APPLICATION OF SELECTED MARINE MICROALGAE IN THERMOPLASTIC STARCH PRODUCTION

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APPLICATION OF SELECTED MARINE MICROALGAE IN THERMOPLASTIC STARCH PRODUCTION

by

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS

$(NH_4)_6Mo_7O_{24}$	Ammonium Molybdate
HCO_3^-	Bicarbonate
H_3BO_3	Boric acid
$CaCl_2 \bullet 2H_2O$	Calcium chloride dihydrate
Cu	Copper
Cu_2SO_4	Copper(I) Sulfate
℃	Degree Celcius
K_2HPO_4	Dipotassium hydrogenphosphate
$Na_2EDTA \bullet 2H_2O$	Disodium ethylenediaminetetraacetate dihydrate
Fe	Iron
$FeSO_4 \bullet 7H_2O$	Iron(II) sulfate heptahydrate
$MgSO_4 \bullet 7H_2O$	Magnesium Sulfate Heptahydrate
$MnCl_2 \bullet 4H_2O$	Manganese(II) Chloride Tetrahydrate
%	Percentage
-	Minus
+	Plus
±	Plus-minus
K_2SO_4	Potassium sulfate
Na	Sodium
Zn	Zink
$NaHCO_3$	Sodium bicarbonate
NaCl	Sodium chloride
$NaNO_3$	Sodium nitrate
v/v	Volume per volume

w/v	Weight per volume
w/w	Weight over weight
$ZnSO_4 \bullet 7H_2O$	Zink sulphate heptahydrate

LIST OF ABBREVIATIONS

MOPS	3-(N-morpholino) propanesulfonic acid
ADP	Adenosine diphosphate
AGPase	ADP-glucose pyrophosphorylase
ANOVA	Analysis of variance
DMSO	Dimethyl sulphoxide
GOPOD	Glucose oxidase/peroxidase
g	Gram
h	Hour
L	Liter
mg	Miligram
mL	Mililiter
mm	Milimeter
min	Minute
µL	Microliter
nm	Nanometer
N	Nitrogen
OD	Optical Density
PAR	Photosynthethic active radiation
PCL	Polycaprolactone
PHA	Polyhydroxybutyrate
PLA	Polylactic acid
PCR	Polymerase Chain Reaction
pH	Potential of hydrogen
rpm	Revolutions per minute
sp.	Species
µ, day^{-1}	Specific growth rate

TPS	Thermoplastic starch
TFA	Trifluoroacetic acid

APLIKASI MIKROALGA MARIN TERPILIH DALAM PENGHASILAN KANJI TERMOPLASTIK

ABSTRAK

Kanji merupakan polisakarida yang boleh dibiodegradasikan sepenuhnya telah digunakan secara meluas dalam aplikasi perindustrian dan juga sebagai sumber tenaga yang boleh diperbaharui. Dalam kajian ini, sepuluh jenis mikroalga (air tawar dan marin) dari Malaysia, dikultur dan dianalisis kandungan biokimia mereka. Hasil kajian menunjukkan bahawa *Chlorella salina* mengandungi kanji tertinggi iaitu 4.92±0.33%, diikuti oleh *Spirulina* sp. 2.58±1.18%. Justeru, *C. salina* dan *Spirulina* sp. dipilih untuk dikaji kesan faktor fizikal dan kimia. *C. salina* menghasilkan kanji yang lebih tinggi berbanding dengan *Spirulina* sp., (69.46 mg/L dan 16.13mg, masing-masing), dalam keadaan pertumbuhan optimum iaitu: 32℃, gelombang merah, 24L:0D (fotokala) dan 39 ppt (saliniti), oleh itu, *C. salina* digunakan untuk penyelidikan lebih lanjut (faktor kimia). Selain itu, didapati bahawa kaedah pemecahan sel yang paling berkesan adalah kaedah etanol panas (0.46g kanji/g pengeringan sejuk beku) jauh lebik baik daripada empat kaedah (ricih cecair ultrasonik, rendaman air panas, lisis alkali dan *bead beating*) yang lain. Di samping itu, hasil kanji tertinggi dan terendah dengan kaedah lisis sel etanol panas dan alkali masing-masing adalah sebanyak 46.4% dan 12.8%. Analisis termogravimetri (TGA) menunjukkan, filem kanji jagung (CS) komersial menunjukkan kehilangan jisim yang lebih tinggi (81.24%) berbanding dengan filem kanji alga (AS) (76.53%) dan suhu lebur (kalorimetri imbasan pembeza) diperhatikan sebagai yang tertinggi untuk filem AS pada 320.24 ℃ sedangkan CS menunjukkan yang terendah pada 317.91 ℃.

Oleh itu, kajian ini menyarankan bahawa filem kanji mikroalga merupakan bahan mentah yang berpotensi untuk digunakan dalam pembangunan teknologi mesra alam.

APPLICATION OF SELECTED MARINE MICROALGAE IN THERMOPLASTIC STARCH PRODUCTION

ABSTRACT

Starch is increasingly used in many industrial applications and as a renewable energy resource. In this study, ten indigenous microalgae (freshwater and marine) were cultured and analyzed for their biochemical content. The results showed that *Chlorella salina* contained the highest starch of 4.92±0.33%, followed by *Spirulina* sp. (2.58±1.18%). Thus, *C. salina* and *Spirulina* sp. were selected to study the effects of physical and chemical factors on their starch production. *C. salina* produced a higher starch yield compared to *Spirulina* sp., (69.46 mg/L and 16.13 mg/L respectively), under optimum growth conditions of 32 ℃, red wavelength, 24L:0D (photoperiod) and 39 ppt (salinity), hence, *C. salina* was selected for further investigation (chemical factors). On the other hand, the efficiency of different starch extractions methods indicated that the ethanol boiling method (0.46g starch/g freeze-dry biomass) was significantly better than the four other methods (ultrasonic liquid shear, hotwater bath, alkaline cell lysis and bead vortexing) used. In addition, the highest and the lowest starch yield of 46.4% and 12.8% were obtained by the ethanol boiling method and alkaline cell lysis method, respectively. Thermoplastic starch (TPS) film preparation was developed using the casting technique. Thermogravimetric analysis indicated that commercial corn starch (CS) film showed higher mass loss (81.24%) compared with the algae starch (AS) film (76.53%) and melting temperatures differential scanning calorimetry (DSC) were observed to be the highest for AS film at 320.24 ℃ whereas CS showed the lowest at 317.91 ℃.

Therefore, this study suggests that algae starch film is a promising raw material with the potential to be used in the development of environmentally friendly technologies.

CHAPTER 1

INTRODUCTION

Plastic is durable, immune to moisture, lightweight, strong and cheaper. These are the enticing attributes that have driven us around the world to such a voracious appetite and overconsumption of plastic products. Even plastic is durable and very slow to degrade. However, durable and very slow to degrade, plastic materials that are used in the production of so many products all, ultimate, become waste with staying power. Our enormous exposure to plastics, combined with an undeniable behavioural propensity to continually over-consume, waste, litter and thus pollute, has become a mixture of lethal nature (American Chemistry Council, 2016).

In 2008, our global plastic consumption throughout the world was measured at 260 million tonnes, and plastic consumption is projected to hit 297.5 million tonnes by 2015 according to a 2012 study by Global Industry Analysts. It has been estimated that approximately 2.45 Metric Tonne (MT) of plastics resins are manufactured locally per annum in Malaysia (MPMA, 2018). However, knowledge of plastics waste and plastics recycling activities in the manufacturing sector is very limited, as the majority of solid waste studies conducted focus on municipal solid waste (MSW) in general, where plastics waste is the third-largest tonnage of waste, alongside putrescible waste and paper (Sreenivasan *et al.*, 2012).

1.1 Environmental impact of macro and microplastic

Consumers today need materials that are cheap, flexible and comfortable, making plastics typically used for plenty of use. Petroleum primarily based plastics, a significant component of global plastic use, getting the benefits of manufacturing

from wide economies of scale and superior technology (Iles and Martin, 2013). These plastics use stems from their strength while maintaining low weight, water, chemicals, sunlight, and bacteria resistant to degradation, and their ability to provide electrical and thermal insulation. While these functions make traditional plastics ideal for plenty of applications, they have environmental and economic problems as well. Polystyrene is one of the commonly used plastics but is very sluggish in environmental biodegradation. HIPS (High impact polystyrene), a polystyrene and polybutadiene copolymer, is low cost, simple to process, and quick to produce plastics (Katančić *et al*., 2011).

A HIP requires 99.8 gigajoules (GJ) of energy to generate 1000 kg of resin, mainly coming from a natural source. This aid consumption is similarly inflated by the fact that traditional plastics are manufactured from crude oil, an increasingly dwindling resource, as a chemical precursor in this costly energy process which yields HIPS resin. In addition to these consumption problems, the resin processing process further affects the environment by creating waste materials, resulting in pollution of the air, water and land. Some of these waste products are known pollutants which can leach over time leading to pollution of groundwater reservoirs (Franklin Associates, 2013). In addition, the properties which make traditional plastics appealing for commercial applications are also harmful to the environment by preventing biodegradation, increasing demand and landfill length to handle the increasing volumes of plastics coming into the municipal solid waste system. Therefore, greater emphasis has been put on the use of biodegradable additives in disposable and other consumer goods (majority made from polyethene and polypropylene) to minimise reliance on petroleum substances and fixing the atmospheric CO_2 (Zhang *et al*., 2000).

Interest in the production of alternatives to synthetic petroleum polymers for different industrial uses has been generated by environmental conversion policies, the critical use of natural resources and the depletion of supplies of hydrocarbons. One strategy to these concerns is plastics recycling, which may reduce landfill filling, and thus leaching of chemicals by increasing a commodity's use, but this has inherent energy consumption issues. Several studies have concentrated on developing, at least partially, biodegradable materials that would replace conventional plastics. Natural biopolymer materials, such as polysaccharides, are a fascinating option that makes starch one of the most promising substances for the improvement of biodegradable plastics (Ma *et al*., 2009).

Plastic will give a negative impact on the environment and human health. As some studies about plastics, these polymers are also the most commonly found plastics in the environment (Tokiwa *et al*., 2009). Macroplastic (>0.5 cm) pollution threatens (aquatic) species through entanglement and ingestion. In Urban water systems, blockage of hydraulic infrastructure by macroplastics lead to more severe and faster water level increased compared to organic debris (Honingh *et al*., 2020). Once in river systems, macroplastics break down into micro and nanoplastics, and can leak toxin additives (van Emmerik and Schwarz, 2020). Also, river plastic is assumed to be one of the main sources of marine plastic pollution (Schmidt *et a*l., 2017).

Microplastics are smaller plastic with size less than 5 mm, have recently drawn attention because microplastics not only make their way into the marine environment but are also more easily ingested by marine organisms, it’s made microplastics may thus act as vectors for the chemical transfer of pollutants within

the food chain (Thompson *et al*., 2009). Microplastics have received significant attention in media and research, but there is little information on people's perceptions of microplastics and their risks. This could be due to the size of microplastic particles- which cannot be seen by the naked eye and the fact that it cannot be easily recovered from the environment (Heidbreder *et al*., 2019) and hence are not within the direct reach of most people. This could be one of the reasons why the environmental problems posed by microplastics are not considered by most people to be as serious, as those posed by larger plastic materials (Anderson *et al*., 2016). However, microplastics are known to pose significant negative effects on terrestrial and sea animals as well as on human health, be it directly or indirectly (Proshad *et al*., 2018; Wong *et al*., 2020).

1.2 Microalgae for potential commercial applications

Microalgae are small, unicellular species capable of transforming solar energy through photosynthesis into chemical energy. They contain various bioactive compounds that may be commercially harnessed. Primary metabolites produced by microalgae during photosynthesis are useful compounds to be used for different purposes since they are more effective in using sunlight as an energy source compared to higher plants (Gibbs, 1992).

Algae are a convenient word that refers to a group of extremely diverse organisms conducting photosynthesis and/or plastids (Keeling, 2004). The prokaryotic cyanobacteria in algae are also included by many scholars since they show a very similar lifestyle to their eukaryotic counterparts and also share the same environment with eukaryotic algae. The origin of plastids is cyanobacteria; plastids are plant organelles and eukaryotic algae harbouring photosynthesis and synthesising

several chemical compounds that are also essential for other biochemical pathways (McFadden, 2001; Palmer, 2003; Keeling, 2004).

Microalgae can be used to create an extensive form of metabolites that can be used for hygiene, food and feed additives, cosmetics and energy production, such as proteins, lipids, carbohydrates, carotenoids or vitamins. The first human use of microalgae dates returned to 2000 years ago in China, when Nostoc (Prokaryote) was used to survive hunger. But biotechnology for microalgae has only really started emerging in the mid-last century. There at moment are diverse commercial applications of microalgae, inclusive of microalgae, which may be used because of their chemical composition to increase the dietary value of meals and animal feed; they play an important function in aquaculture and can be used in cosmetics (Adams *et al*., 2009).

Furthermore, they are being cultivated as a source of great value molecules. For instance, polyunsaturated fatty acid oils like DHA and EPA are added to infant formulas, and nutritional supplements and pigments are important as natural colourings. There are three important characteristics of microalgae that can be converted into technical and commercial benefits. Genetically diverse, they are a very complex group of species with a wide range of physiological and biochemical properties, containing a lot of distinct and unusual fats, carbohydrates, bioactive compounds, etc. (Luiten *et al*., 2003).

However, none of the studies was performed using starch from marine microalgae as a feedstock for the development of bioplastics. Starch is a natural polymer accumulated in plants for the storage of carbohydrates. It is one of the most available renewable resources and is a fully biodegradable polysaccharide. Due to its

abundance, biodegradability and low cost, it has been considered an excellent candidate for partial replacement of synthetic polymer in packaging and other low-cost applications (Doane *et al*., 1992). To transform starch into thermoplastic starch (TPS), primarily water and glycerol (as plasticizer) are used in combination with starch at high temperature and shear (Gomez and Aguilera, 1984;Zdrahala, 1997).

1.3 Problem statements

Bioplastics are plastic produced from renewable resources such as starch and cellulose from crops, oils and protein. Out of this, bioplastics from thermoplastic starch/blends have contributed to about 40% of the total global bioplastics market share. Raw materials for bioplastics production are sugars and starches harvested from crops that otherwise might be grown for food. Large scale production of raw materials could have negative impacts on land use and cause possible deforestation to grow suitable crops and plants. For this, starch from marine microalgae become an alternative feedstock for bioplastics production (Oh *et al*., 2018).

Bio-based plastics from natural feedstocks are a biodegradable alternative to traditional plastics which greatly minimises environmental stress and leverages reserves of fossil fuel. Biomass, starch and cellulose fractions of carbohydrates from maize, wheat, rice and potato have been used as the basis for conversion into bio-based plastics, such as polylactic acid (PLA), cellulose acetate (CA), and thermoplastic starch (TPS) (Jerez *et al*., 2007). Many of these plastics based on starch that has been studied come from crops like maize and potato. The key drawback of traditional bio-based plastics is that the biomass source competes with food and feed applications and large quantities of petroleum products are needed by these agro-crops in their life cycle. These terrestrial crops need large quantities of

fertile soil, irrigation water and fertilisers and take time to grow between harvests to generate the quantities of biomass needed to replace traditional plastic feedstock markets. Starch from marine and freshwater microalgae is thus an efficient feedstock for bioplastic processing.

There are many prominent features compared to plants and crops that make microalgae excellent candidates for the accumulation and processing of starch (the feedstock for the production of bioplastics). Microalgae have higher photosynthetic ability, rapid growth and high biomass, non-polluting and friendly to climate. Microalgae can thus be harvested within a short time compared to plants and crops, and can therefore meet the increasing demand for feedstock (Harun *et al*., 2010). In addition to the simple growth requirement of microalgae, it is possible to grow easily in various aquatic environments such as freshwater, saline water or municipal wastewater (Shilton *et al*., 2008; Sheehan, 2009).

1.4 Research scope and objectives

Microalgae strains, five marine (*Chlorella salina*, *Tetraselmis* sp., *Isochrysis maritima*, *Nitzschia panduriformis* and *Navicula distans)* and 5 freshwater microalgae (*Spirulina* sp., *Ankistrodesmus* sp., *Microcystis* sp., *Chlorococcum* sp., and *Chlorella vulgaris)* with potential high carbohydrates (starch) content cultures were obtained from Microalgal Culture Laboratory of School of Biological Sciences (USMACC) and School of Industrial Technology, Universiti Sains Malaysia. Marine and freshwater microalgae were cultivated and cultured in Conway/Walne's (Walne, 1970) and BG-11 (Stanier *et al*., 1971) respectively. *Spirulina* sp. used for this study was grown with the Zarrouk media (Zarrouk, 1966). For the first stage, all the marine

and freshwater microalgae strains were screened for high starch production and their biochemical composition was analyzed.

At the second stage, *C. salina* and *Spirulina* sp. (strains were selected based on high starch accumulation findings from stage one) were studied for starch accumulation under various laboratory conditions (physical and chemical factors). Firstly, *Spirulina* sp and *C. salina* were studied on the effect of physical factors which comprised of temperature, light wavelength and light duration (photoperiod) on growth, biomass, starch and carbohydrate accumulation. Secondly, *C. salina* was selected based on high starch accumulation for further investigation on the effect of chemical factors which comprised of salinity and nutrient limitation (phosphorus, sulfur and nitrogen). Experiments were conducted using 2 L flasks and cultivated in a growth chamber (Hitec, Malaysia) to control the condition.

For the third stage of the research, the effects of five methods for inducing cell disruption have been investigated on *C. salina*. These included: hot water bath, ultrasonic-liquid shear, acid and alkaline lysis, bead beating and hot ethanol. The efficiency of each cell disruption method was determined using percentages of extracted starch. Using the most suitable method, starch been extracted from *C. salina* and characteristics of starch were investigated to formulate thermoplastic starch (TPS) films.

Finally, commercial corn starch and algae starch films were prepared by a casting technique. Films were cast using glycerol (G) as plasticizers at a concentration ranging from 0-45% (w/v). An aqueous solution containing 5% (w/w) of starch (commercial and algae starch) was prepared by heating the film-forming solutions at 95±2℃ for 15 min under continuous stirring using a hot plate. Films

with optimum concentration of glycerol were further studied, to characterize and compare the properties of thermoplastic starch produce from marine microalgae, *C. salina* and corn starch.

Microalgae possess many advantages as feedstock (starch) for bioplastics production compared to starch from plants/crops. Thus, the aims of this research work are to screen, determined and optimized the cultivation conditions of microalgae for high starch production and accumulation. Therefore, the specific objectives of this research were:

Objective (s) of the research

1. To determine the high starch producer (fresh and marine water) microalgae strain
2. To optimize the cultivation conditions of high starch producer microalgae
3. To compare cell disruption methods and characterization of starch for its suitability used as a feedstock in thermoplastic starch (TPS) production process.
4. To compare and characterize the properties of thermoplastic starch (TPS) produced using commercial corn starch and starch from microalgae

Starch-based bioplastics from marine microalgae can play a vital role as an environmentally friendly, biodegradable alternative compared to conventional plastics. The technology routes for the production of bioplastics using starch from microalgae as feedstock are still under the research phase and are far from commercialization.

CHAPTER 2

LITERATURE REVIEW

2.1 Microalgae

Microalgae are defied as prokaryotic microorganisms which are classified as cyanobacteria (Cyanophyceae) and eukaryotic microorganisms such as green algae (Chlorophyceae) and red algae (Rhodophyceae) with rapid growth rate and due to their unicellular and simple multicellular structure, living in rough conditions (Brennan and Owende, 2010; Mata *et al*., 2010). For most phycologists, they described microalgae as microscopic organisms which contain chlorophyll *a* and thallus roots, stem and leaves that are not differentiated into including oxygenic photosynthetic bacteria (cyanobacteria) (Richmond, 2004; Lee, 2008). However, the simplest and general definition of microalgae is microscopic unicellular organisms with the ability to convert solar energy into chemical energy through photosynthesis.

Microalgae have various types of cell organization such as unicellular (coccoid), colonial and filamentous (Richmond, 2004; Barsanti *et al*., 2008) and the prominent cell organization is the unicellular type. Some unicellular and colonial algae like *Chlamydomonas* sp. and *Platydorina* sp. are propelled by flagella to aid them in locomotion (Graham *et al*., 2009). The taxonomic classification of microalgae described by biologists was based on pigmentation, life cycle, and basic cellular structure. There are nine divisions of microalgae including Chlorophyta, Cyanophyta, Dinphyta, Glaucophyta, Heterokontophyta, Euglenophyta, Rhodophyta, Crytophyta and Haptophyta (Barsanti *et al*., 2008; Lee, 2008). According to Demirbas (2008), the three most accessible classes of microalgae are the diatoms

(Bacillariophyceae), the green algae (Chlorophyceae), and the golden algae (Chrysophyceae).

2.2 Carbohydrate (starch) metabolism of microalgae

The accumulation of carbohydrates in microalgae is due to CO_2 fixation during the photosynthetic process. Photosynthesis is a biological process utilizing ATP/NADPH to fix and convert CO_2 captured from the air to produce glucose and other sugars through a metabolic pathway known as the Calvin cycle (Lehninger *et al*., 2005).

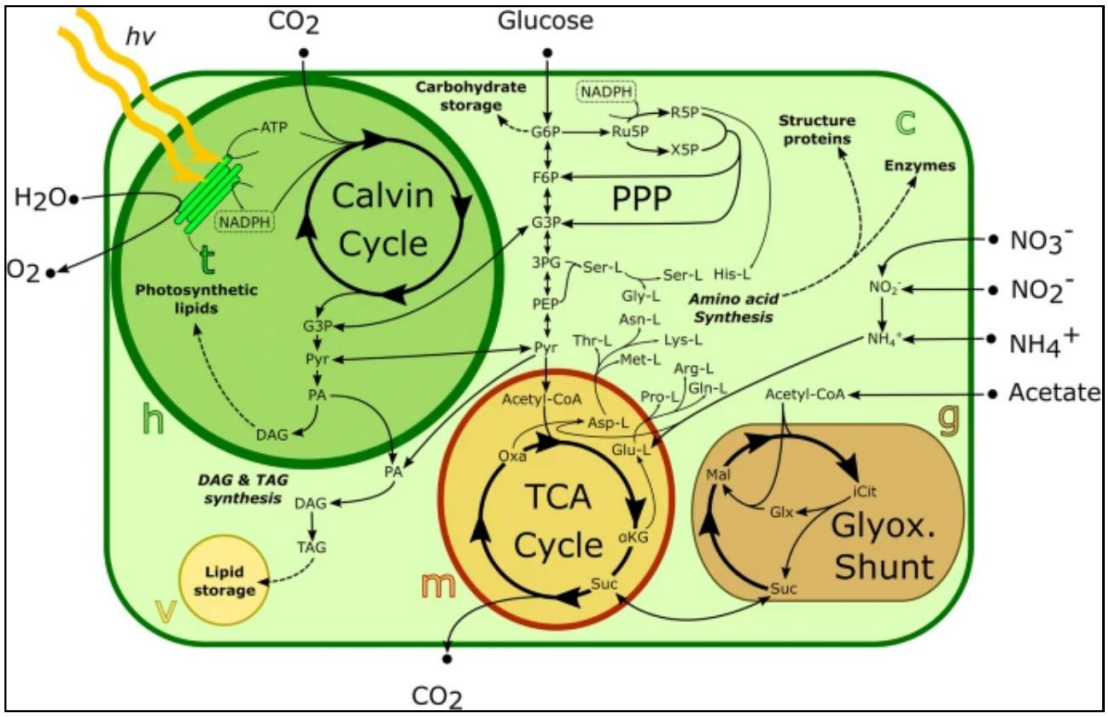

Figure 2.1 Central metabolism in eukaryotic microalgae. The main compartments of active metabolism are shown, i.e., the chloroplast (h), thylakoid lumen (t), vacuole (v), mitochondrium (m), glyoxyssome (g), and cytosol (c) (Tibocha-Bonilla *et al*., 2018).

The metabolic pathways of energy-rich molecules (e.g., carbohydrate and lipid) are closely linked (Figure 2.1). Some studies demonstrated that there was a competition between lipid and starch synthesis because the major precursor for

triacylglycerol (TAG) synthesis is glycerol-3-phosphate (G3P), which is produced via catabolism of glucose (glycolysis) (Ho *et al*., 2012; Rismani-Yazdi *et al*., 2011). Thus, to enhance biofuel production from microalgae-based carbohydrates, it is vital to understand and manipulate the related metabolisms to achieve higher microalgae carbohydrate accumulation via strategies like increasing glucan storage and decreasing starch degradation (Radakovits *et al*., 2010). The starch forms around crystallizing nucleus and is present as an amorphous starch grain. When a chloroplast gathers enough starch, it may become an amyloplast. However, the detailed changes in enzymatic activity and metabolic flux of carbohydrate biosynthesis of microalgae, and a better understanding of the biochemistry of microalgae carbohydrate metabolisms, superior strains for carbohydrate accumulation could be developed.

Except for the starch in plastids, microalgal extracellular coverings (e.g., cell wall) are another carbohydrate-rich part that could be transformed into biofuel (Harun *et al*., 2010). However, the compositions of microalgal extracellular coverings are diverse by species (Domozych *et al*., 2012). Among them, cellulose is one of the main fermentable carbohydrates in most green algae (Radakovits *et al*., 2002). Cellulose synthesis is a complicated process that includes many enzymatic reactions. The starting substrate for cellulose synthesis is UDP-glucose, which is formed from the reaction of UDP and fructose catalyzed by sucrose synthase (Kimura and Kondo, 2002).

2.3 Freshwater microalgae

For microscopic appearance, the freshwater algae can be grouped into 10 predominant divisions (phyla). Any measure of the ecological and taxonomic diversity of these groups is the number of constituent species of freshwater and

terrestrial algae in standard, with green algae and diatoms representing their substantial existence and potential to live in different environments (Randrianarison and Aqeel Ashraf, 2017). Especially diatoms (over 1600 species) are ecologically efficient, both as planktonic organisms and as benthic. Furthermore, John *et al*. (2002) also list other phyla, while they have a taxonomic and phylogenetic interest in these minor phyla, they have less influence in the freshwater climate.

2.3.1 *Spirulina* sp.

Spirulina is a microalgae of photosynthesis, filamentous, spiral-shaped, multicellular blue colour, typically found in freshwater. *Spirulina maxima*, and *Spirulina platensis* are the two most significant plants. It comprises pigments of carotenoids, chlorophyll, and significant phycocyanin. It belongs to Cyanophyceae and is characterised by spiral chains of the cells in a thin sheath (James *et al*., 2006).

This microorganism was named "spirulina" because of its spiral filament-like appearance (and is known as cyanobacterium) under the microscope. Nutritional conditions can differ according to the growing conditions of *Spirulina* sp. It should be remembered that the *Spirulina* sp. cell wall is consists of protein, fat and carbohydrates, not from indigestible cellulose. Therefore, the bioavailability of nutrients from other sources of food, in particular from sources of plant food (Ciferri, 1983).

Starch extracted from *Spirulina* sp. based on previous research is used for health care, skin hazard protection caused by sunlight and hair care products. Recent research by scientists indicates that green algae protein and starch can serve as an HIV treatment vaccine, indicating that by consuming these algae, people are immunised against these diseases (Vo and Kin, 2010; Kasgari *et al*., 2007).

2.3.2 *Microcystis aeruginosa*

Microcystis is a genus of blue-green algae (also called cyanobacteria) and is the primary group of phytoplankton in eutrophic freshwater bodies (Davidson, 1959; Negri *et al*., 1995). Phylum Cyanobacteria; Class Cyanophyceae; Order Chroococcales and Family Microcystaceae includes single, planktonic freshwater cyanobacteria. *Microcystis aeruginosa* is one of the major cyanobacteria categorized by mucilage, with a cell size of 3 to 4 μm and varying colonies ranging from just a few to a hundred cells, among all the species identified (Biswas, 1949).

Microcystis aeruginosa is the most abundant and widespread cyanobacterial species found in freshwater environments ranging from tropical to sub- cold zones (Harke *et al*., 2016). The blooms of *M. aeruginosa* cause many environmental problems, including bad odour and bottom-layer hypoxia; however, the development of hepatotoxic cyanotoxins called microcystins is the issue of greatest concern (Harke *et al*., 2016). The blooms have been a significant eco-environmental issue so far, and the mechanism of the outbreak of the bloom should be further investigated. Because of this, minimum or no study was performed using *Microcystis* sp.

2.3.3 *Chlorococcum* sp.

Chlorococcum sp. with spherical or slightly oblong cells of different sizes, is unicellular. The cells may be solitary or in irregular clumps, forming films often on surfaces that are moist or submerged. The mucilage is slender and unnoticeable. A cell has a single parietal chloroplast shaped cup with a single pyrenoid.

Given changing energy scenario for renewable energy sources, *Chlorococcum* sp. is one of the best known for its capacity to generate biodiesel. Large-scale *Chlorococcum* biomass production depends on many factors, the most

important of which are the availability of nutrients, salinity, temperature, and light. These factors affect the growth and composition of the biomass formed by *Chlorococcum*, causing changes in metabolism. The biomass of algae species consists primarily of proteins, lipids, and carbohydrates (Spolaore *et al*., 2006).

According to Watanable and Lewis (2017), *Chlorococcum* sp. TISTR8583 is ellipsoidal (with varying size and rough cell wall) and is solitary in nature with thin mucilage. Furthermore, the Chlorococcum sp. was investigated for its excellent self-flocculating nature, which facilitates the easy processing of biomass and the removal of sulphur and nitrogen from wastewater as a bioremediating agent (Lv *et al*., 2017).

2.3.4 *Chlorella vulgaris*

Chlorella vulgaris is a 2-10 μm diameter spherical microscopic cell (Illman *et al*., 2000; Yamamoto *et al*., 2004; Yamamoto *et al*., 2005), which has several related structural elements to plants.

C. vulgaris is one of the fastest-growing green microalgae, known as freshwater microalgae. Pulz (2001) estimated that the annual production of 130-150 tonnes of *Chlorella* dry biomass could be produced using an industrial-scale (700 m^3) tubular system in a glasshouse area of 10,000 m^2. In addition, the lipid content in *C. vulgaris* could be significantly increased during the nutrient starvation stage, i.e. between 50 and 70% (Yeh and Chang, 2012).

Cheap enhanced starch biomass can be generated from highly efficient *Chlorella* cultures grown in sufficient outdoor photobioreactors, where the source of photosynthesis of carbon dioxide is obtained from organic waste combustion, fermentation processes or other sources (Doucha *et al*., 2005; Douskova *et al*., 2009; Mann *et al*., 2009).

2.3.5 *Ankistrodesmus* sp.

Ankistrodesmus unicells, or loosely attached colonies of unicells. Cells that are fibre-shaped are smooth, twisted, or spiral; needle-like, curving occasionally. Unicellular, but can be located or twined around one another in clusters (Guiry and Guiry, 2013).

Ankistrodesmus sp. is unicellular green microalgae with total lipid content of up to 24 per cent (Mata *et al*., 2010). The high lipid content and high polyunsaturated FAME levels of *Ankistrodesmus fusiformis* and *Ankistrodesmus falcatus* are used in biodiesel production. In addition, *Ankistrodesmus* sp. can efficiently use CO_2 for its growth (Salim, 2013).

Because of their rapid growth rate, resistance to adverse conditions, nutritional quality and also as a model organism for cell growth and division study, microalgae such as *Ankistrodesmus gracilis* were used in aquacultures. However, in terms of availability of literature is limited (Salim, 2013).

2.4 Marine microalgae

The largest primary biomass, marine microalgae, has been attracting attention as a tool for new metabolites and biotechnologically useful genes. A wide number of microalgae are present in the diversified marine climate. Documented species of microalgae live at least 30,000. Microalgae are classified as mostly unicellular photosynthesis cells, though some complex associations offer colonies that have larger structures. This is a rather heterogeneous group that includes bacteria-like prokaryotic species (cyanobacteria, also known as blue-green algae) and eukaryotic organisms, such as diatoms. The number of species of blue-green is very high and possibly not thoroughly explored (Randrianarison and Ashraf, 2017).

2.4.1 *Isochrysis maritima*

Isochrysis maritima has pairs of apical subequal homodynamic flagella (6 and 8 μm) and normally travels backwards. Includes single yellow-green chromatophore and stigma. For older cells, the scale of this species is about 3 x 7 μm (Billard and Gayral, 1972).

Microalgae of marine origin, *Isochrysis* sp. due to their potential to provide value in the fields of experimental genetic material, has earned immense interest in research studies. These resources contribute to the manufacture of a variety of nutritional and pharmaceutical products, proteins (essential amino acids), energy, other essential nutrients such as vitamins, essential PUFAs and pigments transferred via the food chain. These resources contribute to the development of various nutritional and pharmaceutical products, proteins (essential amino acids), energy, vitamins, essential PUFAs, and food chain pigments, as well as other essential nutrients (Brown, 2002). *Isochrysis maritima* is a mobile golden brown microalga, placed together with *Isochrysis galbana* and *Isochrysis littoralis* in the genus *Isochrysis*.

Previous analysis of *Isochrysis zhangjiangensis* (Haptophyta) showed that carbohydrates accumulated rapidly under depleted conditions of nitrogen, rather than lipids (Wang *et al*., 2014). Sulfur starvation has also been used to increase the production of starch (Yao *et al*., 2012). Multiple stress conditions were used to increase the development of carbohydrates, for example, under high light intensity and nitrogen starvation conditions carbohydrates or microalgae rich in starches were obtained (Aikawa *et al*., 2012; Ho *et al*., 2012; Sun *et al*., 2014; Jerez *et al*., 2015).

2.4.2 *Nannochloropsis* sp.

Nannochloropsis is a marine genus with a picoplanktonic nature. The cells are small (Diameter 2-4 μmin), spherical to slightly ovoid, non-flagellate (Guiry and Guiry, 2014) (Figure 2.7).

Nannochloropsis sp. are common species of microalgae that have promising potential, especially in the application of the aquaculture industry. Furthermore, its high nutritional value, ease of cultivation, lack of toxicity, the correct size of cells and digestible cell wall satisfy the specifications of choice for aquaculture purposes (Hemaiswarya *et al*., 2011). About Nannochloropsis sp. it is capable of producing significant amounts of fatty acid triglycerides (TFA) and polyunsaturated fatty acid (PUFA) lipids up to 65-70 % of the total dry weight (Radolfi *et al*., 2009), making it a species with great potential for biofuel production and feeding larval and juvenile hatcheries of bivalves and fish.

Nannochloropsis sp. is known for its oleaginous properties with a particularly high content of eicosapentaenoic acid (EPA) and has been investigated as larval feed and biodiesel feed for aquaculture applications because of its high content of triacylglycerol (TAG) (up to 60 % of its dry weight, DW) (Rodolfi *et al*., 2008; Mata *et al*., 2010; Pal *et al*., 2011). The production and accumulation of carbohydrates and starches have not been thoroughly studied Li *et al*., 2010.

2.4.3 *Tetraselmis subcordiformis*

The euryhaline marine green microalgae widely distributed in coastal waters (Kirst, 1989), *Tetraselmis subcordiformis* (synonym: *Platymonas subcordiformis*), has been shown to accumulate large amounts of starch under nitrogen, sulphur, or phosphorus starvation (Yao *et al*., 2012).

Tetraselmis subcordiformis is a marine green microalga that has been shown to have an excellent capacity to produce starch under conditions of nutrient limitation and low salinity (Yao *et al*., 2012). *Tetraselmis subcordiformis* is a marine green microalga that has been shown to accumulate intracellular starch above 50 per cent DW under nitrogen deprivation (Yao *et al*., 2012).

2.4.4 *Chlorella salina*

Beijerinck (1890) described the genus *Chlorella* for tiny (< 10 μm) unicellular green cocoid algae. *Chlorella salina* has a cell size of 3.0-6.0 μm that can exceed 8.0 μm and has a pyrenoid-shaped chloroplast surrounded by starch grains (Butcher, 1952). *C. salina*, found mostly in seawater. The *Chlorella* genus consists of small microalgae, spherical to ovoid, nonmotile, unicellular or colonial with a single pyrenoid chloroplast (Bock *et al*., 2011). Over the last 20 or so years, biochemical and molecular studies have led to a significant reassessment of algae called "Chlorella" (Krienitz *et al*., 2015), with many relocating to other genera. 44 *Chlorella* species are currently recognized (Guiry and Guiry, 2017).

Chlorella sp. owing to its peculiar characteristics, due to their characteristics, including the high nutritional value in terms of natural antioxidants (Matsukawa *et al*., 2000; Rodriguez-Garcia and Guil-Guerrero, 2008; Hajimahmoodi *et al*., 2010; Sawant *et al*., 2014), high productivity in terms of lipid and carbohydrate content (Del Campo *et al*., 2004; Goiris *et al*., 2012; Zhu *et al*., 2014; Goiris *et al*., 2015), and a thick cell wall that protects their nutrient content, scientists have become one of the most widely researched microalgae classes (Iwamoto, 2004).

2.4.5 *Nitzschia* sp.

*Nitzschia c*ells are normally long, straight and narrow, but maybe ovoid or even slightly sigmoid. They normally occur individually, but in mucilage tubes, they can form stellate colonies or live. *Nitzschia* is a relatively large genus with hundreds of marine and freshwater species (Lowe, 2003).

Nitzschia species are of ecological importance as live feed for bioindicators (Maznah and Mansoor, 2002), endosymbionts (Lee, 2011) and also aquaculture (Chu *et al*., 1996). Certain tropical *Nitzschia* species are toxic. The first detection of toxic *Nitzschia* species was reported from prawn pond samples in Vietnam (Lundholm and Moestrup, 2000), while others were collected from estuarine sites such as in Malaysia (Suriyanti and Gires, 2015) and lagoon samples from the Southwest Mediterranean Sea (Smide *et al*., 2014). However, previous studies about *Nitzschia* sp. are generally limited up to the current date.

2.5 Microalgae growth profile

The most common technique for cultivating microalgae is batch culture. A limited amount of complete culture medium and microalgae inoculums are put in a culture vessel in a simple batch culture system to be incubated for growth under certain culture conditions. The culture of microalgae can grow rapidly under ideal conditions until the rate of cell division begins to decline, signalling the phase of transition from the exponential to the stationary level. The culture is fully harvested at that stage (Lavens and Sorgeloos, 1996) and the washed container has been refilled with sterilised and enriched medium and inoculated to launch a new culture, vessels of culture may be from a simple conical flask (Richmond, 2004).

There are five relatively well-established phases of the growth of microalgae in batch culture. It starts with the lag period, followed by the exponential phase, the decreasing phase of growth rate, the stationary phase and the phase of death (Fogg, 1975; Lavens and Sorgeloos, 1996), as shown in Figure 2.2.

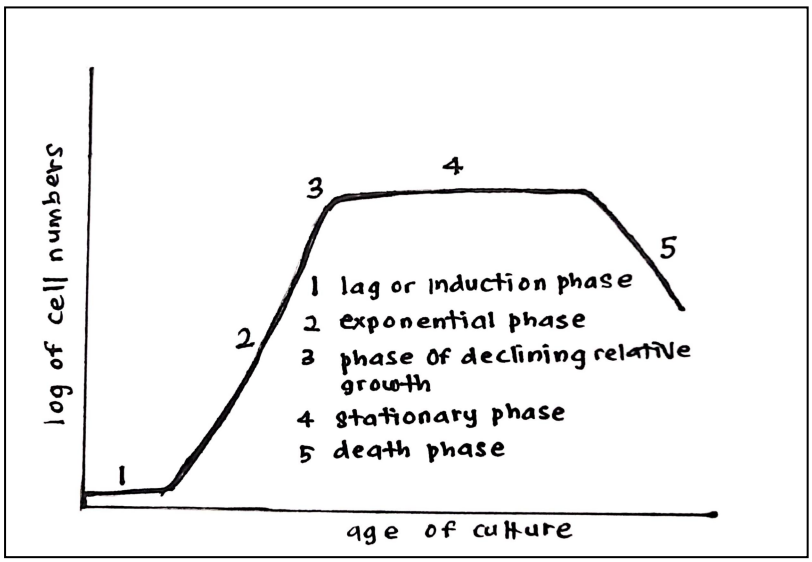

Figure 2.2 General pattern of microalgae growth in batch cultures (Lavens and Sorgeloos, 1996)

The lag phase is the stage where less cell growth occurs. The existence of nonviable cells may be the explanation for this (Fogg, 1975). After adapting to the new environment, the cells begin to enter the exponential (or logarithmic) phase of development. As long as necessary nutrients and light energy are available the cells can replicate rapidly and directly proportional to time (Richmond, 2004). Also in this stage the basic growth rate of a population of microalgae is calculated (Andersen, 2005).

The declining growth phase or early stationary phase typically occurs in the cultures when precise cell division requirements are restricted. Culture density in this process is usually very high. The stationary phase occurs when the factor limit and growth rate is equal, resulting in a relatively linear density of cells as time. Cell

biochemical composition can undergo drastic changes. The death period in the growth process of microalgae. This phase generally occurs when the cell metabolism can no longer be vindicated due to the several limiting factors that led to a decrease in cell density rapidly and the culture eventually collapses (Bold and Wynne, 1985).

2.6 Factors affecting microalgae growth and biochemical contents

Many factors, including physical and chemical factors, may influence the growth and biochemical composition of microalgae (Pruvost *et al*., 2002; Sun and Wang, 2009; Guides *et al.,* 2010). Light (intensity, exposure time, light wavelength), pH, salinity (marine water) and temperature were physical factors, while chemical factors included nutrient requirements for the growth of microalgae, such as nitrogen, carbon, sulphur, phosphorus, potassium, and manganese.

2.6.1 Temperature

Numerous studies have studied the effect of temperature on the development of microalgae. The ideal temperature for the growth of microalgae is usually between 20 °C and 24 °C (Laven and Sorgeloos, 1996). The outcome may however be different with the quality of the culture medium and the cultured species (Renaud *et al*., 1995; Durmaz *et al*., 2008). The effects of temperature on cell cultures are correlated with the temperature dependence of cell structural components (particularly lipids and proteins) and the temperature coefficients of the reaction rate (Sandnes *et al*., 2005). The implications of these primary effects are important for changes in metabolic regulatory mechanisms, specificity of the enzyme reaction, cell permeability and cell composition (Richmond, 2004).

In addition to growth, temperature changes have a significant impact on the biochemical compounds of certain microalgae (Renaud *et al*., 2002). Araújo and Garcia Research (2005) on *Chaetoceros* cf. *wighanii* demonstrated that the content of carbohydrates and lipids was higher at lower temperatures. *Diacronema vlkianum* analysis by Durmaz *et al*. (2008) also yielded similar findings. In *Isochrysis galbana* TK1, especially C18:3 (n-3) and C22:6 (n-3), the polysaturated fatty acid content was higher at low temperatures (Zhu *et al*., 1997). Temperature also influences the carbohydrate levels in microalgae, such as the carbohydrate content in *Spirulina* sp. The temperature rose by 50% from 25 to 40 °C (Ogbonda *et al*., 2007).

2.6.2 Light wavelength

Light plays an important role in microalgae cultivation, as it is a photoautotrophic organism (Pruvost *et al*., 2002) which, like many other plants, possesses photosynthetic pigments. Every photosynthetic pigment such as chlorophyll a, chlorophyll b, carotene, and phycocyanin can absorb some colour and give the algae the colour (Campbell *et al*., 2006). As electricity, microalgae absorb light and transform inorganic carbon into organic carbon and water (Carvalho and Macata, 2005).

For the growth of various algal species, it is known that different wavelengths of light are needed. Microalgae usually use wavelengths for photosynthesis ranging from 400 to 700 nm. Species vary in wavelength of light absorbed by microalgae (Blair *et al*., 2014). One of the fundamental parameters to be investigated is the determination of the optimal wavelength of light for the growth of microalgae. Using sunlight has an outstanding effect on algal growth. The development of microalgae, however, is negatively affected by changes in weather conditions. In microalgae

cultures, artificial light sources are commonly used to improve productivity and meet energy needs under managed conditions. Strength and light wavelength are two of the most critical parameters for the growth of microalgae. In order to achieve the optimum rate of photosynthesis, microalgae require optimal lighting conditions (Zhao *et al*., 2013).

The optimum wavelength, the best wavelength for the growth of most algae species, is a red light with a narrow range of 600-700 nm. This is largely because chlorophylls that can more easily absorb red light relative to other light wavelengths are the most abundant pigments in most organisms (Matthijs *et al*., 1996). For example, light with a shorter wavelength, blue light, has a higher likelihood of triggering photo-inhibition by hitting the light-harvesting complex of cells at its peak electrical energy (Das *et al*., 2011).

2.6.3 Photoperiod

In the natural environment, all life is exposed to a daily cycle of light and dark fluctuation of light intensities and seasonal oscillation of daylight length as a result of the rotation of the planet. Eukaryotic and prokaryotic cells have evolved to respond to the rhythmic changes in environmental conditions and synchronize their cellular processes to the most appropriate time of the day (Dixon et al., 2014). Research on green microalga *Chlamydomonas reinhardtii* shows that a wide range of biological processes including cell division, phototaxis, chemotaxis, cell adhesion, and nitrogen metabolism can be regulated by the natural clock and environmental conditions (Matsuo and Ishiura, 2011). Apart from the regulation of biological processes, both the yield and the composition of algal biomass are dependent on environmental light conditions (Sorokina *et al*., 2011).