondary structure prediction

S.No.	Structural elements	Number with percentage
1	Alpha helix (Hh)	814 is 46.01\%
5	Extended strand (Ee)	346 is 19.56\%
6	Beta turn (Tt)	0 is 0.00\%
7	Bend region (Ss)	O is 0.00\%
8	Random coil (Cc)	609 is 34.43\%
9	Ambiguous states (?)	0 is 0.00\%
10	Other states	0 is 0.00\%

Table 4.4 shows that the secondary structure of Hybrid ORF proteins contains 814 Alpha helices which is 46.01% of overall structure, 346 extended strands which is 19.56% and 609 other coils which is 34.43%. No ambiguous state was found in the structure.

4.11 Template-based Homology Modelling of Hybrid ORF Protein Using SWISS-MODEL Tool on Expasy portal

Homology modeling is also known as comparative modelling of protein which refers to Construction of an Atomic-Resolution model of the target protein from its amino acid sequence and an experimental three-dimensional structure based

on comparison to its homologous proteins which are used as template. SWISS-MODEL is a web-based integrated service dedicated to protein structure homology modelling. Building a homology model comprises four main steps:

1. identification of structural template(s),
2. alignment of target sequence and template structure(s),
3. model-building, and
4. model quality evaluation.

3-D structure of protein was obtained by SWISS-MODEL tool and then it was subjected for structure validation through Ramachandran plot. Input for SWISS-MODEL tool is amino acid sequence and it searches for templates which have resemblance to the query sequence. One template is selected for protein modelling. The protein model is shown in figure 4.9.

The template used for Protein modeling was 2wu9.1.A. The query sequence showed 68.54% sequence identity with the template, which is the amount of characters that exactly match between two sequences

FIGURE 4.9: Result of Protein Modelling by using SWISS-MODEL.

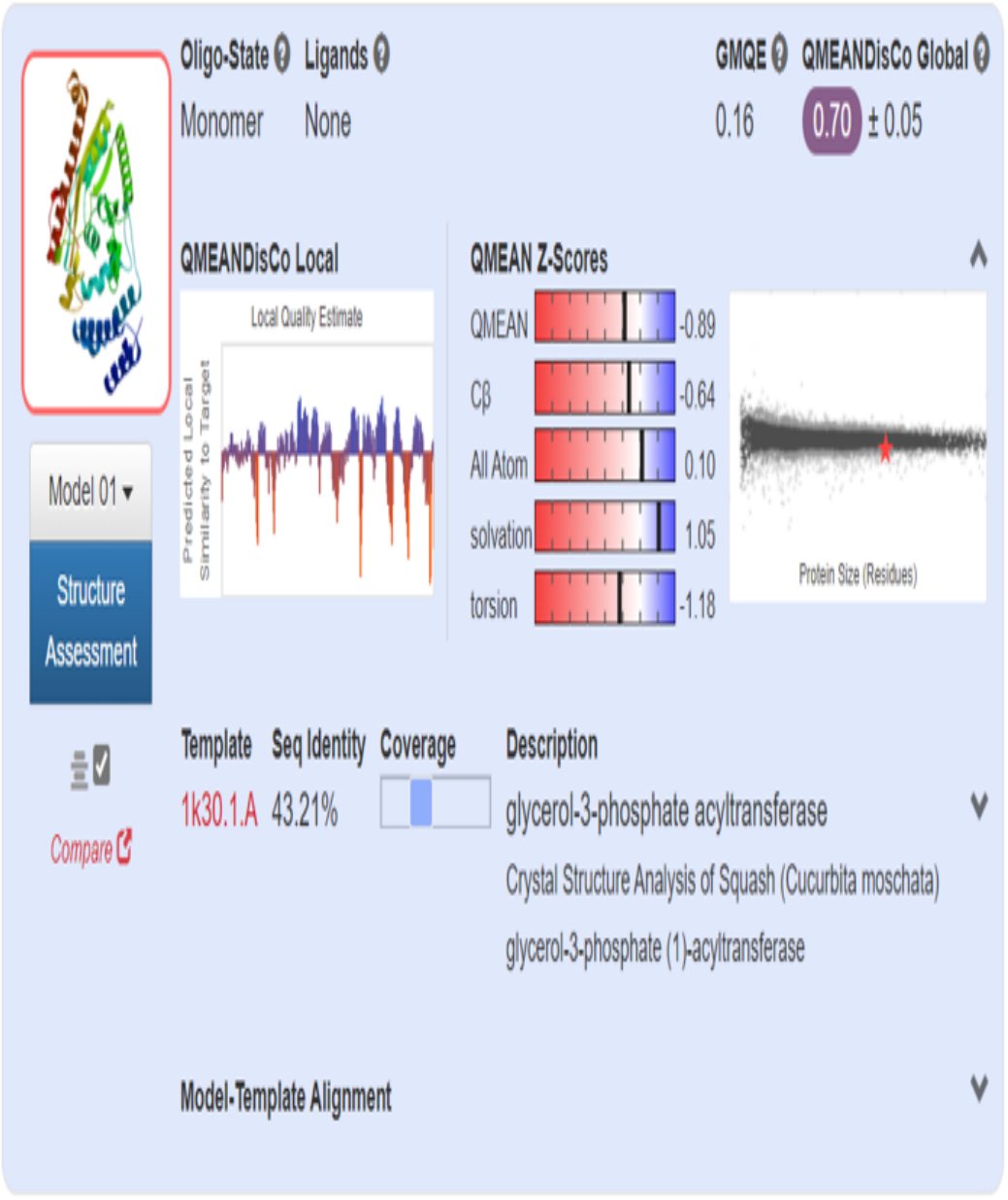

FIGURE 4.10: Result of Protein Modelling by SWISS-MODEL tool showing the template used for modeling was 2wu9.1.A, Identity is 68.54%, QMEANDisCo score is 0.80 which is ideal.

GMQE stands for Global Model Quality Estimate which is the quality estimation that combines properties by alignment of target to template. This score helps in selecting optimal template for modelling. This score must be between 0 and 1. For the protein under study GMQE score is 0.16.

QMEANDisCo global score is average per-residue score which has been correlated with IDDT score and error estimation is based on QMEANDisCo for large set of

models and represents the root mean squared difference between QMEANDisCo global score and IDDT. It must be near to zero. In this model the QMEANDisCo global score is 0.70 ± 0.05 which is good score. QMEAN Z-Score provides estimation of absolute quality of predicted model by relating to reference structures which were solved by X-ray crystallography. It is an estimate of the "degree of nativeness" of the structural features observed in a model in comparison to other experimental structures of high resolution. It must be around zero and ideal is ⩽-4.

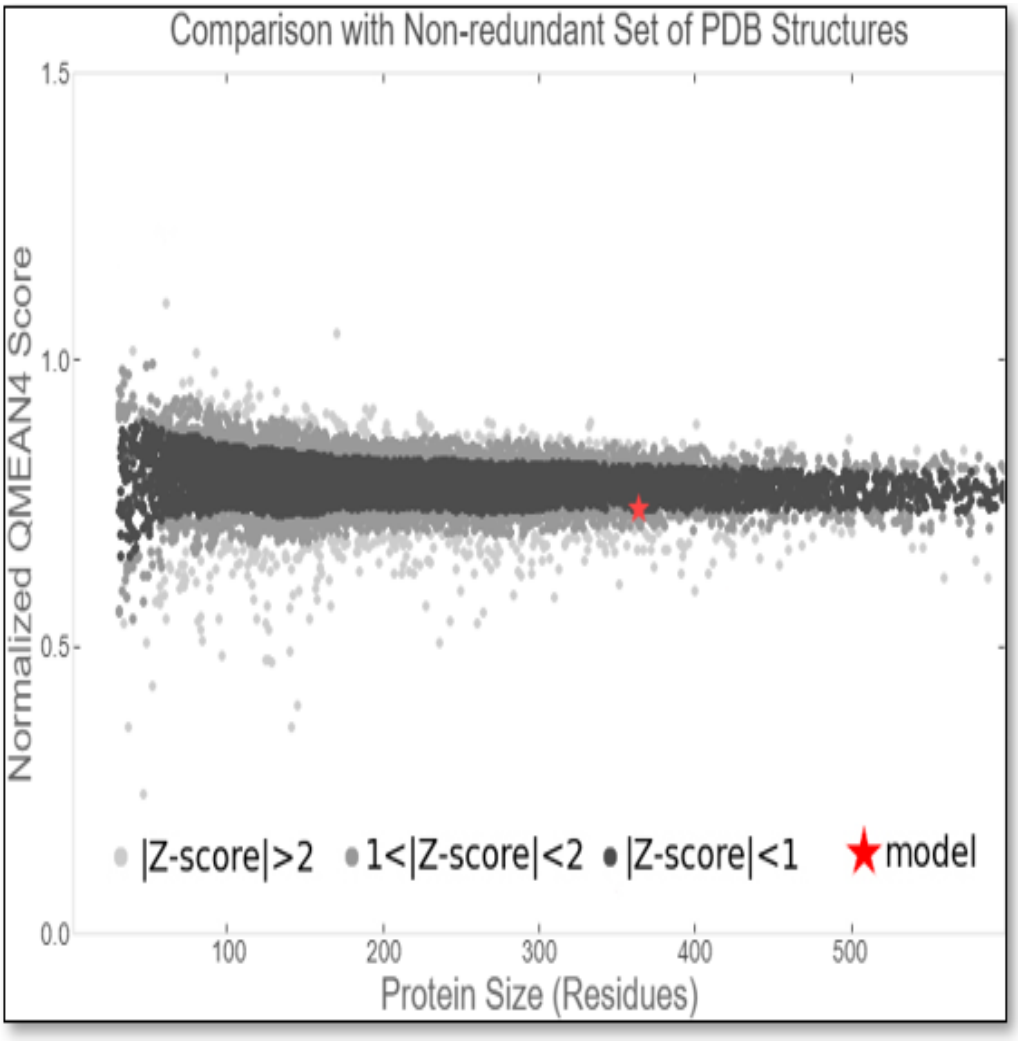

FIGURE 4.11: Comparison plot of QMEAN score and number of residues.

The quality comparison shows protein length (number of residues) on x-axis and QMEAN score on y-axis shown in figure 4.10. Every dot represents one experimental protein structure. Black dots show the experimental structure with QMEAN

score within 1 standard deviation of the mean (z-score between 0 and 1) and grey dots show the structures with z-score between 1 and 2. Light grey show those structures which are even further from mean. The actual model is shown as a red star.

4.12 Structure Assessment

The structure assessment was done through Ramachandran Plot.

4.12.1 Ramachandran Plot

A Ramachandran plot (also known as a Rama plot) is a method in biochemistry for visualizing energetically allowed regions for backbone dihedral angles phi against psi of amino acid residues in a protein structure. It was developed in 1963 by G. N. Ramachandran, C. Ramakrishnan, and V. Sasisekharan. PROCHECK is a suite of programs for analysis of stereochemical properties of a protein structure. Ramachandran plot was obtained by using PROCHECK suite as shown in figure 4.12. It shows 91.8% residues in highly favorable region, 7.5% in added allow region, 0.6% in generously allowed region and no in disallowed region.

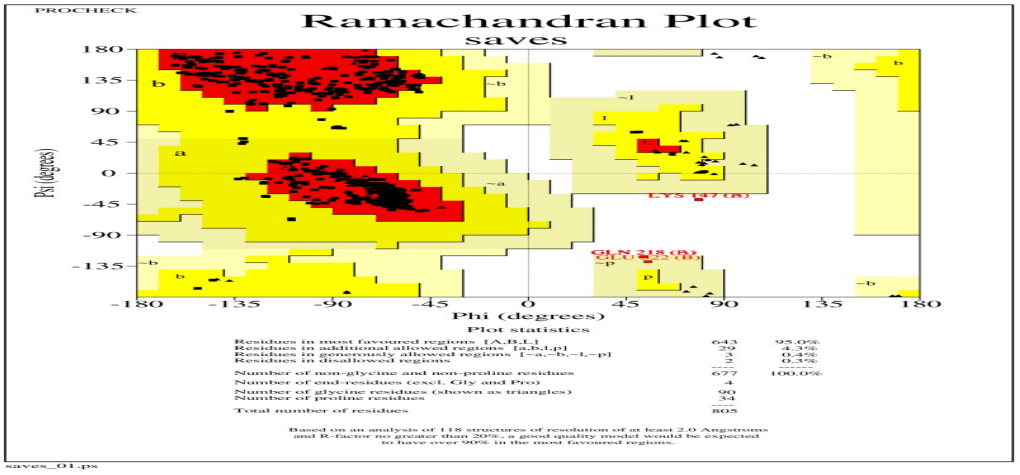

FIGURE 4.12: Ramachandran Plot of modelled protein shows that 95.0% residues are in most favoured regions, 4.3% in extra allowed region, 0.4% in generously allowed region and only 0.3% residues in disallowed regions

4.13 Metabolic Pathway Analysis of Selected Algal Genes Used for Designing Hybrid ORF

KEGG PATHWAY Database is an array of manually illustrated pathways of molecular interaction, reaction and relation networks for metabolism, genetic information processing, environmental information processing, cellular processes, organismal systems, human diseases and drug development. KEGG can be utilized in bioinformatics studies for analysis of data in genomic, metagenomics, metabolomics and other omic studies and in system biology. The metabolic pathways of the target microalgal genes were analyzed by the use of KEGG database. Pathways were studied for identification of function and molecular actions of targeted genes. All the genes were found to be involved in pathways which were leading to synthesis of fatty acids that produces lipids in microalgae.

TABLE 4.5: Metabolic pathways of selected genes of microalgae involved in lipid production.

S. No.	**Microalagal Species**	**Gene**	**Protein**
1.	*C. vulgaris*	ACCD	acetyl-CoA
2.	*C. reinhardtii*	CHLRE	Glycerol
3.	*C. reinhardtii*	CHLRE	Glycerol 3-phosphate
4.	*C. reinhardtii*	CHLRE	Diacylglycerol
5.	*P. tricornutum*	PHATR	Monogalactosyldi
6.	*P. tricornutum*	ACC1	acetyl-CoA

TABLE 4.6: Metabolic pathways of selected genes of microalgae involved in lipid production

S. No.	**Microalagal Species**	**Gene**	**Function of protein**
1	*C. vulgaris*	ACCD	Converts to Malonyl-CoA
2	*C. reinhardtii*	CHLRE_06	Synthesis of Triacylglycerol
3	*C. reinhardtii*	CHLRE_02	Synthesis of Triacylglycerol

Table 4.6 continued from previous page

S. No.	Microalagal Species	Gene	Function of protein
4	*C. reinhardtii*	CHLRE_03	Synthesis of Triacylglycerol
5	*P. tricornutum*	PHATR	Synthesis of Triacylglycerol
6	*P. tricornutum*	ACC1	Fatty acid and biosynthesis

TABLE 4.7: Metabolic pathways of selected genes of microalgae involved in lipid production with KEGG Id

S. No.	Microalagal Species	Gene	KEGG Id
1	*C. vulgaris*	ACCD	cre0061
2	*C. reinhardtii*	CHLRE_06	cre00561
3	*C. reinhardtii*	CHLRE_02	cre00561
4	*C. reinhardtii*	CHLRE_03	cre00561
5	*P. tricornutum*	PHATR	pti00561
6	*P. tricornutum*	ACC1	pti0061

TABLE 4.8: Metabolic pathways of selected genes of microalgae involved in lipid production with the pathway

S. No.	Microalagal Species	Gene	Pathway
1	*C. vulgaris*	ACCD	Fatty acid biosynthesis
2	*C. reinhardtii*	CHLRE_06	Pyruvate metabolism
3	*C. reinhardtii*	CHLRE_02	Unsaturated fatty acid
4	*C. reinhardtii*	CHLRE_03	Fatty acid and biosynthesis
5	*P. tricornutum*	PHATR	Glycerolipid metabolism
6	*P. tricornutum*	ACC1	Fatty acid and biosynthesis

Table 4.5,4.6,4.7 and 4.8 is presenting the pathways identified along with mechanism. ACCD gene is involved in fatty acid biosynthesis and converts acetyl-CoA to malonyl-CoA, CHLRE-06g273250v5 is involved in synthesis of Triacylglycerol, CHLRE-02g143000v5 is involved in synthesis of Triacylglycerol, CHLRE-03g205050v5/ CHLREDRAFT-190539 is involved in synthesis of Triacylglycerol, PHATRDRAFT-14125 is involved in glycerolipid metabolism and ACC1, PHATRDRAFT -54926 is involved in fatty acid biosynthesis shown in above tables.

Chapter 5

Conclusions and Recommendations

The motive of the present research was to design a Hybrid ORF protein which may produce more oil content in microalgae in order to produce more. This study would play a potential role while designing genetically engineered microalgae, and it will also enhance the ability to produce more amount of biofuel. The first objective of this study was to explore the oil producing genes of microalgae through literature survey. 6 genes from 3 species of microalgae were identified. Their conserved regions were identified and protein information was obtained.

The second objective of this study was to construct Hybrid ORF protein for obtaining more oil. Conserved regions of targeted proteins were used to construct Hybrid ORF sequence which was then translated into protein sequence. The protein structure of the target protein was modelled and it was verified by using Ramachandran plot which showed that 95% residues were in most favored region. The stability of the designed Hybrid ORF protein was also checked by Thermodynamic analysis which showed that GC content is 63.6%. This value shows the stability of constructed Hybrid protein.

The third objective of the study was the analysis of selected genes for their metabolic pathways to confirm that all the proteins were involved in synthesis of lipid. The metabolic pathway analysis also verified that all the genes used

in designing Hybrid are particularly involved in those pathways which synthesize lipids.

These results validate the designing of hybrid ORF protein, which would have the capability to be used as an efficient tool for designing a genetically engineered organism which will have the characteristic to produce high cellular oil content in microalgae. This study would provide a great assistance while working for genetically modified microalgae, in invitro studies.

5.0.1 Future Prospects

- This study can be used as base for wet laboratory experiments to design more genetically modified microorganisms.
- As no invitro experiment was carried out during present study, so further invitro studies are highly encouraged to verify the constructed Hybrid ORF protein.
- Variety of genes of other microalgae would be another chance to enhance the oil content in microalgae.
- Similar protocol can be used to construct Hybrid ORF protein for more carbohydrates and other metabolites which are useful in pharmaceutical industry.

Bibliography

[1] M. Cobos et al., "Isolation and Characterization of Native Microalgae from the Peruvian Amazon with Potential for Biodiesel Production," *Energies*, vol. 10, p. 224, Feb. 2017, doi: 10.3390/en10020224.

[2] J. Liu, Y. Song, and W. Qiu, "Oleaginous microalgae Nannochloropsis as a new model for biofuel production: Review andanalysis," *Renew. Sustain. Energy Rev.*, vol. 72, pp. 154–162, 2017, doi: https://doi.org/10.1016/j.rser.2016.12.120.

[3] Y. Su, K. Song, P. Zhang, Y. Su, J. Cheng, and X. Chen, "Progress of microalgae biofuel's commercialization," *Renew. Sustain. Energy Rev.*, vol. 74, pp. 402–411, 2017, doi: https://doi.org/10.1016/j.rser.2016.12.078.

[4] B. Bharathiraja et al., "Aquatic biomass (algae) as a future feed stock for bio-refineries: A review on cultivation, processing and products," *Renew. Sustain. Energy Rev.*, vol. 47, pp. 634–653, 2015, doi: https://doi.org/10.1016/j.rser.2015.03.047. [5]

[5] F. Cherubini and A. H. Strømman, "Life cycle assessment of bioenergy systems: State of the art and future challenges," *Bioresour. Technol.*, vol. 102, no. 2, pp. 437–451, 2011, doi: https://doi.org/10.1016/j.biortech.2010.08.010.

[6] Y. C. Sharma and V. Singh, "Microalgal biodiesel: A possible solution for India's energy security," *Renew. Sustain. Energy Rev.*, vol. 67, pp. 72–88, 2017, doi: https://doi.org/10.1016/j.rser.2016.08.031.

[7] P. L. Show, M. S. Y. Tang, D. Nagarajan, T. C. Ling, C.-W. Ooi, and J.-S. Chang, "A Holistic Approach to Managing Microalgae for Biofuel Applications," *Int. J. Mol. Sci.*, vol. 18, no. 1, p. 215, Jan. 2017, doi: 10.3390/ijms18010215.

[8] S. P. Singh and D. Singh, "Biodiesel production through the use of different sources and characterization of oils and their esters as the substitute of diesel: A review," *Renew. Sustain.* Energy Rev., vol. 14, no. 1, pp. 200–216, 2010, doi: https://doi.org/10.1016/j.rser.2009.07.017.

[9] P. D. Álvarez-Díaz, J. Ruiz, Z. Arbib, J. Barragán, M. C. Garrido-Pérez, and J. A. Perales, "Freshwater microalgae selection for simultaneous wastewater nutrient removal and lipid production," *Algal Res.*, vol. 24, pp. 477–485, 2017, doi: https://doi.org/10.1016/j.algal.2017.02.006.

[10] A. Aslam, S. R. Thomas-Hall, T. A. Mughal, and P. M. Schenk, "Selection and adaptation of microalgae to growth in 100% unfiltered coal-fired flue gas," *Bioresour. Technol.*, vol. 233, pp. 271–283, 2017, doi: https://doi.org/10.1016/j.biortech.2017.02.111.

[11] A. Abid, F. Saidane, and M. Hamdi, "Feasibility of carbon dioxide sequestration by Spongiochloris sp microalgae during petroleum wastewater treatment in airlift bioreactor," *Bioresour. Technol.*, vol. 234, pp. 297–302, 2017, doi: https://doi.org/10.1016/j.biortech.2017.03.041.

[12] J. de S. Castro, M. L. Calijuri, P. P. Assemany, P. R. Cecon, I. R. de Assis, and V. J. Ribeiro, "Microalgae biofilm in soil: Greenhouse gas emissions, ammonia volatilization and plant growth," *Sci. Total Environ.*, vol. 574, pp. 1640–1648, 2017, doi: https://doi.org/10.1016/j.scitotenv.2016.08.205.

[13] M. Veillette, A. Giroir-Fendler, N. Faucheux, and M. Heitz, "Esterification of free fatty acids with methanol to biodiesel using heterogeneous catalysts: From model acid oil to microalgae lipids," *Chem. Eng. J.*, vol. 308, pp. 101–109, 2017, doi: https://doi.org/10.1016/j.cej.2016.07.061.

[14] S. Sahay and V. Braganza, "Microalgae-based biodiesel production: Current and future scenario," . *Exp. Sci.*, vol. 7, pp. 31–35, 2016.

[15] L. Brennan and P. Owende, "Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products," *Renew. Sustain. Energy Rev.*, vol. 14, no. 2, pp. 557–577, 2010, doi: https://doi.org/10.1016/j.rser.2009.10.009.

[16] C.-M. Kuo et al., "Simultaneous microalgal biomass production and CO2 fixation by cultivating Chlorella sp. GD with aquaculture wastewater and boiler flue gas," *Bioresour. Technol.*, vol. 221, pp. 241–250, 2016, doi: https://doi.org/10.1016/j.biortech.2016.09.014.

[17] T. Shirvani, "The environmental feasibility of algae biodiesel production," *Appl. Petrochemical Res.*, vol. 2, no. 3, pp. 93–95, 2012, doi: 10.1007/s13203-012-0015-5.

[18] Y. Chisti, "Constraints to commercialization of algal fuels," *J. Biotechnol* ., vol. 167, no. 3, pp. 201–214, 2013, doi: https://doi.org/10.1016/j.jbiotec.2013.07.020.

[19] M. Saber, B. Nakhshiniev, and K. Yoshikawa, "A review of production and upgrading of algal bio-oil," *Renew. Sustain. Energy Rev.*, vol. 58, pp. 918–930, 2016, doi: 10.1016/j.rser.2015.12.342.

[20] R. Luque et al., "Biofuels: A Technological Perspective," *Energy Environ. Sci.*, vol. 1, pp. 542–564, Nov. 2008, doi: 10.1039/B807094F.

[21] L. D. Zhu, E. Hiltunen, E. Antila, J. J. Zhong, Z. H. Yuan, and Z. M. Wang, "Microalgal biofuels: Flexible bioenergies for sustainable development," *Renew. Sustain.* Energy Rev., vol. 30, pp. 1035–1046, 2014, doi: https://doi.org/10.1016/j.rser.2013.11.003.

[22] P. K. Naraharisetti, P. Das, and P. N. Sharratt, "Critical factors in energy generation from microalgae," *Energy*, vol. 120, pp. 138–152, 2017, doi: https://doi.org/10.1016/j.energy.2016.12.117.

[23] Y. Chisti, "Biodiesel from microalgae," *Biotechnol. Adv.*, vol. 25, no. 3, pp. 294–306, 2007, doi: 10.1016/j.biotechadv.2007.02.001.

[24] P. D. Patil, H. Reddy, T. Muppaneni, and S. Deng, "Biodiesel fuel production from algal lipids using supercritical methyl acetate (glycerin-free) technology," *Fuel*, vol. 195, pp. 201–207, 2017, doi: https://doi.org/10.1016/j.fuel.2016.12.060.

[25] T. M. Mata, A. A. Martins, and N. S. Caetano, "Microalgae for biodiesel production and other applications: A review," Renew. Sustain. *Energy Rev.*, vol. 14, no. 1, pp. 217–232, 2010, doi: https://doi.org/10.1016/j.rser.2009.07.020.

[26] N. I. Chernova and S. V Kiseleva, "Microalgae biofuels: Induction of lipid synthesis for biodiesel production and biomass residues into hydrogen conversion," *Int. J. Hydrogen Energy*, vol. 42, no. 5, pp. 2861–2867, 2017, doi: https://doi.org/10.1016/j.ijhydene.2016.05.302.

[27] Z. L. Ogburn and F. Vogt, "Microalgae as embedded environmental monitors," *Anal. Chim. Acta*, vol. 954, pp. 1–13, 2017, doi: https://doi.org/10.1016/j.aca.2016.11.058.

[28] T. Suganya, M. Varman, H. H. Masjuki, and S. Renganathan, "Macroalgae and microalgae as a potential source for commercial applications along with biofuels production: A biorefinery approach," *Renew. Sustain.* Energy Rev., vol. 55, pp. 909–941, Mar. 2016, doi: 10.1016/j.rser.2015.11.026.

[29] S. A. Razzak, M. M. Hossain, R. A. Lucky, A. S. Bassi, and H. de Lasa, "Integrated CO2 capture, wastewater treatment and biofuel production by microalgae culturing—A review," *Renew. Sustain. Energy Rev.*, vol. 27, pp. 622–653, 2013, doi: https://doi.org/10.1016/j.rser.2013.05.063.

[30] A. E.-F. Abomohra, M. El-Sheekh, and D. Hanelt, "Screening of marine microalgae isolated from the hypersaline Bardawil lagoon for biodiesel feedstock," *Renew. Energy*, vol. 101, pp. 1266–1272, 2017, doi: https://doi.org/10.1016/j.renene.2016.10.015.

[31] M. Ali and I. A. Watson, "Microwave treatment of wet algal paste for enhanced solvent extraction of lipids for biodiesel production," Renew. Energy, vol. 76, pp. 470–477, 2015, doi: https://doi.org/10.1016/j.renene.2014.11.024.

[32] I. Moreno-Garrido, "Microalgae immobilization: Current techniques and uses," *Bioresour. Technol.*, vol. 99, no. 10, pp. 3949–3964, 2008, doi: 10.1016/j.biortech.2007.05.040.

[33] A. Demirbas, "Use of algae as biofuel sources," *Energy Convers. Manag.*, vol. 51, no. 12, pp. 2738–2749, 2010, doi: https://doi.org/10.1016/j.enconman.2010.06.010.

[34] A. Richmond, "Biological Principles of Mass Cultivation," Handbook of Microalgal Culture. pp. 125–177, Nov. 25, 2003, doi: https://doi.org/10.1002/9780470995280.ch8.

[35] M. D. Guiry, "C. VAN DEN HOEK, D. G. MANN and H. M. JAHNS. Algae. An Introduction to Phycology. Cambridge University Press, Cambridge. 1995, pp. xiv+623. ISBN: 0 521 30419 9 (hardback); 0 051 31687 1 (paperback). Price: £70.00 (hard); £24.95 (soft).," *Eur. J. Phycol.*, vol. 32, no. 2, pp. 203–205, 1997, doi: DOI: undefined.

[36] X. Zhang, J. Rong, H. Chen, C. He, and Q. Wang, "Current Status and Outlook in the Application of Microalgae in Biodiesel Production and Environmental Protection ," *Frontiers in Energy Research* , vol. 2. p. 32, 2014, [Online]. Available: https://www.frontiersin.org/article/10.3389/fenrg.2014.00032.

[37] S. Varfolomeev and L. Wasserman, "Microalgae as Source of Biofuel, Food, Fodder, and Medicines," *Appl. Biochem. Microbiol.*, vol. 47, Dec. 2011, doi: 10.1134/S0003683811090079.

[38] S. P. Cuellar-Bermudez, J. S. Garcia-Perez, B. E. Rittmann, and R. Parra-Saldivar, "Photosynthetic bioenergy utilizing CO2: an approach on flue

gases utilization for third generation biofuels," *J. Clean. Prod.*, vol. 98, pp. 53–65, 2015, doi: https://doi.org/10.1016/j.jclepro.2014.03.034.

[39] R. Che et al., "Effect of fulvic acid induction on the physiology, metabolism, and lipid biosynthesis-related gene transcription of Monoraphidium sp. FXY-10," *Bioresour. Technol.*, vol. 227, pp. 324–334, 2017, doi: https://doi.org/10.1016/j.biortech.2016.12.017.

[40] S. Wahidin, A. Idris, and S. R. M. Shaleh, "Rapid biodiesel production using wet microalgae via microwave irradiation," *Energy Convers. Manag.*, vol. 84, pp. 227–233, 2014, doi: https://doi.org/10.1016/j.enconman.2014.04.034.

[41] Q. Hu et al., "Microalgal triacylglycerols as feedstocks for biofuel production: Perspectives and advances," *Plant Journal*, vol. 54, no. 4. pp. 621–639, 2008, doi: 10.1111/j.1365-313X.2008.03492.x.

[42] P. T. Vasudevan and M. Briggs, "Biodiesel production—current state of the art and challenges," J. Ind. Microbiol. Biotechnol., vol. 35, no. 5, p. 421, 2008, doi: 10.1007/s10295-008-0312-2.

[43] G. Knothe and L. F. Razon, "Biodiesel fuels," *Prog. Energy Combust. Sci.*, vol. 58, pp. 36–59, 2017, doi: https://doi.org/10.1016/j.pecs.2016.08.001.

[44] H. Pereira et al., "Isolation of a euryhaline microalgal strain, Tetraselmis sp. CTP4, as a robust feedstock for biodiesel production," *Sci. Rep.*, vol. 6, no. 1, p. 35663, 2016, doi: 10.1038/srep35663.

[45] R. A. I. Abou-Shanab et al., "Cultivation of a new microalga, Micractinium reisseri, in municipal wastewater for nutrient removal, biomass, lipid, and fatty acid production," Biotechnol. *Bioprocess Eng.*, vol. 19, no. 3, pp. 510–518, 2014, doi: 10.1007/s12257-013-0485-z.

[46] R. Piloto-Rodríguez, Y. Sánchez-Borroto, E. A. Melo-Espinosa, and S. Verhelst, "Assessment of diesel engine performance when fueled with biodiesel from algae and microalgae: An overview," *Renew. Sustain. Energy Rev.*, vol. 69, Mar. 2017, doi: 10.1016/j.rser.2016.11.015.

[47] M. N. Nabi, M. S. Akhter, and M. M. Zaglul Shahadat, "Improvement of engine emissions with conventional diesel fuel and diesel–biodiesel blends," *Bioresour. Technol.*, vol. 97, no. 3, pp. 372–378, 2006, doi: https://doi.org/10.1016/j.biortech.2005.03.013.

[48] M. A. Borowitzka and N. R. Moheimani, Algae for biofuels and energy. 2013.

[49] X. Deng, Y. Li, and X. Fei, "Microalgae: A promising feedstock for biodiesel," *African J. Microbiol.* Res., vol. 3, pp. 1008–1014, Dec. 2009.

[50] D. Chiaramonti, M. Prussi, M. Buffi, A. M. Rizzo, and L. Pari, "Review and experimental study on pyrolysis and hydrothermal liquefaction of microalgae for biofuel production," *Appl. Energy*, vol. 185, pp. 963–972, 2017, doi: https://doi.org/10.1016/j.apenergy.2015.12.001.

[51] E. Del Río, E. García-Gómez, J. Moreno, M. G. Guerrero, and M. García-González, "Microalgae for oil. Assessment of fatty acid productivity in continuous culture by two high-yield strains, Chlorococcum oleofaciens and Pseudokirchneriella subcapitata," Algal Res., vol. 23, pp. 37–42, 2017, doi: https://doi.org/10.1016/j.algal.2017.01.003.

[52] E. Angles, P. Jaouen, J. Pruvost, and L. Marchal, "Wet lipid extraction from the microalga Nannochloropsis sp.: Disruption, physiological effects and solvent screening," Algal Res., vol. 21, pp. 27–34, 2017, doi: 10.1016/j.algal.2016.11.005.

[53] T. Řezanka, L. Nedbalová, J. Lukavský, A. Střížek, and K. Sigler, "Pilot cultivation of the green alga Monoraphidium sp. producing a high content of polyunsaturated fatty acids in a low-temperature environment," Algal Res., vol. 22, pp. 160–165, 2017, doi: https://doi.org/10.1016/j.algal.2016.12.017.

[54] Z.-Y. Liu, G.-C. Wang, and B.-C. Zhou, "Effect of iron on growth and lipid accumulation in Chlorella vulgaris," Bioresour. Technol., vol. 99, no. 11, pp. 4717–4722, 2008, doi: https://doi.org/10.1016/j.biortech.2007.09.073.

[55] L. F. Wu, P. C. Chen, A. P. Huang, and C. M. Lee, "The feasibility of biodiesel production by microalgae using industrial wastewater," Bioresour. Technol., vol. 113, pp. 14–18, 2012, doi: 10.1016/j.biortech.2011.12.128.

[56] D. Song, J. Fu, and D. Shi, "Exploitation of Oil-bearing Microalgae for Biodiesel," Chin. J. Biotechnol., vol. 24, no. 3, pp. 341–348, 2008, doi: https://doi.org/10.1016/S1872-2075(08)60016-3.

[57] M. J. GROOM, E. M. GRAY, and P. A. TOWNSEND, "Biofuels and Biodiversity: Principles for Creating Better Policies for Biofuel Production," Conserv. Biol., vol. 22, no. 3, pp. 602–609, Jun. 2008, doi: https://doi.org/10.1111/j.1523-1739.2007.00879.x.

[58] P. Longhurst, J. Gift-Onyesoh, and V. Manovic, "Biomass resources and biofuels potential for the production of transportation fuels in Nigeria," Renew. Sustain. Energy Rev., vol. 2016, pp. 172–179, May 2016, doi: 10.1016/j.rser.2016.05.050.

[59] A. E.-F. Abomohra, W. M., M. El-Sheekh, and H. Dieter, "Lipid and total fatty acid productivity in photoautotrophic fresh water microalgae: screening studies towards biodiesel production," J. Appl. Phycol., vol. 25, pp. 931–936, Jan. 2013.

[60] J. L. Csavina, B. J. Stuart, R. Guy Riefler, and M. L. Vis, "Growth optimization of algae for biodiesel production," J. Appl. Microbiol., vol. 111, no. 2, pp. 312–318, Aug. 2011, doi: https://doi.org/10.1111/j.1365-2672.2011.05064.x.

[61] S. Manigandan, A. E. Atabani, V. K. Ponnusamy, A. Pugazhendhi, P. Gunasekar, and S. Prakash, "Effect of hydrogen and multiwall carbon nanotubes blends on combustion performance and emission of diesel engine using Tagu

[62] R. Sims, M. Taylor, J. Saddler, and W. Mabee, "From 1st-to 2nd-generation biofuel technologies," Paris Int. Energy Agency Organ. Econ. Co-Operation Dev., pp. 16–20, 2008.

[63] FAO/WHO, The State of Food Insecurity in the World, http://www.fao.org/publications/sofi/en/. 2003.

[64] C. H. Wu C., Z. X., Zhou F., "The Analysis of Biomass Energy UseTechnology development," 2007.

[65] C. V. P. Pascoal, A. L. L. Oliveira, D. D. Figueiredo, and J. C. C. Assunção, "Optimization and kinetic study of ultrasonic-mediated in situ transesterification for biodiesel production from the almonds of Syagrus cearensis," Renew. Energy, vol. 147, pp. 1815–1824, 2020, doi: https://doi.org/10.1016/j.renene.2019.09.122.

[66] M. R. Miladinović et al., "Valorization of walnut shell ash as a catalyst for biodiesel production," Renew. Energy, vol. 147, pp. 1033–1043, 2020, doi: https://doi.org/10.1016/j.renene.2019.09.056.

[67] S. Manigandan, A. E. Atabani, V. K. Ponnusamy, A. Pugazhendhi, P. Gunasekar, and S. Prakash, "Effect of hydrogen and multiwall carbon nanotubes blends on combustion performance and emission of diesel engine using Taguchi approach," Fuel, vol. 276, p. 118120, 2020, doi: https://doi.org/10.1016/j.fuel.2020.118120.

[68] G. Goga, B. S. Chauhan, S. K. Mahla, and H. M. Cho, "Performance and emission characteristics of diesel engine fueled with rice bran biodiesel and n-butanol," Energy Reports, vol. 5, pp. 78–83, 2019, doi: https://doi.org/10.1016/j.egyr.2018.12.002.

[69] M. Sarno and E. Ponticorvo, "A new nanohybrid for electrocatalytic biodiesel production from waste Amalfi coast lemon seed oil," Fuel, vol. 267, p. 117178, 2020, doi: https://doi.org/10.1016/j.fuel.2020.117178.

[70] S. Thiyagarajan, M. R. Herfatmanesh, V. E. Geo, and Z. Peng, "Experimental investigation into the effect of magnetic fuel reforming on diesel combustion and emissions running on wheat germ and pine oil," Fuel Process. Technol., vol. 186, pp. 116–124, 2019, doi: https://doi.org/10.1016/j.fuproc.2018.12.017.

[71] A. Kolakoti and B. V. A. Rao, "Effect of fatty acid composition on the performance and emission characteristics of an IDI supercharged engine using neat palm biodiesel and coconut biodiesel as an additive," Biofuels, vol. 10, no. 5, pp. 591–605, Sep. 2019, doi: 10.1080/17597269.2017.1332293.

[72] L. A. Raman, B. Deepanraj, S. Rajakumar, and V. Sivasubramanian, "Experimental investigation on performance, combustion and emission analysis of a direct injection diesel engine fuelled with rapeseed oil biodiesel," Fuel, vol. 246, pp. 69–74, 2019, doi: https://doi.org/10.1016/j.fuel.2019.02.106.

[73] M. A. Hazrat, M. G. Rasul, M. M. K. Khan, N. Ashwath, and T. E. Rufford, "Emission Characteristics of Polymer Additive Mixed Diesel-Sunflower Biodiesel Fuel," Energy Procedia, vol. 156, pp. 59–64, 2019, doi: https://doi.org/10.1016/j.egypro.2018.11.090.

[74] C. Patel, N. Tiwari, and A. K. Agarwal, "Experimental investigations of Soyabean and Rapeseed SVO and biodiesels on engine noise, vibrations, and engine characteristics," Fuel, vol. 238, pp. 86–97, 2019, doi: https://doi.org/10.1016/j.fuel.2018.10.068.

[75] A. Sharma, P. Kodgire, and S. S. Kachhwaha, "Biodiesel production from waste cotton-seed cooking oil using microwave-assisted transesterification: Optimization and kinetic modeling," Renew. Sustain. Energy Rev., vol. 116, p. 109394, 2019, doi: https://doi.org/10.1016/j.rser.2019.109394.

[76] Kelly, "Plant Systematics.org." http://www.plantsystematics.org/.

[77] T. A. M. Lira et al., "Performance of agricultural tractor consuming diesel and biodiesel derived from babassu ('Orbinya martiana')," Aust. J. Crop Sci., Sep. 2021, doi: 10.3316/informit.751771562734262.

[78] A. A. Espitia Cubillos, A. E. Delgado Tobón, and W. A. Aperador Chaparro, "Oleoresin from copaiba as raw material for the production of biodiesel," Rev. Int. Contam. Ambient., vol. 34, no. 2, pp. 317–329, 2018, doi: 10.20937/RICA.2018.34.02.12.

[79] S. V Khandal, N. R. Banapurmath, and V. N. Gaitonde, "Effect of exhaust gas recirculation, fuel injection pressure and injection timing on the performance of common rail direct injection engine powered with honge biodiesel (BHO)," Energy, vol. 139, pp. 828–841, 2017, doi: https://doi.org/10.1016/j.energy.2017.08.035.

[80] N. Acharya, P. Nanda, S. Panda, and S. Acharya, "A comparative study of stability characteristics of mahua and jatropha biodiesel and their blends," J. King Saud Univ. - Eng. Sci., vol. 31, no. 2, pp. 184–190, 2019, doi: https://doi.org/10.1016/j.jksues.2017.09.003.

[81] A. Sandouqa and Z. Al-Hamamre, "Energy analysis of biodiesel production from jojoba seed oil," Renew. Energy, vol. 130, pp. 831–842, 2019, doi: https://doi.org/10.1016/j.renene.2018.07.015.

[82] T. A. Mallah and A. R. Sahito, "Optimization of castor and neem biodiesel blends and development of empirical models to predicts its characteristics," Fuel, vol. 262, p. 116341, 2020, doi: https://doi.org/10.1016/j.fuel.2019.116341.

[83] C. Samart, S. Karnjanakom, C. Chaiya, P. Reubroycharoen, R. Sawangkeaw, and M. Charoenpanich, "Statistical optimization of biodiesel production from para rubber seed oil by SO3H-MCM-41 catalyst," Arab. J. Chem., vol. 12, no. 8, pp. 2028–2036, 2019, doi: https://doi.org/10.1016/j.arabjc.2014.12.034.

[84] O. Ogunkunle and N. A. Ahmed, "Performance evaluation of a diesel engine using blends of optimized yields of sand apple (Parinari polyandra) oil biodiesel," Renew. Energy, vol. 134, pp. 1320–1331, 2019, doi: https://doi.org/10.1016/j.renene.2018.09.040.

[85] M. V. Kumar, A. V. Babu, and P. R. Kumar, "Influence of metal-based cerium oxide nanoparticle additive on performance, combustion, and emissions with biodiesel in diesel engine," Environ. Sci. Pollut. Res., vol. 26, no. 8, pp. 7651–7664, 2019, doi: 10.1007/s11356-018-04075-0.

[86] M. Arunkumar, M. Kannan, and G. Murali, "Experimental studies on engine performance and emission characteristics using castor biodiesel as fuel in CI engine," Renew. Energy, vol. 131, pp. 737–744, 2019, doi: https://doi.org/10.1016/j.renene.2018.07.096.

[87] "Nature's Power Nutraceuticals®.," Nutraceuticals World, 2020. http://login.ezproxy.ub.unimaas.nl/login?url

[88] M. Nigam, R. Yadav, and G. Awasthi, "In-silico construction of hybrid orf protein to enhance algal oil content for biofuel," Lect. Notes Bioeng., pp. 67–89, 2021, doi: 10.1007/978-981-15-6329-48.

[89] G. Lu and E. N. Moriyama, "Vector NTI, a balanced all-in-one sequence analysis suite.," Brief. Bioinform., vol. 5, no. 4, pp. 378–388, 2004, doi: 10.1093/bib/5.4.378.

[90] B. A. Evans, E. S. Pickerill, V. K. Vyas, and D. A. Bernstein, "Crispr-mediated genome editing of the human fungal pathogen candida albicans," J. Vis. Exp., vol. 2018, no. 141, 2018, doi: 10.3791/58764.

[91] M. R. Wilkins et al., "Protein identification and analysis tools in the ExPASy server.," Methods Mol. Biol., vol. 112, pp. 531–552, 1999, doi: 10.1385/1-59259-584-7:531.

[92] C. Geourjon and G. Deléage, "Sopma: Significant improvements in protein secondary structure prediction by consensus prediction from multiple alignments," Bioinformatics, vol. 11, no. 6, pp. 681–684, 1995, doi: 10.1093/bioinformatics/11.6.681.

[93] A. Waterhouse et al., "SWISS-MODEL: Homology modelling of protein structures and complexes," Nucleic Acids Res., vol. 46, no. W1, pp. W296–W303, 2018, doi: 10.1093/nar/gky427.

[94] G. N. Ramachandran, C. Ramakrishnan, and V. Sasisekharan, "Stereochemistry of polypeptide chain configurations," J. Mol. Biol., vol. 7, no. 1, pp. 95–99, 1963, doi: 10.1016/S0022-2836(63)80023-6.

[95] M. Kanehisa and S. Goto, "KEGG: Kyoto Encyclopedia of Genes and Genomes," Nucleic Acids Res., vol. 28, no. 1, pp. 27–30, 2000, doi: 10.1093/nar/28.1.27.

[96] M. Kanehisa, M. Furumichi, M. Tanabe, Y. Sato, and K. Morishima, "KEGG: New perspectives on genomes, pathways, diseases and drugs," Nucleic Acids Res., vol. 45, no. D1, pp. D353–D361, 2017, doi: 10.1093/nar/gkw1092.

[97] A. Khamoushi, V. Tafakori, M. A. Zahed, S. E. Gayglou, and S. A. Angaji, "Augmenting the expression of accD and rbcL genes using optimized iron concentration to achieve higher biomass and biodiesel in Chlorella vulgaris," Biotechnol. Lett., vol. 42, no. 12, pp. 2631–2641, 2020, doi: 10.1007/s10529-020-02973-3.

[98] L. Kirchner et al., "Identification, characterization, and expression of diacylgylcerol acyltransferase type-1 from Chlorella vulgaris," Algal Res., vol. 13, pp. 167–181, 2016, doi: 10.1016/j.algal.2015.10.017.

[99] S. K. Dutcher et al., "Whole-genome sequencing to identify mutants and polymorphisms in chlamydomonas reinhardtii," G3 Genes, Genomes, Genet., vol. 2, no. 1, pp. 15–22, 2012, doi: 10.1534/g3.111.000919.

[100] S. S. Merchant et al., "The Chlamydomonas genome reveals the evolution of key animal and plant functions," Science (80-.)., vol. 318, no. 5848, pp. 245–251, 2007, doi: 10.1126/science.1143609.

[101] C. Bowler et al., "The Phaeodactylum genome reveals the evolutionary history of diatom genomes," Nature, vol. 456, no. 7219, pp. 239–244, 2008, doi: 10.1038/nature07410.

An Appendix

5.1 Sequence

>hybrid_ORF_length_5322bp

ATGACCCTCGGCTCATGCTTTCCGGCACGGCTGATGTGCCAGGATTCTTCGACAAGGGGAGCTGGAAGGAATATCTC
AGTGGATGGGGTAAGAGTGTTGTCATTGGACGCGGTCGCCTTGGAGGTATCCCTATGGGAGCCATTGCTGTCGAGAC
TCGCCTCGTGGACAAAGTCATCCCTGCCGACCCGGCTGACCCCAACTCGCGAGAAGCCATTCTTCCCCAGGCCGGCC
AGGTACTCTTCCCTGACTCTTCGTACAAGACTGCCCAAGCTCTTCGTGACTTCAACAAGGAAGGCCTTCCGGTTATG
AATGCCGCTCGCAAAGCTGCGAAACGTGGTGCTGGAGTACGCGGCCATAGCCATCTACGTCAGCGCCATCTACACCT
CGGTGGTGCTGCTGCCCTCGGCGCTCGCGCTGTTCTACCTGTTTGGGGCCACCAGCCCCTCGGCCTGGCTGCTGCTA
GCCGCCTTCCTGGCCCTCACCTTCACGCCGCTGCAGCTGACCACCGGTGCGCTGTCGGAGCGGTTCGTGCAGTTCAG
TGTGGCGCGGGCGGCGGCCTACTTCCCCACCCGCGTGGTGGTCACGGACCCGGAGGCCTTCCGCACTGACCGCGGCT
ACTTGTTCGGATTCTGCCCGCACTCGGCTCTGCCCATCGCACTGCCCATCGCCTTCGCCACCACCTCGCCGCTGCTG
CCCAAGGAGCTGCGCGGCCGCACACACGGCTTGGCGTCGTCCGTGTGCTTCAGCGCGCCCATAGTGCGGCAGCTGTA
CTGGTGGCTGGGCGTGCGGCCCGCCACGCGGCAGAGCATCAGCGGCCTGTTGCGGGCGCGCAAGGTGGCGGTGCTGG
TGCCGGGGGGCGTGCAGGAGGTGCTCAACATGGAGCACGGCAAGGAGGTGGCCTACCTCTCCAGCCGCACCGGCTTC
GTGCGACTGGCCGTGCAGCACGGCGCGCCGCTGGTGCCAGTGTGGGCGTTCGGCCAGACGCGCGCGTACAGCTGGTT
CCGGCCGGGGCCGCCGCTCGTGCCCACGTGGCTCGTGGAGCGCATCTCACGTGCCGCCGGCGCCGTACCCATCGGCA
TGTTTGGGCAGTACGGCACGCCCATGCCGCACCGCGAGCCCCTCACCATTGTGGTGGGTCGCCCCATCCCGGTGCCG
GAGCTGGCGCCGGGCCAGCTCGAGCCCGAGCCCGAGGTGCTGGCGGCGCTCCTCAAGCGCTTCACGGACGACCTGCA
GGCGCTGTACGACAAGCACAAGGCGCAGTTCGGCAAGGGCGAGGAGCTGGTCATAATGTAGATGCTGCACGCGACTC
AGCAGCGCGCGGTCGCTGGCCGTCGCCCGTTCTCGGGTGCGCGCGCGTCGAACCGCGTTGTTGCTCACGCGGCTGCG
ACCGTCGCCACCAGTCTGCCGACCGTTGACGTCCAGTTCCACCAGCCTAAGCTGGCGGGCGTGACCAACGAGCAGCA
GTTCAAGGCGGTAATCAAGGGGCTGGTCGCTCAGGGCAAGTTCCCTCCGCAGCTGGAGCCCGCTTGGGATTACTTCT
ATGACAACTACAAGAAGGCTGTCACCAGCAGTGGCGTCGCTGGGGCCGATGAGAAGCTTGTCACCCAGGTGCAAGCC
AGCATTCTGGACAATGTCCTGAACCAGGCGGTGAACCCCTACACCTTCCCCTCTTTCCACACCCGCCTAATTGAGCC
CTACAACTACTATGACTTCGGTCAGCGCTACGTCGCGACCCTCATCGACTTCCAGAACTCCGTGCTGGGTTTCCGCG
AGCGTTTCGACCGCGTTCAGGAGCTGCTGGACCAGAAGCACAACGTTGTTATCCTCGCGAACCACCAGACGGAGGCC
GACCCCGGTGTGTTTGCCCATATGCTGGCGAAGACGCACCCTAAGCTGGCGACGGATGTGATCTACGTCGCTGGCGA
CCGCGTTGTCACCGATCCGATGTGCAAGCCCTTCTCCATGGGCCGCAACCTCTTCTGCGTGCACTCCAAGAAGCACA
TGGACGACGCTCCGGAGCTGAAGGCCGCAAAGATGGAGACCAACCGCAAGACGCTGGTCGCCATGCAACGCAAGCTG
AACGAGGGCGGCACGCTCATGTGGATCGCCCCCAGCGGCGGCCGCGACCGCCCCAACGCCAACGACGAGTGGGTGCC
CGATAACTTTGATCCCGCCGCCGTGGAGCTGATGCGCAACCTGGTGCAGCGCGCCAAGCAGCCGGGCCACCTGATGC
CCATGTCCATGTTCAGCTACCCCATGATGCCGCCGCCCAAGACCGTGGACAAGTCCATTGGCGAGCGCCGCCTCACG
GCCTTCACGGGCGTGGGCATCTCCCTGTGCGAGGAGCTGGACGTGGCGGCCATCATCGCGGCCAGCGGCTCGGAGGA
GAAGGAGGTGCAGCAGAAGGCTCTGGCCAAGGCCGCGCACGACGCGGTGAAGGAGTCGTACGCGGTGCTGTCCAAGG
CCATCCAGGATCCCGCCTTCCGCGCCACCCGCAAGGAGTTCACACAGCCCTGGATGGCGTAAATGGTAGAAACATCT
CGTGCTCCTGACGGGACGAACCGGAGGAACTCGCTATCGGCCCGTCCTGCGGCAAACCTTCCCGGTTTGCGGAACGT
TGCTTCTGTAAGCGCTGACAAGGACCAGGAACCAGCACTGGTGACAAGGGAGGGGCCGACCAAGGACAACGCGGAAA
TCGACCAGCTGTACGCGGATGCGGAGGATCAGCGGTTGTCGAAAACGTCGCTGGTAGACGACGTGCTGAACATCAGC
AATGTGCTCACGGACGGCGTGTCGGCGATGGTGGACGACTCCTTCAACAAGTGCTTTACCAGCACGCGCCCGGAGCC
CTGGAACTGGAACATCTACCTGTTCCCCATCTGGGTGGTGGGCGTGCTGGTCCGGTATTTCATCCTGTTCCCGGTGC

FIGURE 5.1: Complete sequence of constructed Hybrid ORF

CCTCTTGTTCCTGGTGTTCGACATCTCACTGCCGCGCGGCAGACGGAAGATGGCGATTCAGCGCAAGCTGGTGCAGT
GGATGTGCTGCGCCTGGGTGGCGGCGTGGCACGGGGTGATCAGGTACCACGGCCCCAAGCCCACGCCCGGCCCCAAC
CGCATCTGGGTGTCCAACCACACCTCCATGATTGACTACGTGGTGCTGTGTAGCTACAGCCCCTTCGCCGTCATCAT
GCAGCTGCACCACGGCTGGATCGCGTTCCTGCAGAAGCGCATCCTGTCCTCGCTGGGCTGCCTGTGGTTCAACAGGA
CGGAGGTGAACGACCGCGCAGTGGTGGCGACGCGCATGCGCGAGCACGTGAACAACCCGGACGGCATCCCGCTGCTC
ATCTTCCCCGAGGGCACCTGCGTCAACAACGAGTACACGGTCATGTTTAAACGTGGCGCGTTCGACATCGGCGCCAC
GGTGTGCCCTGTGGCCATCAAGTACAACAAGATCTTTGTGGACGCCTTCTGGAACAGCCGCCGCGAGTCGTTCGGCA
AGCACCTGTTCCGCCTGCTGACCAGCTGGGCTCTGGTGTGCGACATCTACTTCCTGGAGCCGCAGGCGCTGCGGGAG
GGCGAGACGCCGCAGGAGTTCGCGGGGCGGGTGCAGGCGATGATTGCCAAGTATGCCAACCTGCGCATTGTGCCCTG
GGACGGCTACCTCAAATACTACAACCTAGGAGAGAAGAACCCGGGGCTTATCGAGAAGCGGCGCCGCGTGCTTGCGG
ACGTGCTGCGTGGATATCTGGGAAAGCAGGTGCAGCAGCCGGCGGCGGCGGCAGCGGCAGGAGGCGGAGAGAAGGCT
GCGAAAGGTGTGGCGGACAAGAGCGGCTCAGAAGAGCCCACCGGCTGGAAGAAGGTGGCGGCAGGCGCGCAGGTGCA
CCCGCAGTGAATGTCCGACACTGGCGGGGGTCACAGGGCGTCCGCCAACGCCTTACGAGACGCCTTTGATACACTGC
ATCCCGGCAGAATACAGTGCGATATTGTCGATATTTATACAGAGTACGGACCATTTTGGCCGTACGATTCCTATATT
GAACTCTATAAATTCGCGGCCAAATATCCGATCACTTGGGATATTTTTTATCATTTCGGCGCAACCGATTTTGGTAT
CTGGTTGAATCGACTCATGCTGGAGTTGTTTTGTTTTGAACCCTTCAAAACTTGCTTGTCTCGACCATCGGGGAATT
CCGGGAAAAAGGCCGATATGGTGGTGTCCGTACATCCTCTTACCCAAGATATCCCGCTACGGATATTGGCGGAACTG
GATTCGAACGGAGCGACGCGGGAACGGACCGGGCGGAAAACGCCGTTTTGTACCGTCGTCACTGATCTCGGGAGTGC
CCATCCAACTTGGTTCAACAAAGATGTCGACAAATGCTTTGTTCCGTCCGATGCATTGTACCTGGCTGCCAAAAAGC
GACAGCTCCAAGACTCGCAAATCGTGCAATACGGATTGCCAATCCGACAAGGGTTTTGGGCAAACAGCGAGTCGGCG
CATGTGGCGCCAGAAAAGGTACGTAAATCGCTTCGCCGTCAATTGGGTTTGGACGAAAATCTTCCGACCGTCTTGAT
CGTCGGTGGCGGGGATGGAATGGGAGGAATTGTTGAGATTTCGAAAAGTCTGGGTGTTGCTTTAGGCACAGCCAGTA
CCACTACACAAATGGTTGTTGTTTGCGGCAACAACCAAGAAGCCAAGGCAAGTTTAGAGAAGGAATCCTGGGGTACC
ACAGTGCGAGTCAACGTTCAAGGCTTCGTCGAGAACATGGACGAATGGATGAAGGCTTCGGATGCTTTGGTGACCAA
AGCCGGCCCCGGAACAATCGCTGAAGCATCAATCTGTGGACTACCTTGCATGCTCTTTTCGTATCTCCCCGGCCAAG
AAGAAGGCAATATTCCGTTCGTCGAAGAGGCTGGTTTTGGAAAGTACAGTGGCGACGCCTCCGTGATTGCCAATACT
GTGAGCTCGTGGCTGCTGTCCCCCGAGAAGCTTGAAGCAATGCGAAATGCTGCGCTAGCCGCCGCGCGGCCACAGGC
TACGCTAAATATTGCCAAAGACTTGATGTCTGCTTTAACTCGTACTACTCGTGTGCTCTCATTAGGCGAGCTTCGTC
TCTGTATCACTAGTAATCAACAGGATGAGAAAGGGAATGATCCAATGACGTGGCGTCCCTTTGATGATGGAATGGCA
CCATGTGATCTACTAGATTTTCGAGAGCATAATGCCTTGACTGACCGTTTATCCGACGCCCCAGAACGAACAGGTCT
GCAAGATCCTGTTCGACAGGGGGAGGACTGCGGGACGGTATGCCGATAG

FIGURE 5.2: Sequence of constructed Hybrid ORF

Table 5.1: Conserved regions of superfamilies of selected genes

S.No.	Organism	Protein	Superfamily	ORF Start Position	ORF Stop Position	Length of ORF
1.	*C. vulgaris*	acetyl-CoA	Crotonase	22	276	25584
2.	*C. reinhardtii*	glycerol	LPLAT	117	1487	1371456
3.	*C. reinhardtii*	glycerol 3-phosphate	PLN02349	1	1233	1233410
4.	*C. reinhardtii*	diacylglycerol	LPLAT	1	984	984327
5.	*P. tricornutum*	acetyl-CoA	PycA	5242	5550	309102
6.	*P. tricornutum*	Monogalacto synthase	PLN02605	13	1182	1170389