Halochlorella rubescens CULTIVATION IN PHOTOBIOREACTORS FOR OPTIMUM LIPID PRODUCTION

LAVANYA A/P ANBALAGAN

UNIVERSITI SAINS MALAYSIA

2022

Halochlorella rubescens CULTIVATION IN PHOTOBIOREACTORS FOR OPTIMUM LIPID PRODUCTION

by

LAVANYA A/P ANBALAGAN

Thesis submitted in fulfilment of the requirements
for the degree of
Master of Science

September 2022

ACKNOWLEDGEMENT

This dissertation which has been a work for the past three years would not have been possible without the hands of my lecturers, coursemates, friends and family, hence I would like to thank the Almighty for having everything fall into place for me to complete this thesis on time.

I would like to express my deepest gratidude to my project supervisor, Dr Noor Aziah Serri and co-supervisor, Dr Mohd Asyraf Kassim for their inexplicable support and guidance in ensuring that I complete my work.

In addition, I am also grateful towards my lab mates Charles, Ramizah and Mazni who supported and guided me throughout this venture. Also, a ton of gratitude to my friends Thivineshvaren, Vanetha and Moganes for always supporting me during my project. Thank you forbeing my pillars of strength. To lab assistants Mr Azmaizan and Mrs Najmah, I would like to extend my heartfelt thanks for their endless cooperation and commitments throughout the laboratory sessions.

Last but not least, I would like to thank my family for the undying love and support. The tremendous courage, strength and spirit they gave me have been a huge turnover during my hard times. This dissertation has been the journey of a tribe, and I sincerely thank everyone who has sailed with me to this point, for it not only made me a person with more knowledge, but also a better one as a whole.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS

α	Growth associated coefficient
β	Non growth associated coefficient
^{o}	Degree
%	Percentage
$\pm$	Plus, minus
Δt	Time interval

LIST OF ABBREVIATIONS

Accase	Acetyl-CoA Carboxylase
ACP	Acyl carrier protein
ALA	Alpha linolenic acid
AOAC	Association of Official Agricultural Chemists
ARA	Arachidonic acid
ATP	Adenosine triphosphate
ATR	Attenuated total reflectance
BBM	Bold basal medium
$CaCl_2 2H_2O$	Calcium chloride dehydrate
CLPBR	Centralized Light Photobioreactor
cm	Centimetre
CO_2	Carbon dioxide
CO_3^{2-}	Carbonate ion
CoA	Coenzyme A
$Co(NO_3)_2. 6H_2O$	Cobalt (ll) nitrate hexahydrate
CTPBR	Cubic Tank Photobioreactor
$CuSO_4. 5H_2O$	Copper sulfate pentahydrate
d^{-1}	Per day
DAG	Diacylglycerol
DGAT	Diacylglycerol acyltransferase
DGDG	Digalactosyldiacylglycerol
DHA	Docosahexaenoic acid
dH_2O	Distilled water
DMSO	Dimethyl sulfoxide
ENR	Enoyl-ACP reductase
EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
FAD	Fatty acid desaturase
FAS	Fatty acid synthase

FAT	Fatty acyl-ACP thioesterase
$FeSO_4.7H_2O$	Ferruos suplohate heptahydrate
FTIR	Fluorescence transform infrared spectroscopy
G3P	Glycerol 3-phospahte
G3PDH	Glycerol 3-phosphate dehydrogenase
GC	Gas chromatography
GC FID	Gas chromatography flame ionization detector
gL^{-1}	Gram per litre
$gL^{-1}d^{-1}$	Gram per litre per day
GPAT	Glycerol 3-phosphate acyltransferase
H_2CO_3	Carbonic acid
H_3BO_3	Boric acid
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
HCO^-_3	Bicarbonate
HD	3-hydroxyacyl-ACP dehydratase
ITS	Internal transcribed space
K_2HPO_4	Dipotassium hydrogen phosphate
KH_2PO_4	Potassium dihydrogen phosphate
KAR	3-ketoacyl-ACP reductase
KAS	3-ketoacyl-ACP synthase
KOH	Potassium hydroxide
L	Litre
L/min	Litre per minute
LED	Light emitting diode
LPAAT	Lyso-phosphatidic acid acyltransferase
LPAT	Lysophosphatidylcholine acyltransferase
M	Metre
M	Molar

µm	Micrometre
MAT	Malonyl-CoA ACP transferase
Mg	Milligram
$mgL^{-1}d^{-1}$	Milligram per litre per day
MGDG	Monogalactosydiacylglycerol
$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate
µL	Microlitre
mL	Millilitre
mL/min	Millilitre per minute
$MnCl_2.4H_2O$	Manganese (II) Chloride tetrahydrate
MoO_3	Molybdenum trioxide
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
$NaNO_3$	Sodium nitrate
NaOH	Sodium hydroxide
Nm	Nanometre
O_2	Oxygen
OD	Optical density
OVAT	One variable at time
PA	Phosphatidic acid
PAP	Phosphatidate phosphatase
PBR	Photobioreactor
PDH	Pyruvate dehydrogenase complex
PG8	Phosphatidylglycerol
Ph	Potential of hydrogen
PUFA	Polyunsaturated fatty acids
PVC	Polyvinyl chloride
Rpm	Revolutions per minute
SE	Standard error

SQDG	Sulfoquinovosyldiacylglycerol
SPSS	Statistical Package for the Social Sciences
TAG	Triacylglycerol
TPBR	Tubular photobioreactor
v/v	Volume per volume
vvm	Volume of air per volume of liquid per minute
w/w	Weight per weight
Xg	Times gravity
$ZnSO_4.7H_2O$	Zinc sulphate heptahydrate

LIST OF APPENDICES

PEMBIAKAN *Halochlorella rubescens* DALAM FOTOBIOREAKTOR UNTUK PENGHASILAN LIPID YANG OPTIMUM

ABSTRAK

Lipid daripada mikroalga telah dikaji untuk potensinya dalam pelbagai industri. Penghasilan lipid dari mikroalga dipengaruhi oleh kaedah pembiakan dan keadaan persekitaran. Kajian ini menyiasat kandungan lipid dan kinetik penghasilan lipid pada keadaan pembiakan berbeza yang melibatkan pH, gabungan sumber nitrogen dan fosforus dalam kepekatan berbeza, kepekatan karbon dioksida dan keamatan cahaya ke atas *Halochlorella rubescens*, mikroalga air tawar dalam fotobioreaktor dengan konfigurasi berbeza seperti fotobioreaktor tangki kiub (CTPBR), fotobioreaktor berpusat cahaya (CLPBR) dan fotobioreaktor tiub (TPBR) menggunakan satu pembolehubah pada satu masa (kaedah 'OVAT'). Kepentingan perbezaan antara setiap pembolehubah ujian ditentukan menggunakan ANOVA sehala di mana ($p < 0.05$) dianggap signifikan secara statistik. Secara kualitatif pengumpulan lipid sel ditentukan melalui kaedah pewarnaan 'Nile red'. Analisis Spektroskopi inframerah fourier transformasi (FTIR) pada parameter berbeza mengesahkan pengeluaran lipid. Model Leudeking-Piret mendedahkan bahawa pengeluaran lipid *Halochlorella rubescens* adalah berkadar langsung dengan pertumbuhan sel untuk kondisi yang berbeza. Kandungan lipid tertinggi ($39.42 \pm 0.426\%$) dan ketumpatan biojisim maksimum (0.3662 ± 0.002 g L^{-1}) dicapai pada keadaan optimum (pH 9, tanpa sumber nitrogen, 15% CO_2, 4000 lux) dalam 10L isu padu menggunakkan TPBR. Analisis lipid yang diekstrak daripada sel yang dibiak dalam TPBR menunjukkan bahawa asid lemak utama yang terdapat pada keadaan

optimum iaitu asid palmitik (C16:0), asid linolenik (C18:3n3), asid oleik (C18:1) cis dan asid linoleik (C18:2) cis. Kesimpulannya, TPBR sesuai untuk penghasilan lipid *Halochlorella rubescens* pada keadaan optimum di mana asid lemak yang dihasilkan sesuai untuk penghasilan biodiesel, bahan kosmetik dan sumber terbaik asid lemak tak tepu yang boleh digunakan untuk makanan manusia.

Halochlorella rubescens CULTIVATION IN PHOTOBIOREACTORS FOR OPTIMUM LIPID PRODUCTION

ABSTRACT

Lipids from microalgae have been studied for their potential application in various industries. The microalgae lipid production is mainly influenced by the cultivation methods and environmental conditions. This study investigates the effects of lipid content and lipid formation kinetics at different cultivation conditions involving pH, combination of nitrogen and phosphorus sources of different concentrations, carbon dioxide concentrations and light intensity on *Halochlorella rubescens,* a freshwater microalgae in photobioreactors with different configurations such as the cubic tank photobioreactor (CTPBR), centralized light photobioreactor (CLPBR) and tubular photobioreactor (TPBR) using one variable at a time (OVAT) method. The significance of differences between each test variable was determined using one way ANOVA where ($p < 0.05$) is considered statistically significant. Qualitatively the lipid accumulation of cells was determined via Nile red staining method. The analysis of Fourier Transform Infrared Spectroscopy (FTIR) at different parameters confirmed the functional groups of lipids. The Leudeking-Piret model revealed that the lipid production of *Halochlorella rubescens* is growth-associated at all different cultivation conditions. The highest lipid content (39.42 ± 0.426%) and maximum biomass density (0.3662 ± 0.002 gL^{-1}) was achieved at optimized conditions (pH 9, nitrogen-depleted medium, 15% CO_2, 4000 lux) in 10L volume using TPBR. The analysis of lipids extracted from cells cultivated in TPBR shows that the major fatty acids present at optimum conditions are palmitic acid (C16:0),

linolenic acid (C18:3n3), oleic acid (C18:1) cis and linoleic acid (C18:2) cis. In conclusion, TPBR is suitable for lipid production of *Halochlorella rubescens* at an optimum condition where the fatty acids produced indicates that it is suitable for biodiesel production, cosmetic ingredient and also a good source of polyunsaturated fatty acids which could be used for human consumption.

CHAPTER 1

INTRODUCTION

1.1 Background

Microalgae are unicellular organisms that can be found in aquatic habitats such as the ocean, rivers, lakes, wastewater and desserts (Khan et al., 2018). Microalgae are classified into eukaryotic (diatoms, green algae) or prokaryotic organisms (cyanobacteria) based on their defined cellular structure (Hachicha et al., 2022). Microalgae are known as photosynthetic organism that synthesizes biochemical compounds such as proteins, lipids and carbohydrate (Orejuela-Escobar et al., 2021; Tan et al., 2020). Over the past decade, the research studies about biology and applications of microalgae increased because it is a potential source of commercial products such as healthy food, vitamins, proteins, fine chemicals, fertilizers, and animal feed and renewable fuels. Microalgae such as *Chlorella*, *Dunaliella*, *Botryococcus*, *Nanochlorosis*, *Schizochytrium*, *Tetraselmis*, *Nitzschia* and *Poryphyridium* are some examples that have commercial benefits (Priyadarshani & Rath, 2012). The studies on microalgae metabolism such as primary metabolites and secondary metabolites lead to potential application in different fields of interest.

Among the primary metabolites, lipids composition can reach up to 90% of dry weight in microalgae (Pal et al., 2019). Lipids are composed of phospholipids, glycolipids, non-polar glycerolipids, saturated fatty acids and unsaturated fatty acids. During photosynthesis, microalgae capture solar energy from sunlight in the presence of water and oxygen to accumulate lipids in the biomass. Microalgae accumulate neutral storage lipids known as triacylglycerol in high amounts which contains saturated and monosaturated fatty acids (Alishah Aratboni et al., 2019; Zhu et al., 2017). The studies on triacylglycerol from microalgae are often directed

towards biofuel production, cosmetics and pharmaceuticals for commercial productions.

Lipids from microalgae were found as an alternative source compared to other feedstocks the large scale production of traditional crops resulted in food prices, land usage and carbon emissions problems (Kumar et al., 2015). In addition, the large requirements of water and fertilizers for the crops provoke to find another substitute for these feedstocks. Since they are highly resistant organisms, they are capable of living in different water sources such as brackish water, fresh and salty water (Mallick et al., 2016). Moreover, less water and fertilizer requirement than land crops, more cost effective and high growth rate are the reliable reasons for considering microalgae as an effective feedstock for lipid production (Alam et al., 2015). Thus, microalgae were found to be a sustainable source of lipid. Carbon dioxide which is released from the combustion process balances saving from carbon capture during microalgae growth (Kumar & Sharma, 2015; Sun et al., 2019; Kim et al., 2018). Furthermore, fuel produced from microalgae has similar properties to petroleum and biodiesel from crops (Khan et al., 2017).

Few methods were employed for the cultivation of algae such as open ponds, photobioreactors and hybrid systems. Photobioreactor was highly recommended for the cultivation of microalgae because it has low risk contamination and the environmental conditions can be controlled compared to the open cultivation system. The common types of photobioreactors used for cultivation are tubular, flat panel, bag PBR, stirred tank PBR, fermenter type PBR and hybrid PBR (Chen et al., 2011; Ting et al., 2017; Zhu et al., 2017). The design of photobioreactors and the productivity of microalgae in the photobioreactor are greatly influenced by several

factors such as temperature, pH, salinity, nutrients, light intensity, flow characteristics, aeration, contamination, power, gaseous transfer and mixing.

The study of kinetics is carried out for growth and product formation in a specific analysis. The model that is commonly used for product formation is Leudeking- Piret model. According to the Leudeking-Piret model, the rate of product formation is linearly dependent on instantaneous biomass concentration and growth rate (Baskar Gurunathan, 2018). The development of the Leudeking-Piret model gives a better understanding to predict the behaviour of cells at certain phases and enables shortening of the harvesting period where the product formation is higher. Fatty acid profiling is mainly used to identify the presence of fatty acid in a sample. Fatty acid profiling is useful to determine the quantity of certain fatty acids present in a sample and to provide assistance to process desirable fatty acids in commercial applications. Fatty acid profiling can be done using gas chromatography (GC) to study the oil composition of a sample.

So in this study, three different closed photobioreactors (Cubic tank photobioreactor, Centred light photobioreactor and Tubular photobioreactor) were tested for the efficiency of lipid production of *Halochlorella rubescens,* a freshwater microalgae. Parameters such as pH, light intensity, carbon dioxide concentration and nutrients were optimized to determine the highest lipid production. The product formation was studied using the Leudeking-Piret model at different parameters. The highest lipid content from the selected photobioreactors was used to characterize the fatty acids profile of *Halochlorella rubescens.*

1.2 Problem Statement

The production of lipids from various sources for commercial use is a vastly studied area in the bioprocess industry. The flexibility of lipids to be used in many forms for cosmetic, industrial and food industries render it significant to be studied, especially when the amount of extracted lipids highly affect the production in the industries. Studies have shown the possibility of lipid extracted from microalgae, though their plausibility highly depends on factors such as the species of microalgae, cultivation methods, the environmental conditions and the medium in the cultivation content. The microalgae strain used for this study is a freshwater microalgae, *Halochlorella rubescens*. This strain was chosen due to its ability to tolerate environmental stress and rapid growth rate. There are limited studies on this type of strain for lipid production, especially for *Halochlorella rubescens*. However, the microalgae strains that belong to the same class as *Halochlorella rubescens* such as *Dunaliella*, *Scenedesmus* and *Chlamydomonas* produced lipid contents from 16% to 77% of cell dry weight (Ahmed et al., 2017), (Sharma et al., 2015) and (Weldy & Huesemann, 2007). Commercial application of triacylglycerol requires high lipid production from microalgae, which is highly influenced in the process of cultivation.

Cultivation using an open pond has some drawbacks such as low mixing rate, poor temperature control, water evaporation and contamination issues (Biłos et al., 2016). In this regard, cultivation in photobioreactors grabbed the attention of researchers since the risk of contamination was lowered and they were able to control abiotic parameters. Although, the main challenge of cultivation using photobioreactors is the low biomass and lipid production efficiency. This may be due to the designs and configurations of the photobioreactors themselves influence biomass and lipid production. This scenario triggers the approach of comparing the

efficiency of three different designs of photobioreactors for biomass and lipid production. In this study, the different structures and components of three different photobioreactors are being studied for their ability for lipid accumulations. The three closed photobioreactors observed are the Cubic Tank Photobioreactor (CTPBR), Centralized Light Photobioreactor (CLPBR) and Tubular Photobioreactor (TPBR), all of which were used to compare their effects on *Halochlorella rubescens* growth, biomass and lipid composition based on their different designs.

The oil accumulation in microalgae varies according to the species. Low oil accumulation of microalgae is one of the problems that hinder the exploration of microalgae for commercial applications. The lipid accumulation of microalgae is very crucial to determine its suitability for the downstream process. Hence, to manipulate the environmental conditions for optimal lipid accumulation is required. Although, there has been no research conducted to collectively deduce the optimal conditions for maximum lipid production, and despite the availability of various studies on the different parameters that support lipid production from microalgae, a complete, overall research from bioreactor design until cultivation parameters study that produces the final, most optimum conditions for commercial microalgae production is yet to be found. However, the accumulation of lipid can be increased by introducing stress conditions to the microalgae. This approach is usually done by manipulating environmental conditions such as pH, salinity, nutrients, light intensity, heavy metals, temperature and carbon dioxide. Manipulating environmental conditions lead to the alteration of the biosynthetic pathway for microalgae that will favour the production of certain metabolites. In this study, the effect on lipid accumulation of *Halochlorella rubescens* was investigated by manipulating the

environmental conditions to determine potential fatty acids produced at optimum conditions for commercial production.

The Leudeking-Piret model is important to establish the relationship between cell growth and product. The parameters obtained are useful for scaling up and commercial production. The importance of this research is to bridge the gap for determining suitable closed photobioreactor to enhance biomass and lipid production, besides optimizing parameters for the cultivation of *Halochlorella rubescens* which can result in high production of biomass and lipid based on extensive reviews conducted prior to executing this research and from executing the experimental procedures repeatedly with altered variables. The outcome would be to determine a photobioreactor that would result in a high production of lipid, thus establishing a complete model for optimum lipid production commercially.

1.3 Scope of the study

This research is conducted to optimize the cultivation of *Halochlorella rubescens* using three different closed photobioreactors such as Cubic Tank Photobioreactor (CTPBR), Centralized Light Photobioreactor (CLPBR) and Tubular Photobioreactor (TPBR). These photobioreactors are designed with different configurations and modes of operation. CTPBR is cube shaped closed reactor with the capacity of 15L. It is made of acrylic material. The reactor does not have any interior parts and equipped with a draining port at the outer bottom part. Light is provided for the reactor externally. The CLPBR is a cuboid shaped reactor. CLPBR consists of three units of plastic containers. The interior part of CLPBR is complex. The capacity of each container is 4.5L. Light is provided for this reactor internally by placing it in the centre of the container inside the transparent pipes. It is made up of polypropylene with drainage port for each container. The TPBR is a cylinder-shaped

reactor the capacity of 12L. It is made up of acrylic material. Light is provided for the reactor externally. The benefits of TPBR for microalgae cultivation are low shear stress, good mixing and high surface area to volume ratio. The design of the reactor is not complex and thus easy to clean. It is also suitable for both indoor and outdoor cultivation. The efficiency of the photobioreactors is evaluated based on the high lipid accumulation.

Different cultivation conditions such as pH (4, 7, 8, 9), different nitrogen and phosphorus concentrations, CO_2 concentration (0.04%, 5%, 15%, 25%) and light intensity (1000 lux, 2000 lux, 3000 lux, 4000 lux) was investigated throughout the experiment. One variable at a time (OVAT) approach was used to study the lipid content of *Halochlorella rubescens* at different environmental conditions. This approach was employed to minimize error and to study in detail the parameters that affects the lipid content of microalgae. The optimization of parameters was validated using the One-Way ANOVA analysis and Post Hoc test.

The lipid production kinetics was experimented using the Leudeking-Piret model. This model was evaluated at different cultivation conditions to study if the lipid production is growth associated or non-growth associated. The microalgae cell behaviour was studied using this model to determine the effect of cultivation conditions on mode of lipid production. The growth associated (α) and non-growth coefficients (β) was determined to study the mode of lipid production for *Halochlorella rubescens*. The lipid extraction was carried out using Bligh and Dyer lipid extraction method assisted with sonication. The sonication method was used to increase the efficiency of lipid extraction method.

The presence of lipid was determined using Fluorescence Transmission Infrared Spectroscopy (FTIR) by studying the functional groups related to lipid. Besides, Nile red staining analysis was done to visualize lipid accumulation of *Halochlorella rubescens* at different cultivation conditions. The photobioreactor that gives high lipid content was chosen for mass cultivation at optimized cultivation conditions. The lipid extracts further used for the fatty acids analysis. Finally, the fatty acid present in the *Halochlorella rubescens* was analysed using Gas Chromatography (GC).

1.4 Research objectives

1.4.1 Objectives 1

To evaluate three different types of photobioreactors (CTPBR, CLPBR, TPBR) suitable for lipid production of *Halochlorella rubescens* by optimizing the selected parameters (pH, nutrients, light intensity, carbon dioxide concentration) using OVAT method.

1.4.2 Objectives 2

To determine the kinetics of lipid production from *Halochlorella rubescens* using the Leudeking-Piret model.

1.4.3 Objectives 3

To characterize the fatty acids produced by *Halochlorella rubescens* at optimum conditions via Gas Chromatography (GC) analysis using AOAC 996.06 17^{th} Ed process.

CHAPTER 2

LITERATURE REVIEW

2.1 Microalgae

2.1.1 Microalgae vs Macroalgae

Algae are divided into two distinct groups which are macroalgae and microalgae. Macroalgae is a complex multicellular cell that is visible to naked eyes while microalgae are small unicellular that can be seen under a light microscope (Khan et al., 2018). Classification of algae depends on size, reproduction mode, motility, habitat and composition of pigments. Microalgae are photosynthetic organism that varies upon cellulosic presence, size, colour and shape. Microalgae are very vast in diversity since there are more than 50 000 different types of microalgae species (Sathasivam et al., 2019).

Microalgae are usually found in freshwaters such as ponds, lakes and puddles, marine, wastewater and saline area (Ravindran et al., 2016). Besides, they can also grow in rocks, dessert, humus soil and the building wall. They are mostly found in all environments as highly adaptable to various environmental conditions. The large population of microalgae is visible to naked eyes which can be black, green, brown or red patches (Singh & Saxena, 2015).

Microalgae are usually classified into two groups which are prokaryotic and eukaryotic. Cyanophyta and prochlorophyta belong to prokaryotic division while eukaryotic division consists of Chlorophyta, Rhodophyta, Dinophyta, Glaucophyta, Haptophyta, Euglenaphyta, Ochrophyta, Cryptophytes and Chlorarachinophyte (Hamed, 2016). The taxonomic study first started with classification based on morphology, phenotype, and metabolism and later with the ultrastructural approach.

However, an unsuccessful attempt for the study was further investigated by molecular studies based on ribosomal RNA gene sequencing, Rubisco large subunit gene and Internal Transcribed Spacer (ITS) region (Champenois et al., 2015). The integrative approach is being used now as a taxonomic tool where it combines both classical and modern approaches.

Microalgae do not possess roots, stems and leaves which is similar to cellular organelles. The nuclei are membrane-bound organelles. The microalgae cell wall consists of polysaccharides which is a selective barrier that is porous (Domozych et al., 2012). It has lipid bodies, endoplasmic reticulum, ribosome, Golgi apparatus, mitochondria and also vacuole. The vacuole is able to maintain the cell shape and cell structure. Hemicelluloses and lignin are absent which makes it available for potential commercial products. The composition of microalgae is divided into primary metabolites and secondary metabolites. Primary and secondary metabolites are synthesized in microalgae based on chemical functional group and pathway of biosynthesis (Stark & O'Gara, 2012). The primary metabolites include carbohydrates, proteins and lipids.

Carbohydrates in microalgae are present in form of sugar which is monosaccharide, disaccharides, oligosaccharides and polysaccharides. The most abundant sugar present in microalgae is glucose, mannose, rhamnose and xylose which is synthesized in the cytosol (Ravindran et al., 2016). Proteins of microalgae synthesize all essential amino acids. Lipids are classified into polar lipids (structural) and non polar lipids (neutral). The lipids present are used for various commercial products. Table 2.1 shows the different oil content of different strains of microalgae. These primary metabolites are usually important to maintain the physical components for the survival of cells.

Secondary metabolites maintain the proper function of the physiological system. These metabolites consist of antioxidants such as flavonoids, terpenoids, carotenoids and phenols (Ani Azaman et al., 2017). Light energy is harvested during photosynthesis with the aid of some pigments such as lipophilic chlorophylls, hydrophilic phycobilins and carotenoids present in chloroplast. There are two different carotenoids which are primary (B-carotene, lutein, zeaxanthin) and secondary (astaxanthin, canthaxanthin and adonixanthin) carotenoids.

Table 2.1 The oil content of different microalgae based on its dry weight (Kumar et al., 2015), (Kamyab et al., 2013), (Sharma & Thukral, 2015)

No	Microalgae	Oil content (% dry weight)
1	*Botryococcus braunii*	25 – 75
2	*Chlorella sp*	28 – 32
3	*Chlorella emersonii*	14 – 57
4	*Chlorella protothocoides*	14 -57
5	*Chlorella vulgaris*	5 – 58
6	*Crypthecodiniumcohmi*	20 – 51
7	*Cylindrothecasp*	16 – 37
8	*Dunaliellaprimolecta*	23
9	*Isochrysis sp*	7 – 40
10	*Monallanthusalina*	20
11	*Nanochloropsis sp*	20 – 56
12	*Nanochlorosis sp*	31 – 68
13	*Neochlorosis oleoabundans*	29 – 65
14	*Zitzchia*	45 – 47
15	*Phaeodacltylum*	9 – 57
16	*Schizochytrium sp*	50 – 77
17	*Ttetraselmis suecica*	15 – 23
18	*Scenedesmus obliquus*	11 – 55
19	*Scenedesmus dimorphus*	16 – 40

2.1.2 Products from microalgae

2.1.2 (a) Bioenergy

Different feedstocks are used for bioenergy production which can be classified according to three different generations. Figure 2.1 shows the different generations of bioenergy feedstocks used till now.

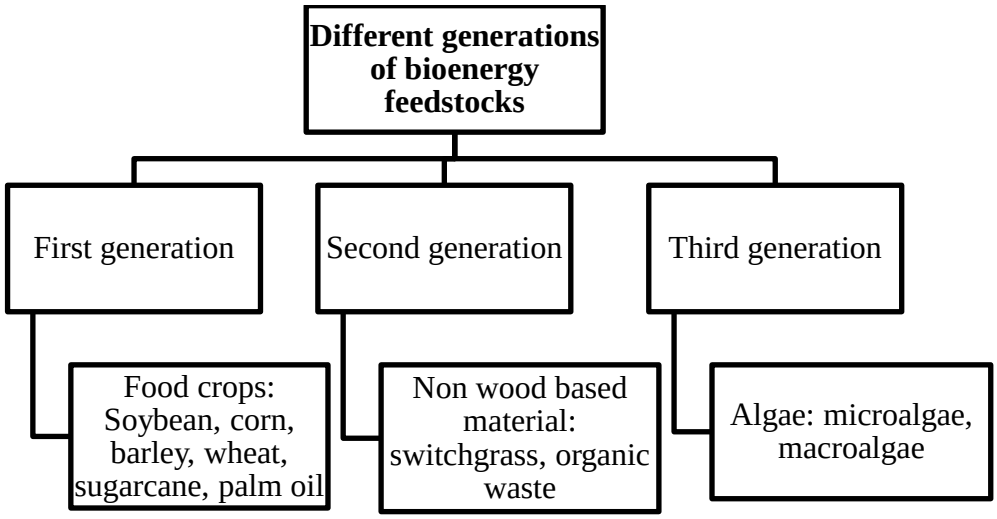

Figure 2.1 Different generations of feedstocks involved in bioenergy production (Bardhan et al., 2015), (Lee & Lavoie, 2013) and (Singh et al., 2020)

Microalgae captured the attention to be used as a feedstock for bioethanol production due to their properties such as high carbohydrate content and thin cellulose walls. Bioethanol is typically produced using the fermentation and gasification method (Harun et al., 2010). The process of fermentation starts by converting starch into sugar using enzymes, then the sugars are converted to ethanol in the presence of yeast (John et al., 2011). Microalgae do not have lignin which is easier during the pretreatment stage as it is cost effective.

Table 2.2 The comparison of oil content between different sources based on their dry weight (Kamyab et al., 2013), (Demirbas & Fatih Demirbas, 2011), (Fatani et al., 2018), (Ahmad et al., 2011), (Chisti, 2007)

No	Sources	Oil content (L/hectare)
1	Corn	172
2	Soybean	446 – 636
3	Canola	1190
4	Jatropha	1892
5	Coconut	2689
6	Oil palm	5366 – 5950
7	Safflower	779
8	Sunflower	952 – 1074
9	Castor	1307 – 1413
10	Borbadosnut	1500 -2000
11	Rapeseed	974
12	Microalgae	58 700 -136 900

Biodiesel is produced when vegetable oils or animal fats are converted into monoalkyl esters of long chain fatty acids (Mujeeb et al., 2016). Different feedstocks are being used for the production of biodiesel. Table 2.2 shows the oil content of different feedstocks. Microalgae are regarded as a potential feedstock for biodiesel due to some of their advantages compared to terrestrial crops (Mondal et al., 2017). For example, the microalgae growth rate is faster and it contains high lipid content (Amin & Prabandono, 2017). Triglycerides accumulated in microalgae lipids can be transesterified into fatty acid alkyl esters. Biodiesel produced from microalgae does not have sulphurs. In addition, the amount of carbon monoxide, hydrocarbons and sulphur oxides are reduced by using biodiesel microalgae (Sarkar & Bhattacharyya, 2012).

Biogas production is one of the biofuels that is being studied and investigated extensively for future applications such as transportation, appliances, power generation and industrial use (Arun & Singh, 2012), (Marcin et al., 2013). The fuel produced from microalgae is clean and environmentally friendly. Biogas consists of

methane and carbon dioxide (Zhu et al., 2016). The advantages of using microalgae are where the residues after fermentation can be still used for other commercial value products such as fertilizer and for biodiesel production.

The production of biohydrogen can be carried out using two main methods which are fermentation and photosynthetic. Mostly dark and photo fermentation are being carried out for biohydrogen production (Khetkorn et al., 2017). Biohydrogen produced from microalgae are found to have high energy density and does not emit greenhouse gases or exhaust pollutants (Rashid et al., 2013). Biohydrogen from microalgae is produced where there are no carbon emissions (Limongi et al., 2021). A vast study on microalgae biohydrogen includes strains such as *Chlorella* sp. and *Scenedesmus* sp (Wang & Yin, 2018).

2.1.2 (b) Other Commercial Products

Microalgae are composed of fine chemicals and bioactive compounds which can be processed to be used as human food (Safi et al., 2014). Figure 2.2 shows different commercial products from microalgae. The benefits of using microalgae as a food source include protection against oxidative stress, improving the immune system, prevention of viral infection and cancer (Priyadarshani & Rath, 2012). Microalgae are an excellent source of vitamins and minerals where it used in candies, snacks, cereals, noodles and beverages. *Dunaliella salina* and *Dunaliella terticola* contain high protein content and nutritional value that can be used for human consumption (Krishna et al., 2019). The pigments or carotenoids present in microalgae are being applied on an industrial scale especially in food production (Spolaore et al., 2006), (Barkia et al., 2019). In addition, β-carotene is used as an

orange dye and supplement (Sarkar & Bhattacharyya, 2012). It is used to enhance the margarine colour.

The application of microalgae as human health supplements can be seen in the pharmaceutical and nutraceutical industries. The compounds such as pigments, vitamins and polyunsaturated fatty acids in microalgae become a source of health supplements (Mata et al., 2010). The application of microalgae can be seen mostly for vaccines, pharmacy and human nutrition (Milledge, 2011). Besides, the bioactive compounds which have antibacterial, antiviral, antidiabetic and anticancer properties are useful in the production of supplements (Safi et al., 2014). *Spirulina*, *Chlorella*, *Dunaliella salina* and *Haematococcus pluvialis* are extensively being studied for nutraceutical application (Del Mondo et al., 2020). *Dunaliella* contains proteins, glycerol and β-carotene which can be used in the field of nutraceuticals. They also possess antioxidant activity and are used as a supplement for hepatoprotective effects (Nilesh Hemantkumar & Ilza Rahimbhai, 2020).

The biomass of algae is used to produce animal feed for larvae, crustaceans, molluscs and fish (Safi et al., 2014), (Dani et al., 2016). The compounds present in algae improves the immunity system of the animal (Han et al., 2019), (Mata et al., 2010). The astaxanthin present in microalgae is composed of high carotenoid value which protects the phospholipids membranes of the animals (Barkia et al., 2019). Microalgae are used mainly in the aquaculture industry to feed the animals. *Scenedesmus* is also used for terrestrial and aquatic animals feed (Krishna et al., 2019). *Dunaliella* strains were proven that can be used in fisheries as feed and also for zooplanktons (Dineshbabu et al., 2019).

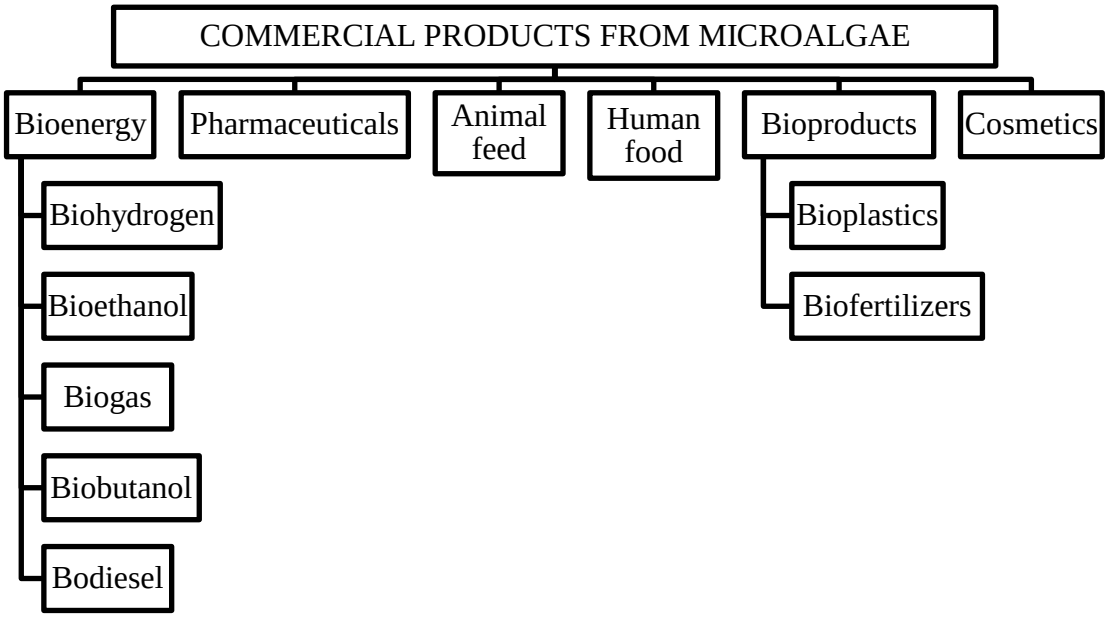

Figure 2.2 The potential products from microalgae

2.1.3 *Halochlorella rubescens*

Halochlorella rubescens strain belong to the green algae group which is one of the large group of algae (Champenois et al., 2015). *Halochlorella rubescens* is a freshwater microalgae. The shape of *Halochlorella rubescens* is spherical where their size ranges from 2 to 10µm diameter. It is also known as *Scenedesmus rubescens* which has high lipid content that varies from 10% to 72% (Tsavatopoulou et al., 2021). Besides, this type of strain can withstand extreme environmental conditions. It is considered green algae because of the presence of pigments responsible for photosynthesis such as chlorophyll a and b with similar proportions in higher plants (Mobin & Alam, 2017). It is unicellular and does not have flagella (Huang et al., 2015). *Scenedesmus* and *Dunaliella* species are found in lakes, ponds, freshwater, marine and brackish water (Beherepatil et al., 2013) and (Phinyo et al., 2017)

The classification of *Halochlorella rubescens* is shown in Figure 2.3. This type of species is described as holotype and previously was assumed to belong to the *Scenedesmus* genus.

Empire:	Eukaryota
Kingdom:	Plantae
Subkingdom:	Viridaieplantea
Infrakingdom:	Chlorophyta
Phylum:	Chlorophyta
Subphylum:	Chlorophytina
Class:	Chlorophyceae
Order:	Chlamydomonnadales
Family:	Chlamydomononadales incertae sadis
Genus:	*Halochlorella*
Species:	*Halochlorella rubescens*

Figure 2.3 The classification of *Halochlorella rubescens*

Figure 2.4 shows the morphology of *Halochlorella rubescens* which is spherical and green in colour. The age cultures of these microalgae will turn into a brick red colour. Besides, introducing stress conditions to the culture medium can also change the colour of microalgae into brown or brick red due to inhibition of growth.

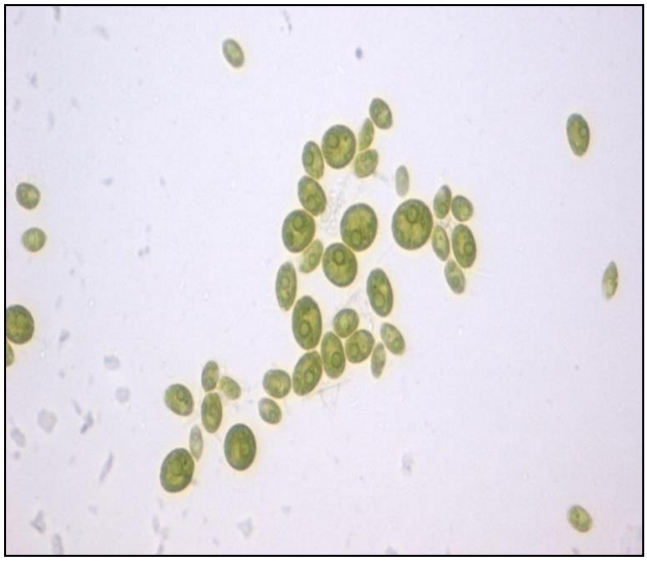

Figure 2.4 Morphology of *Halochlorella rubescens*

2.2 Lipid production from microalgae

Biochemical compositions such as carbohydrates, proteins and lipids are present in microalgae in different proportions at the different growth phases of the cell. Microalgae produces lipid to serve as energy storage to microalgae cells. The role of lipid bodies is important in protein storage, degradation and transport of lipid (Sharma et al., 2012). Lipids are not soluble in water but soluble in organic solvents. The structure of lipid contains a hydrocarbon group and consists of carbon, hydrogen and oxygen. Basically lipids produced by microalgae are comprised of two classes which are storage lipids (non polar) and structural lipids (polar) (Kumar et al., 2015). The non polar lipids are produced in the form of triacylglycerol (TAG) which contains saturated and unsaturated fatty acids (Sun et al., 2018). Some structural lipids will play an important role in cell signalling pathways which act as key intermediates (Alishah Aratboni et al., 2019).

2.2.1 Triacylglycerol biosynthesis of microalgae

In higher plants, the triacylglycerol is synthesized commonly at the endoplasmic reticulum. The lipid synthesis involves various enzymes that lead to TAG formation. The triacylglycerol synthesis starts from the Calvin cycle with the consumption of carbon dioxide (Bilbao et al., 2017). The synthesis of pyruvate to acetyl-CoA is catalysed by the glycolysis process and an enzyme called pyruvate kinase (Sakthivel et al., 2011). The main role of Accase is to convert the acetyl-CoA to malonyl-CoA. Acetyl-CoA is metabolized into malonyl-acyl carrier protein (ACP) by the sequential reaction. The carboxylation of the acetyl unit utilizes one ATP to generate one malonyl unit (Alishah Aratboni et al., 2019). Two molecules of NADPH (2NADPH) are used for the reduction of the keto group.

Malonyl-CoA is then converted into Malonyl ACP catalysed by Malonyl-CoA ACP transacylase (Sun et al., 2018). Malonyl group from CoA is received by protein cofactor on acyl carrier protein. The fatty acid synthesis starts as the 3-ketoacyl-ACP-synthase converts the malonyl-ACP into 3- ketoacyl- ACP which is then converted by 3-ketoacyl-ACP-reductase to form 3-hydroxyacyl ACP. Then, 3-hydroxyacyl ACP is subsequently converted into 3-hydroxyacyl-CP-dehydratase and the trans-enoyl-ACP is converted into acyl-ACP which is catalysed by Enoyl-ACP reductase. The acyl-ACP is then changed into free fatty acids (Zhu et al., 2016).

The fatty acid elongations rely on two core enzyme reactions such as acetyl-CoA carboxylase (ACCase) and fatty acid synthase (FAS). After completing numerous steps of reaction the C_{16} - C_{18} fatty acid thioester formed (Gong & Miao, 2019). The C_{18} fatty acid chain undergoes a desaturation reaction which regularly takes place in different microalgae species. In general, it has been found that

Chlorella sp. has a short chain of fatty chains which are C_{14} - C_{18} which are used for biodiesel production (Lim et al., 2012). The desaturation of the carbon chain of fatty acids involves fatty acid desaturases (FAD) which catalyze a single bond to be converted into a double bond at certain fatty acyl chain positions (Kong et al., 2018). Desaturases and elongases of polyunsaturated fatty acids (PUFA) are identified as important enzymes in elongation and desaturation. Table 2.3 shows all the enzymes involved in the lipid metabolism of microalgae.

The synthesis of triacylglycerol in microalgae is similar to plant cells. Kennedy or glycerol phosphate pathway and monoacylglycerol pathway are two major pathways of triacylglycerol (TAG) synthesis (Zienkiewicz et al., 2016). For the eukaryotic algae, triglyceride synthesis occurs via the Kennedy pathway (Cagliari et al., 2011). Figure 2.5 shows the pathway involved in the lipid metabolism of microalgae cells. The free fatty acid that is released into the cytoplasm will react with Acyl-CoA in the endoplasmic reticulum to produce lipid droplets. (Guschina & Harwood, 2006). In the endoplasmic reticulum (ER), the first reaction where the acyl-CoA react with Glycerol-3-phosphate to form lysophosphatidic acid is catalysed by Glycerol-3-phosphate acyltransferase (Zhu et al., 2016)**.** Again in the second reaction of acyl-CoA with lysophosphatidic acid is catalysed by Lyso-phosphatidic acid acyltransferase to form phosphatidic acid. The phosphatidic acid is eventually converted into diacylglycerol by Phosphatidate phosphatase. The third reaction of acyl-CoA with diacylglycerol is catalysed by diacylglycerol transferase to form TAG. The formation of DAG is a crucial part of TAG synthesis of microalgae.

TAG synthesized deposited in the cytosol as lipid droplets (Kong et al., 2018). Droplets of oil are removed from ER membrane which eventually forms distinctive cell organelles. These oil droplets released into cytoplasm made up of phospholipids and hydrophilic head groups are located on its surface (Bilbao et al., 2017).

Table 2.3 Enzymes involved in triacylglycerol (TAG) synthesis in the microalgae cells (Alishah Aratboni et al., 2019), (Bilbao et al., 2017) and (Sun et al., 2018)

No	Enzyme	Full name
1	ACCase	Acetyl-CoA carboxylase
2	ACP	Acyl carrier protein
3	CoA	Coenzyme A
4	DGAT	Diacylglycerolacyl transferase
5	DHAP	Dihydroxyacetone phosphate
6	ENR	Enoyl- ACP reductase
7	FAT	Fatty acyl-ACP thioesterase
8	G3PDH	Glycerol-3-phosphate dehydrogenase
9	HD	3- hydroxyacyl- ACP dehydratase
10	GPAT	Glycerol-3-phosphate acyltransferase
11	KAR	3-Ketoacyl-ACP-reductase
12	KAS	3-Ketoacyl-ACP-synthase
13	LPAAT	Lyso-phosphatidic acid acyltransferase
14	LPAT	Lysophosphatidylcholine acyltransferase
15	MAT	Malonyl-CoA ACP transacylase
16	PDH	Pyruvate dehydrogenase complex

However, the prokaryotic pathway that takes place in chloroplast releases phosphatidic acid (PA), diacylglycerol (DAG) and lipids such as monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG) (Liu & Benning, 2013), (Zhu et al., 2016). The specific localization of the lipids synthesized in the prokaryotic pathway is complicated.

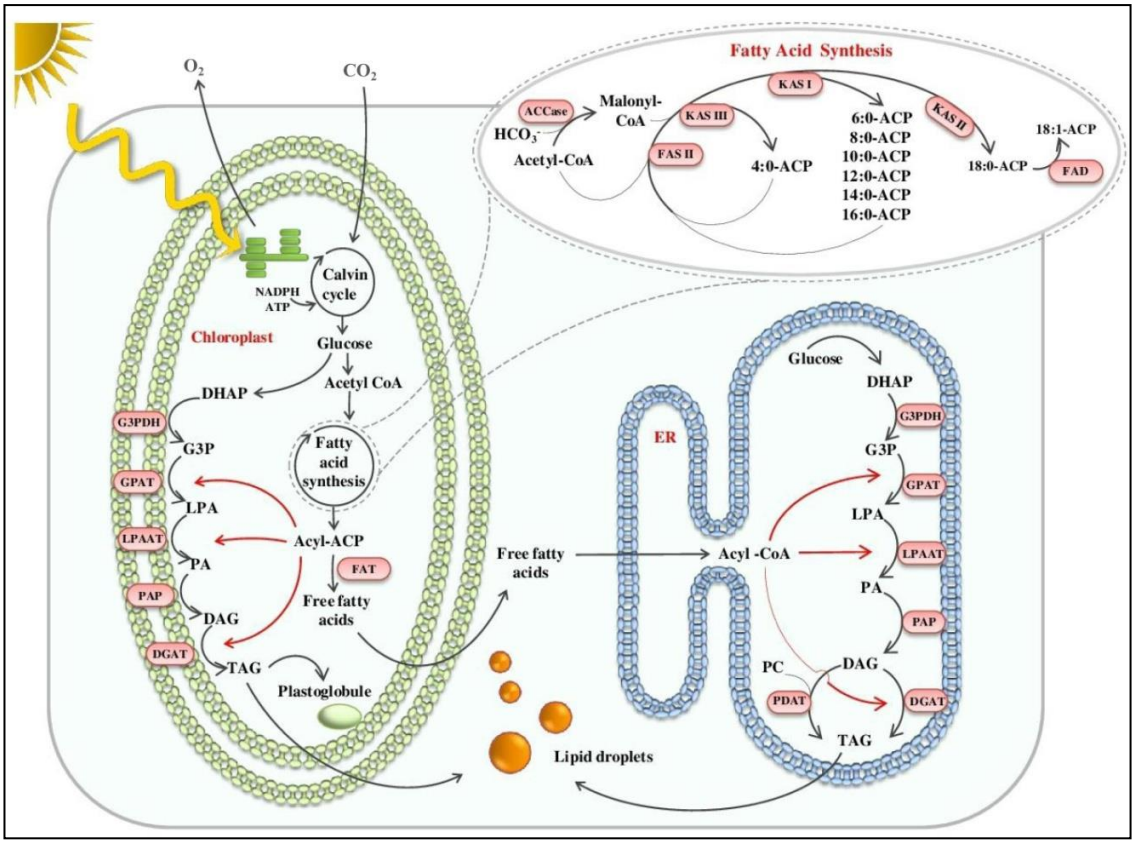

Figure 2.5 The pathway for TAG synthesis in microalgae (Sharma et al., 2018)

2.3 Cultivation mode

Different mode of cultivation is used for microalgae cultivation such as autotrophic, photoautotrophic, heterotrophic, and mixotrophic. The mode of microalgae cultivation employed in the present study is mixotrophic. The mode of cultivation is classified based on the light requirements, type of carbon source used and energy utilized for cultivation of microalgae as shown in Table 2.4 (Wang et al., 2014). Mixotrophic mode is better among all the modes of cultivation as it can utilize both organic and inorganic carbon sources which eventually increase the biomass productivity of microalgae (Prokop et al., 2015). According to Gurumoorthy & Saravanan (2019), the maximum lipid content (65%) was achieved at the mixotrophic condition for *Dunaliella salina* CCAP19. Mixotrophic cultivation also

was found to be suitable for *Scenedesmus quadricauda* according to (Song & Pei, 2018). Besides, the oil accumulation and biomass production of *Scenedesmus dimorphus* was found to be better than in mixotrophic conditions compared to other cultivation modes (Manzoor et al., 2020).

Table 2.4 The comparison between different modes of cultivation (Prokop et al., 2015), (Wang et al., 2014)

Growth mode	Energy	Carbon	Light
Autotrophic	Light	Inorganic	Obligatory
Photoautotrophic	Light	Inorganic	Obligatory
Heterotrophic	Organic carbon source	Organic	Not obligatory
Photoheterotrophic	Light	Organic	Obligatory
Mixotrophic	Light and organic carbon source	Inorganic and organic	Not obligatory

2. 4 Photobioreactor

A photobioreactor is defined as a closed system that comprises of transparent culture vessel optimized for phototrophic cultivation. The thickness of the photobioreactor is usually from 2-4 cm and the diameter can be 10 inches to allow penetration of more light (Hagendijk, 2015). The cultures inside this illuminated vessel are circulated through mixing which enhances the gas exchange and nutrient supply to algal cells (Borowitzka & Moheimani, 2013). Photobioreactors are preferred upon open pond systems due to their advantages. It has higher yields which are approximately five times better yield than the open pond. Table 2.5 shows the comparison of open ponds and photobioreactors.

The common factors that need to be considered in designing a photobioreactor are the type of microalgae strains, efficient light usage, uniform

illumination, reduced mutual shading, less fouling, high illuminated ratio of surface area to volume ratio and fast mass transfer of oxygen and carbon dioxide (Brennan & Owende, 2010). The world's largest photobioreactor is located in the greenhouse in Germany (Prokop et al., 2015). They are horizontal tubular photobioreactors that have a capacity of 700cm^3.

Mostly used photobioreactors are tubular and flat plate photobioreactors. The classification of photobioreactor is based on design, orientation and mode of operation. The materials employed for the construction of the photobioreactor are polycarbonate, polyvinyl chloride, glass and polymethyl methacrylate (Chen et al., 2011). These materials are used for efficient light penetration through photobioreactor barrier to reach the algal cells. The limitations with the photobioreactors are the design, production scale, cost, materials used and environmental conditions fixed (Pruvost et al., 2020).

Usually, the sparging gas bubbles are situated at the bottom for good mixing. However, the limiting factors for photobioreactors are high energy cost, mixing, cooling and oxygen accumulation that is produced during photosynthesis. Photobioreactors are designed with different configurations for better light utilization (Alaswad et al., 2015). Different types of photobioreactors are illustrated in Figure 2.6. Important factors that need to be considered for photobioreactor are better illumination, removal of oxygen, surface area to volume ratio, carbon dioxide supply, easy to clean, cost effective, not complex and can be cultivated both indoor and outdoor (Kunjapur & Eldridge, 2010).